Role of melatonin MT2 receptors in the regulation of sleep and anxiety disorders

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Abstract

The neurohormone melatonin (MLT) is implicated in the regulation of sleep and anxiety. MLT activates two G-protein coupled receptors, MT_1 and MT_2, whose differential roles in sleep and anxiety remain to be defined. The aim of this thesis was to characterize the single role of MT_2 receptors in sleep and anxiety. We have therefore explored the putative hypnotic and anxiolytic-like effects of the novel MT_2 receptor partial agonist UCM765. The systemic administration of different doses of UCM765 in rats produces hypnotic effects with high doses while a low dose elicits anxiolytic-like activity. In particular, in sleep, the activation of MT_2 receptors by UCM765 selectively promotes non-rapid eye movement sleep (NREMS) during the light phase with no changes in sleep architecture in rats. The sleep promoting actions of UCM765 are blocked by the selective MT_2 antagonist (4P-PDOT), thus confirming the role of the MT_2 receptors in the regulation of NREMS. In keeping with this view, in mice, UCM765 induces and maintains NREMS in wild strain (WT) and MT_1 knock-out (MT_1KO) but not in MT_2 knock-out (MT_2KO) mice. In agreement, the basal sleep profile in MT_2KO, but not MT_1KO mice shows a decrease in NREMS compared to WT during the light phase, indicating that the genetic inhibition of MT_2 receptors impairs NREMS. Interestingly, the dual activation of MT_1/MT_2 receptors by MLT or non-selective MT_1/MT_2 analogs slightly reduces NREMS latency with no effects on sleep maintenance, suggesting that the selective activation of MT_2 receptors results in a more incisive effect on sleep onset and NREMS maintenance. In anxiety, the selective activation of MT_2 or dual activation of MT_1/MT_2 receptors by UCM765 or MLT, respectively, induces anxiolytic-like effects in the elevated plus maze test and novelty suppressed feeding test with no sedative effects in the open field test. The enhancement of NREMS and anxiolytic-like effects of UCM765 are nullified by the pharmacological blockade of MT_2 receptors, suggesting that the MT_2 receptor is certainly mediating such effects. Immunohistochemical labeling reveals that MT_2 receptors are localized in sleep and anxiety related brain regions, and notably in the reticular thalamic nucleus (Rt). The behavioural pattern induced by UCM765 was congruent with electrophysiological data. Indeed, UCM765 increases the firing rate and bursting activity of Rt neurons, which is blocked by 4P-PDOT. Moreover, the activation of MT_2 receptors in Rt neurons by UCM765 is involved in its hypnotic effects. These results demonstrate, for the first time, the hypnotic and anxiolytic properties of UCM765 and suggest that MT_2 receptors may be considered a novel target for the development of hypnotic and anxiolytic drugs.

Résumé

La mélatonine est une neurohormone impliquée dans la régulation du sommeil et de l'anxiété. Elle active deux récepteurs MT_1 et MT_2 appartenant à la famille des récepteurs couplés à une protéine G et dont le rôle dans le sommeil et l'anxiété restent à déterminer. L'objective de ce travail de thèse consiste en la caractérisation du rôle des récepteurs MT_2 dans le sommeil et l'anxiété. Par ailleurs, nous avons exploré les rôles hypnotique et anxiolytique putatifs d'un agoniste partiel des récepteurs MT_2, l'UCM765. L'administration systémique chez le rat de différentes doses de ce ligand produit, à fortes doses, un effet hypnotique et à faibles doses un effet anxiolytique. L'activation par cet agoniste partiel des récepteurs MT_2 chez le rat favorise de manière sélective, pendant la phase lumineuse, les mouvements non-rapides des yeux (NREMS) sans changement de l'architecture général du sommeil. Cet effet de promotion du sommeil par l'UCM765 a été ensuite inhibé par l'administration d'un antagoniste des récepteurs MT_2 (4P-PDOT), ce qui confirme le rôle de ce récepteur dans la régulation des NREMS. Dans ce sens, l'UCM765 induit le maintien des NREMS chez des souris control et MT_1KO mais pas chez des souris MT_2KO. Par ailleurs durant la phase lumineuse, le profil du sommeil basal montre une baisse des NREMS chez les souris MT_2KO mais pas les MT_1KO en comparaison avec les souris control. Ce résultat révèle que la désactivation génétique des récepteurs MT_2 provoque l'élimination des NREMS. Cependant, l'activation simultanée des récepteurs MT_1/ MT_2 par la mélatonine ou des ligands non sélectifs MT_1/ MT_2 produit une faible diminution du temps de réponse des NREMS sans toutefois affecter le sommeil réparateur. Cela suggère que l'activation sélective des récepteurs MT_2 a un effet primordial dans l'établissement du sommeil et la réparation des NREMS. Dans le cas de l'anxiété, l'activation respective par l'UCM765 ou la mélatonine du MT_2 ou des MT_1/MT_2 produit un effet anxiolytique dans les tests du labyrinthe (EPMT) et de l'alimentation supprimée par la nouveauté (NSFT) et sans toutefois provoquer d'effet sédatif dans le test d'open-field (OFT). Le renforcement des NREMS et de l'activité anxiolytique de l'UCM765 a été annulé par le blocage pharmacologique des récepteurs MT_2 confirmant ainsi l'implication de ce récepteur dans les effets mentionnés. Par ailleurs, le marquage immunohistochimique a montré la présence de ces récepteurs au niveau des zones du cerveau responsables de l'anxiété et du sommeil, en particulier le noyau thalamique réticulaire (Rt). En effet, le comportement induit par l'administration de l'UCM765 est conforme aux résultats d'électrophysiologie. L'UCM765 augmente l'activité électrique et d'éclatement des

neurones Rt et dont le blocage est induit par l'antagoniste 4P-PDOT des MT_2. Par ailleurs, l'activation de ces récepteurs MT_2 par l'UCM765 au niveau des neurones Rt est responsable de ses effets hypnotiques. Ces résultats démontrent pour la première fois l'implication du récepteur MT_2 dans les effets hypnotiques et anxiolytiques par le biais de l'agoniste partiel UCM765 et présentent ce récepteur comme nouvelle cible pour le développement de nouveaux agents hypnotiques et anxiolytiques.

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Contribution of authors

The present dissertation includes 4 original co-authored manuscripts. Notably, my supervisor Dr. Gabriella Gobbi provided substantial contribution in all aspects involved in this PhD project including conception of the idea, analyses, coordination of different research groups, interpretation of results, writing manuscripts and reviewing this dissertation. The different affiliated institutions are indicated in each publication.

As a first author, I was responsible for designing and conducting all experiments, processing data, and performing statistical analyses associated with the EEGs in rats and mice presented in this dissertation. I solely wrote the chapters, the connecting texts between chapters as well as the final conclusions.

Dr. Stefano Comai provided substantial contribution in data processing, performing analyses and in writing the following manuscripts: Ochoa-Sanchez et al., 2011; Comai, Ochoa-Sanchez et al., 2012 and Ochoa-Sanchez et al., 2014. Additionally, he was an important contributor to the revision of this thesis.

Drs. Gilberto Spadoni, Silvia Rivara, Annalida Bedini, Franco Frachini, Marco Mor and Giorgio Tarzia designed and synthetized the melatonin ligands UCM765, UCM924 and UCM793. Additionally, Dr. Fraschini characterized the affinity and the intrinsic properties of these ligands.

Dr. Debora Angeloni and Dr. Fraschini were responsible for the production of the polyclonal anti-MT_2 antibodies used in immunohistochemical experiments on rat brain tissue.

Dr. Laurent Descarries (1939-2012) and Dr. Babtiste Lacoste were responsible for the immunohistochemical experiments for the localization of MT_2 receptors in the rat brain. They provided the corresponding pictures and notably, their feedback was substantial for the publication of Ochoa-Sanchez et al., 2011.

Dr. Sergio Dominguez-Lopez and Dr. Francis Bambico provided substantial input in the analysis of the EEG in rats in the manuscript Ochoa-Sanchez et al., 2011.

Dr. Quentin Rainer and I conducted the behavioral experiments in anxiety, and we were responsible for design, data processing, statistical analysis and writing the manuscript Ochoa-Sanchez et al., 2012. The writing of the mentioned manuscript also includes the participation of Dr. Comai and Dr. Gobbi.

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Abbreviations

[^{125}I]Mel	2-iodo-melatonin
[^{35}S]GTPγS	Guanylyl 5'-(γ-thio)triphosphate coupled with sulfur-35 radioisotope
4P-PDOT	4-phenyl-2-propionamidotetralin
5-HT	Serotonin (5-hydroxytryptamine) neurotransmitter
5-HT$_{2A}$	Serotonin 2A subtype receptor
5-HT$_{2B}$	Serotonin 2B subtype receptor
5-HT$_{2C}$	Serotonin 2C subtype receptor
ACh	Acetylcholine
AP	Antero-posterior
ATP	Adenosine triphosphate
BZ	Benzodiazepine site/receptor
BZs	Benzodiazepines
CA1	Cornu Ammonis subfield 1
CA3	Cornu Ammonis subfield 3
CA4	Cornu Ammonis subfield 4
cm	Centimeter
CNS	Central nervous system
DR	Dorsal raphe
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4th Edition
DZ	Diazepam
ED$_{50}$	Half-maximal (50%) effective dose
EEG	Electroencephalogram
EMG	Electromyogram
EPMT	Elevated plus maze test
FDA	Food and drug administration (USA)

FFT	Fourier transform
g	Grams
GABA	Gamma-aminobutyric acid neurotransmitter
$GABA_A$	Gamma-aminobutyric acid receptor subtype A
GAD	Generalized anxiety disorder
GH	Growth hormone
GPCR	G-protein-couple receptors
h	Hour
H_3	Histamine receptor subtype 3
HPA	Hypothalamo-pituitary-adrenocortical axis
Hz	Hertz, cycles per second
i.p (ip)	Intraperitoneal (or intraperitoneally)
i.v (iv)	Intravenus (or intravenously)
IIK7	6H-Isoindolo[2,1-a]indoles derivative
K-185	6,7-Dihydro-5H-benzo[c]azepino[2,1-a]indoles derivative
KDa	Kilodalton
kg	Kilograms
L	Lateral
LC	Locus coeruleus
LDT	Laterodorsal tegmental nuclei
mg	Milligrams
min	Minutes
ml	Millilitre
MLT	Melatonin *(*N-acetyl-5-methoxytryptamine)
mm	Millimetre
mRNA	Messenger Ribonucleic acid
ms	Milliseconds

MT_1	Melatonin receptor subtype 1
MT_1/MT_2KO or $MT_1^{-/-}/MT_2^{-/-}$	Melatonin receptor subtypes 1 and 2 knock-out
MT_1KO or $MT_1^{-/-}$	Melatonin receptor subtype 1 knock-out
MT_2	Melatonin receptor subtype 2
MT_2KO or $MT_2^{-/-}$	Melatonin receptor subtype 2 knock-out
NE	Noradrenaline neurotransmitter
Neu-P11	Melatonin MT_1/MT_2 receptor agonist
ng	Nanograms
NREMS	Non-rapid eye movement sleep
NSFT	Novelty suppressing feeding test
OFT	Open field test
PB	Phosphate buffer
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
pKi	Log dissociation constant
PPT	Pedunculopontine
REMS	Rapid eye movement sleep
RNA	Ribonucleic acid
Rt	Reticular thalamic nucleus/neurons
RT-PCR	Reverse transcription-polymerase chain reaction
s	Seconds
S23478	Melatonin MT_1/MT_2 receptor agonist
SCN	Suprachiasmatic nucleus
SERT	Serotonin transporter
SNK	Student Newman Keuls post hoc analysis
SNP	Substantia nigra pars reticulate

SSRIs	Serotonin-specific reuptake inhibitors
SWS	Slow wave sleep (NREMS)
TAK-375	Ramelteon, melatonin MT_1/MT_2 receptor agonist
TBS	Tris-buffered saline
TC	Thalamocortical neurons
TMN	Tuberomammillary nucleus
UCM765	*N*-{2-[(3-methoxyphenyl)phenylamino]ethyl}acetamide
UCM793	*N*-{2-[(3-methoxyphenyl)methylamino]ethyl}acetamide
UCM924	*N*-{2-[(3-bromophenyl)(4-fluorophenyl)amino]ethyl}acetamide
VLPO	Ventrolateral preoptic area
WT	Wild type
μg	Microgram
μl	Microliter
μm	Micrometre

Chapter I

Introduction and objectives

1.1 Introduction

1.1.1 Melatonin

Melatonin (MLT, *N*-acetyl-5-methoxytryptamine) is a widely distributed compound in nature. Its functional role has been demonstrated in a wide range of organisms, from unicellular organisms to plants, animals, and humans (Hirsh-Rodriguez et al., 2007). MLT is involved in numerous physiological functions including sleep, circadian rhythms, mood regulation, anxiety, appetite, immune responses and cardiac functions (Reiter 2003; Hardeland et al., 2011). In humans, MLT is primarily synthesized by the pinealocytes of the pineal gland, an endocrine gland located in the center of the brain but outside the blood–brain barrier. MLT is synthesized from the precursor *L*-tryptophan, which is converted into 5-hydroxytryptophan and then further into serotonin (5-HT). Afterwards, 5-HT is acetylated by aryl alkylamine *N*-acetyltransferase and then converted into MLT by hydroxyindole O-methyltransferase (Benarroch 2008). Once synthesized, MLT is released into the bloodstream where it can cross the blood-brain barrier and enter the central nervous system (CNS) (Longatti et al., 2007; Hardeland 2010).

Circulating MLT has a short half-life of 2–20 minutes; it is mainly metabolized by the liver, where MLT is first hydroxylated by cytochrome P450 mono-oxygenases and thereafter conjugated with sulfate (Benarroch 2008). When MLT is administered orally, more than 80% is excreted in the urine as 6-sulfatoxyMLT while the rest is excreted as *N*-acetylserotonin (Leston et al., 2010).

MLT secretion in the pineal gland follows a marked circadian rhythm. More precisely, the concentrations of MLT in the body are lower during the day and reach maximal levels during the night in both diurnal and nocturnal species. In humans, plasma levels of MLT begin to rise about 2 hours before habitual bedtime and remain elevated during the dark hours. A typical human adult's average daytime and night-time levels of blood MLT are approximately 10 and 60 pg/mL, respectively. These daily changes in circulating levels of MLT have been related to the entrainment of circadian rhythms of several biological functions including sleep. Thus, the MLT signal is part of the system that regulates the sleep-wake cycle and other cyclical bodily activities by acting in the suprachiasmatic nucleus (SCN). The SCN, located in the anterior hypothalamus, is the master clock controlling circadian rhythms in mammals. The SCN contains neurons that

exhibit a circadian pattern of activity in response to the light/dark cycle, which regulate MLT secretion in the pineal gland through a multisynaptic pathway (Benarroch 2008). For instance, the lesioning of the SCN in rats inhibits the difference between day and night in MLT synthesis (Perreau-Lenz et al., 2003).

1.1.2 Melatonin signalling and melatonin binding sites

As mentioned above, MLT is involved in numerous physiological functions (Reiter 2003; Hardeland et al., 2011). Several effects of MLT in the brain are mainly mediated by the activation of two high-affinity G protein-coupled receptors (GPCRs), MT_1 and MT_2 (Duvocovich and Markowska 2005). The activation of MT_1 and MT_2 receptors produce downstream effects such as inhibition of adenylyl cyclase and cAMP production. By using recombinant MLT receptors it has been shown that the MT_1 receptor is coupled to different G-proteins that mediate the adenylyl cyclase inhibition by a pertussis toxin-sensitive G-protein and phospholipase C beta activation (Reppert et al., 1995). The MT_2 receptor is also coupled with inhibition of adenylyl cyclase and additionally, it inhibits the soluble guanylyl cyclase pathway (Petit et al., 1999). Altogether, it appears that multiple and distinct downstream mechanisms induced by the activation of MT_1 or MT_2 receptors, are associated with unique cellular responses responsible for the different physiological actions of MLT (Witt-Enderby et al., 2003) and, in some cases complementary or opposite effects (Doolen et al., 1998; Wan et al., 1999). For instance, at the level of blood vessels, the activation of MT_1 and MT_2 receptors promote vasoconstriction and vasodilatation, respectively (Doolen et al., 1998). In relation to $GABA_A$, a class of receptors that respond to the neurotransmitter gamma-aminobutyric acid (GABA), *in vitro* studies in hippocampal slices showed that the activation of MT_1 or MT_2 receptors potentiates or inhibits the function of $GABA_A$ receptors, respectively, demonstrating the opposite effects of MT_1 and MT_2 receptors (Wan et al., 1999).

As previously mentioned, the effects of MLT are associated with its interaction with the SCN, a brain region that expresses both MT_1 and MT_2 receptor mRNA (Dubocovich et al., 1998), where MLT induces acute neuronal inhibition and phase-shifting (Liu et al., 1997). *In vitro* studies in brain slices from wild strain (WT), MT_1 knock-out (MT_1KO) and MT_2 knock-out (MT_2KO) mice have showed that the MT_1 receptor is responsible for the MLT-induced acute inhibition of SCN firing rate but not for the phase shifting effects. Therefore, the regulation of

SCN firing rate and possibly other limbic areas by MT_1 receptor suggest that the MT_1 receptor might control sleep regulation (Liu et al., 1997). Regarding MT_2 receptors, *in vitro* experiments in rat SCN slices demonstrate that MT_2 is responsible for the phase-shifting effect of MLT (Hunt et al., 2001). Moreover, behavioural experiments in mice demonstrate that phase-shifting effects of MLT are mediated by the MT_2 receptor (Dubocovich et al., 1998). However, these studies concerning the role of MT_2 in phase-shift presented several pitfalls. For instance, the experiments *in vivo* had been performed with relatively low doses of MLT receptor ligands (Dubocovich et al., 1998), and those using SCN slices had inevitably eliminated the whole brain circuitry (Hunt et al., 2001). Therefore, the single role of the MT_2 in the regulation of circadian rhythms remains controversial. In fact, *in vivo* studies have demonstrated that MLT induces phase shifts in WT and MT_2KO mice (Hudson et al., 2005), but not in MT_1KO mice (Dubocovich et al., 2005), whereas circadian rhythms appear to be unaltered in MT_2KO mice (Jin et al., 2003), suggesting that MT_1 but not MT_2 is the receptor subtype involved in circadian rhythms modulation. Nevertheless, the limited number of studies in MT_1KO and MT_2KO mice along with the lack of selective MT_1 or MT_2 agonists does not allow to elucidate the single role of MLT receptors in the sleep-wake cycle and other pathophysiological conditions such as anxiety, mood disorders and neurodegenerative diseases.

MLT receptors also respond to external/internal cues and follow a circadian rhythm with positive expression during the light phase, associated with low concentrations of MLT, but a significant desensitization during the dark phase, associated with high concentrations of MLT (Witt-Enderby et al., 2003). In contrast to the human MT_1 receptor, the human MT_2 receptor has a lower affinity for 2-iodo-melatonin ([^{125}I]Mel) (von Gall et al., 2002). *In vitro* and *in vivo* studies showed the expression of the MT_2 receptor protein in the SCN to be below the detectable level by 2-[^{125}I]-Mel binding (Dubocovich et al., 1998; Liu et al., 1997). In addition, the rat SCN MT_2 receptor, unlike MT_1, desensitizes after exposure to the full agonist MLT, probably due to an internalization mechanism at the beginning of the night, similar to the majority of GPCRs, suggesting a temporal specificity of MLT in modulating circadian rhythms (Witt-Enderby et al., 2003; Gerdin et al., 2004), but it is not clear whether this occurs after short- (minutes) or long-term (5-8 h) exposure to MLT *in vivo* (Dubocovich and Markowska., 2005). Interestingly, such MT_2 receptor desensitization might inhibit or prevent a desired circadian phase shift and other

pharmacological effects by exogenous MLT or analogs to treat sleep and mood disorders (Gerdin et al., 2004).

Besides MT_1 and MT_2 receptors, other binding sites for MLT have been described. For instance, a low-affinity MLT binding protein, named MT_3, is a non-membrane receptor that represents a MLT-sensitive form of the human enzyme quinone reductase 2, known for its detoxifying properties (Nosjean et al., 2000; Hardeland 2009). In addition, binding sites at several transcription factors from the retinoic acid receptor superfamily, at calcium binding proteins and in mitochondria bind to MLT. However, much less is known about these MLT binding sites and further experiments are needed to study the signalling pathway and assess the effects of selective ligands for these binding sites in order to fully understand their functions (Hardeland 2008; Hardeland 2009).

1.1.3 Localization of melatonin receptors

Aside from the different downstream mechanisms promoted by MT_1 and MT_2 receptors, other factors such as overlapped or different localization in the brain may explain the wide range of physiological actions associated with MLT (Morgan et al., 1994). In this context, identification, characterization and localization of MLT binding sites in the brain and in peripheral tissues have been done by quantitative autoradiography *in vitro* using the non-selective agonist [^{125}I]Mel, a high-affinity radioligand (Dubocovich and Takahashi 1987). Moreover, the real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) has also allowed us to detect the levels of RNA expression in different tissues.

In rodents, for instance, RT-PCR has detected MT_1 and MT_2 receptor mRNAs in the hippocampus, hypothalamus, vestibular nuclei, retina and pineal gland (Musshoff et al., 2002; Sallinen et al., 2005; Yerer el al., 2010; Ahn et al., 2012). Similarly, autoradiography with [^{125}I]Mel and *in situ* hybridization histochemistry have detected the expression of MT_1 and MT_2 receptors in the SCN (Hunt et al., 2001). In humans, both RT-PCR and autoradiography with [^{125}I]Mel have detected the expression of MT_1 and MT_2 receptors in the cerebellum, hypothalamus, thalamus, cortex and hippocampus (Mazzucchelli et al., 1996; Al-Ghoul et al., 1998). Also, in humans, experiments using polyclonal specific antibodies have recently localized MT_2 receptors at the level of the SCN, supraoptic nucleus and paraventricular nucleus. In

particular, these receptors were confined to neurons and nerve fibres but not to glial cells (Wu et al., 2013). Furthermore, immunohistochemical experiments using peroxidase-staining have localized MT_1 and MT_2 receptors in pinealocytes throughout the human pineal gland as well as in the pyramidal/non-pyramidal neurons of the occipital cortex (Brunner et al., 2006).

Interestingly, the localization of MT_1 and MT_2 receptors overlaps in some, but not all regions. For instance, in the hippocampus, MT_1 receptors were expressed in CA1, while MT_2 receptors were localized in CA3 and CA4 pyramidal neurons, which received glutamatergic excitatory inputs from the entorhinal cortex (Ekmekcioglu 2006). In addition, our preliminary data regarding the localization of MLT receptors in rat brain suggests that MT_1 receptors are present in the dorsal raphe (unpublished results) whereas no MT_2 labeling was observed in the raphe nuclei (Ochoa-Sanchez et al., 2011). Therefore, the different localization of MT_1 and MT_2 receptors in the brain may play different and complementary physiological roles for each receptor subtype.

Despite all these findings, the distribution of MLT receptors in the mammalian brain has not been completely elucidated; consequently, further advances in the characterization of MLT receptors, including development of novel selective antibodies for MT_2 receptors will contribute to understanding the mechanism of action of MLT in the mammalian brain.

1.1.4 Sleep

During sleep, consciousness and motor activity can be either totally or partially inhibited, and voluntary movements are inactive. It is well known that the quantity and quality of sleep is associated with the quality and effectiveness of our physical and mental activities. However, what sleep is, why we need it and what the real function of sleep is, is still a matter of debate (Siegel 2003). One of the main hypotheses suggests that sleep allows for the reparation of cells that have been damaged by oxidative stress, via elimination of free radicals - extremely reactive chemicals that result from metabolism. Thus, high metabolic rates lead to increased injury in cells, in their nucleic acids and in their proteins (Siegel 2003). In concordance, high rates of metabolism and production of free radicals occur during the day, although they lower at night when we sleep (Katayose et al., 2009). In fact, it has been proven that sleep activates the genes implicated in cellular repair (Bellesi et al., 2013). There is a correlation between an animal's size

and the quantity of sleep needed to cell repair- small animals with a fast metabolism need to sleep more than bigger animals with a slower metabolism (Siegel 2003).

Another implication of sleep includes restoring/refilling energy (Adenosine triphosphate, ATP) used during wakefulness up to the former/optimal level. Consequently, lower levels of ATP tell the body that it is time to sleep (Dworak et al., 2010). The other function associated with sleep is reinforcing newly acquired information in to memory (Diekelmann and Born 2010; Wilhelm et al., 2013). For instance, the consolidation process of new information occurs more effectively during sleep (Diekelmann and Born 2010). In fact, it has been proven that sleep promotes or weakens new synapses generated by the process of learning, mainly during the awake stages. In other words, sleep improves learning by facilitation of relevant synapses and inhibition of superfluous connections (Frank 2012).

In mammals, normal sleep is characterized by an orderly progression from wakefulness to non-rapid eye movement sleep (NREMS) and to rapid eye movement sleep (REMS). Then, in humans, NREMS and REMS cycle throughout the night in an ultradian pattern every 90 minutes. The electroencephalogram (EEG) is the most common tool to study sleep; it is an indicator of brain state where wakefulness, NREMS and REMS show specific electrical activity features. Additionally, the power spectrum, a quantitative analysis of EEG signals, is an important tool to study sleep in more detail. The power spectrum provides information regarding specific frequency features such as slow delta (1-4 Hz) or theta (4-9 Hz) brain waves (Achermann 2009). Wakefulness is characterized by low voltage, fast EEG activity and high muscle tone with phasic electromyogram (EMG) activity. Once in NREMS, the EEG activity begins to decrease in frequency (slow delta waves), increase in voltage, and muscle tone is reduced. In addition, NREMS is characterized by the presence of k-complexes (large electrical sharp EEG waves) and sleep spindles (short-synchronized 7-14 Hz EEG oscillations) (Walker and Stickgold 2004). During REMS, or paradoxical sleep, the voltage decreases, the EEG activity becomes faster with pronounced theta activity and muscle tone is totally inhibited (Walker and Stickgold 2004).

NREMS can be divided in 4 stages. Stage 1 is the initiation of sleep and the transition from wakefulness to sleep that lasts about 5-10 min, it is considered light sleep, characterized by theta waves with high amplitude. Stage 2 is an intermediate sleep that initially lasts approximately 20 min; is characterized by the presence of sleep spindles and K-complexes while

the body temperature and heart rate begin to decrease. Stages 3 and 4 are similar and are considered deep sleep; they are characterized by high amplitude slow waves (delta waves) and a deeper level of uncousiousness (Bryant et al., 2004).

The deep stages of NREMS (stages 3 and 4) are also known as slow wave sleep (SWS), and are thought to be the most "restorative" sleep stage (Walker and Stickgold 2004). For instance, NREMS is characterized by a lower metabolic rate, an effect implicated in cell damage control and protection of functions associated with sleep (Siegel 2003). Additionally, NREMS is responsible for other physiological processes associated with sleep including memory consolidation (Stickgold 2005; Marshall et al., 2006; Diekelmann and Born 2010; Born 2010), metabolic regulation (Madsen 1991; Maquet et al., 1992; Tasali et al., 2008), and drop in blood pressure (Sayk et al., 2010).

1.1.5 Neurotransmitters and melatonin in sleep

Electrophysiological, genetic, neurochemical and neuropharmacological experiments have demonstrated that NREMS, REMS and wakefulness are regulated by neurotransmitters such as 5-HT (Monti 2011), noradrenaline (NE) (Mitchell and Weinshenker 2010), GABA, histamine, dopamine (DA), glutamate (Watson el at., 2010) and acetylcholine (ACh) (Lee et al., 2005).

5-HT is synthesized mostly in the raphe nuclei, a brain area associated with sleep regulation and considered the main reservoir of 5-HT, in the reticular formation. Notably, during wakefulness, the firing rate of the dorsal raphe neurons is tonic but higher than during NREMS, whereas these neurons become virtually silent during REMS (REMS-off cells) (Urbain et al., 2006). This result is consistent with cortical arousal (increase in wave frequency) regulated by higher levels of extracellular 5-HT in cortical and subcortical areas receiving projections from the raphe nuclei during the awake state (Portas et al., 2000). Similarly, the firing rate of NE neurons in the locus coeruleus (LC), the main reservoir of NE, is higher during the awake state, decreases during NREMS, and becomes silent during REMS. In addition, NE-LC neurons fired before the onset of cortical activation during the transition from NREMS to waking, play a significant role in the sleep/wake switch (Takahashi et al., 2010; Gottesmann 2011)(see figure 1).

Accordingly, drugs that inhibit the re-uptake of 5-HT and NE, such as tricyclic antidepressants modify the sleep behavior (Mayers and Baldwin, 2005; Weber et al., 2012).

GABA, the major inhibitory neurotransmitter of the CNS, plays a key role in the neuronal mechanism that regulates sleep. Spatially diverse GABAergic populations are known to be involved in sleep regulation, such as those of the ventro lateral preoptic area (VLPO) in the thalamus and brainstem necessary for normal sleep. VLPO neurons secrete GABA and their firing rate is higher during NREMS than during wake state. VLPO neurons inhibit the arousal system and vice verse functioning like a on-off switch associated with the homeostasis of sleep and wakefulness (Saper et al., 2005; Schwartz and Roth 2008). In particular, VLPO neurons inhibit the firing rate of NE neurons of the LC, 5-HT neurons of the dorsal raphe, and histaminergic neurons of the tuberomammilary nucleus (TMN), therefore activation of VLMO neurons reduces the excitability of the wake/arousal centres (Saper et al., 2005; see figure 1).

In particular, the $GABA_A$ receptor has been implicated in the regulation of sleep and anxiety. Generally speaking, drugs that act as agonists for the $GABA_A$ receptor, such as benzodiazepines (BZs, e.g. diazepam) and non-BZs, induce anxiolytic, sedative and hypnotic effects. These agonists act in the VLPO by enhancing the actions of GABA at the receptor to silence the arousal system (Saper et al., 2005).

The GABAergic cells in the brainstem are involved in the mechanism of REMS generation. During NREMS and wakefulness, GABAergic neurons (REMS-off cells) from the brainstem ventrolateral periaqueductal gray (vIPAG) inhibit glutamatergic neurons (REMS-on cells) in the pontine sublaterodorsal tegmental nucleus during NREMS and wakefulness. The inhibition of GABAergic neurons in the vIPAG can then promote the onset and maintenance of REMS (Luppi et al., 2011). Nevertheless, the physiological and pharmacological effects of GABA are present in almost all structures of the nervous system, including other brain regions such as the dorsal raphe, LC and pountine reticular formation. For instance, in rats, the local administration of GABA into the dorsal raphe decreases the firing rate of 5-HT neurons, whereas this effect is inhibited by the pharmacological blockage of $GABA_A$ receptors (Gallager and Aghajanian, 1976). It has been proposed that the GABAergic input to 5-HT neurons regulates the firing rate in the dorsal raphe inducing wakefulness, NREMS or REMS (Gervasoni et al., 2000). Similarly, the GABAergic input has been also associated with the regulation of the firing rate of

LC neurons. For instance, intracellular recordings in rat tissue show that the administration of GABA decreases the firing rate of LC neurons, while this effect was blocked by the $GABA_A$ antagonist bicuculline (Osmanović and Shefner 1990). Moreover, experiments in unanaesthetized rats showed that the microinfusion of bicuculline into the LC during NREMS or REMS restore a tonic firing rate of NE-LC neurons suggesting a GABAergic inhibitory tone that modulates the vigilance states (Gervasoni et al., 1998). Meanwhile, in the pontine reticular formation endogenous GABA decreases NREMS and promotes wakefulness. In concordance, the pharmacological blockage of $GABA_A$ receptors increase sleep, mainly REMS (see Vanini el al., 2011).

DA is also involved in the sleep-wake cycle, in particular in regulating wakefulness. For instance, experiments in DA-transporter KO mice showed increased wakefulness and decreased NREMS. The administration of D_1 agonists increase wakefulness and reduce sleep, while opposite effects were found with the administration of D_1 antagonists. In this case, the firing rate of DA neurons in the ventral tegmental area (VTA) and substantia nigra pars compact (SNc), the main two areas that regulate DA and arousal states, do not change across the sleep-wake cycle as observed with 5-HT neurons in the dorsal raphe and NE neurons in the LC. Instead, DA neurons in the VTA show a temporal pattern with increased bursts of activity during wakefulness and REMS, an effect that increases DA (Monti and Jantos 2008).

Regarding ACh, a neurotransmitter also involved in REMS and wakefulness regulation, the cholinergic neurons from the reticular formation in the brain stem induce an arousal state via stimulation of cells in the basal forebrain. The levels of cortical ACh released increase during REMS and wakefulness/alertness, whereas they decrease during NREMS (Vazquez and Baghdoyan 2001). In concordance, the maximal firing rate, burst and rhythmic theta discharge of cholinergic neurons are observed during REMS and wakefulness whereas the firing rate is decreased during NREMS (Lee et al., 2005).

Recently, the orexin system (also know as hypocretins) has been implicated in the regulation of the sleep-wake cycle, in particular in the mechanism that maintains wakefulness and controls the "flip-flop" switch that stabilizes the transition between sleep and wakefulness. The orexin system activates monoaminergic and cholinergic neurons in the hypothalamus and brainstem regions to maintain wakefulness whereas during NREMS these orexin neurons are inhibited by VLPO neurons which release the inhibitor neurotransmitter GABA (Slats et al., 2013). In particular, orexin neurons projects to the dorsal raphe, LC and TMN, all regions involved in arousal states (Inutsuka and Yamanaka 2013)(figure 1). In fact, the deficiency of orexin has been linked to fragmented wakefulness and narcolepsy in humans and rodents (Tsujino and Sakurai 2009). Additionally, sleep is modulated by a distinct secretion pattern of hormones, including growth hormone (GH) and cortisol. For instance, peptide growth hormone-releasing hormone and the GH secretions are positively correlated with NREMS, whereas corticotropin-releasing hormone and cortisol induce opposite effects (Van Cauter et al., 2000).

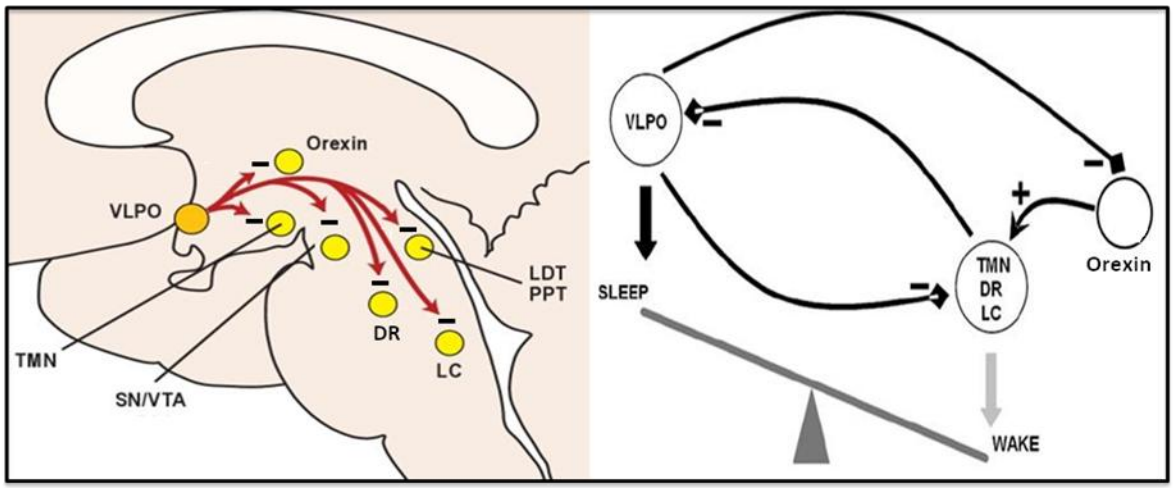

Figure 1. Left, Schematic anatomic of the sleep prompting system (NREMS), ventrolateral preoptic (VLPO) neurons inhibit cell groups of the arousal system including tuberomammillary nucleus (TMN), locus coeruleus (LC), substantia nigra (SN), ventral tegmental area (VTA), dorsal raphe (DR), latero dorsal tegmental nuclei (LDT), pedunculopontine (PPT) and perifornical orexin neurons. Right, the sleep and wakefulness circuits have reciprocal inhibitory connections, "flip-flop" switching system. During wakefulness, orexin neurons project to cell groups of the arousal system (activation) whereas during sleep, orexin neurons are inhibited by VLPO neurons, on which they have no influence. Excitatory and inhibitory signs are indicated by + and –, respectively (modified from Saper et al., 2005; Slats et al., 2013).

Interestingly, the secretion and actions of MLT in the brain have been associated with changes in the activity of the previously mentioned brain regions and neurotransmitters and there is evidence for the interaction of MLT and these neurotransmitters in sleep-wake regulation and other physiological conditions. For instance, the pharmacological blockage of BZ-$GABA_A$ receptors by the antagonist flumazenil prevents the sleep inducing effects of MLT (Wang et al., 2003a), suggesting that MLT may exert its effects through the BZ-$GABA_A$ receptors. In addition, the pre-treatment with flumazenil or picrotoxin, channel blockers for the $GABA_A$ receptor, inhibits the anxiolytic and anti-oxidant effects of MLT in sleep deprived mice (Kumar and Singh 2009). In pinealectomized rats, the circadian rhythm of GABA and BZ binding is disrupted, whereas the binding of GABA and BZ is increased after chronic treatment with MLT (Rosenstein and Cardinali 1990). Microdialysis studies in rats have shown that the variation in the levels of GABA between night (high) and day (low) is linked to endogenous MLT secretion (Marquez de Prado et al., 2000). Together, these findings demonstrate that the GABAergic system constitutes a target for MLT actions.

In relation to 5-HT, MLT is able to stimulate 5-HT metabolism in hypothalamic areas. For instance, the pinealectomy in rats decreases the levels of 5-HT content in anterior and ventromedial hypothalamic nucleus whereas the chronic treatment with MLT reverses such an effect. In contrast, in the SCN, the pinealectomy increases the synthesis of 5-HT and the chronic treatment with MLT does not reverses that effect, thereby suggesting that the effects of MLT on the 5-HT system are region specific (Miguez et al., 1996). The interaction with the 5-HT system also depends on the light-dark cycle. MLT decreases the firing rate of the dorsal raphe 5-HT neurons during the light phase, whereas pinealectomy increases the firing activity during the dark phase (Dominguez-Lopez et al., 2012). Besides, 5-HT immunoreactivity is decreased during the dark phase when levels of MLT are elevated (Birkett and Fite, 2005).

NE has also been involved in arousal state mechanisms related to MLT. MLT affects the NE system and the activity of NE, in turn, regulates the synthesis of MLT. More precisely, NE influences the synthesis and release of MLT via the activation of β-adrenergic receptors in the pineal gland, whereas the administration of a β-adrenergic antagonist suppresses MLT synthesis promoting wakefulness (Stoschitzky et al., 1999). One of the brain regions involved in the sleep/wake switch effect of NE is the SCN (Takahashi et al., 2010). Interestingly, MLT and non-

selective MT_1/MT_2 agonists decrease the firing rate of SCN neurons that suppress wake drive and increase sleep. Nevertheless, the effects of NE on the sleep-wake cycle might be due to NE activation per se, although there is no evidence regarding the role of MLT on the activity of LC and sleep (Mitchell and Weinshenker 2010).

DA is also involved in the synthesis and release of MLT. DA receptors in the pineal gland interact with NE receptors to form heteromers. Furthermore, the activation of DA receptors by DA inhibits the effects of NE on the pineal gland, decreasing the synthesis of MLT, an effect associated with wakefulness in the early morning. In concordance, the expression of DA receptors is higher at the end of the night, leading to adrenergic and MLT synthesis inhibition, which prepares the body to awake in the morning (González et al., 2012). Regarding the effects of MLT on DA neurotransmission, MLT inhibits DA release in brain regions involved in sleep such as the hypothalamus, the hippocampus, the medulla-pons, and the striatum (Zisapel 2001).

MLT also modifies cholinergic neurotransmission and interacts with ACh interneurons from different brain regions related to sleep-wake regulation including the nucleus accumbens (Qiu et al., 2012), where MLT increases ACh release via MLT receptors and results in the activation of the nucleus accumbens (Paredes et al., 1999). Consequently, such activation may promote an inhibition of arousal systems, an effect associated with sleep induction (Qiu et al., 2012). Regarding the orexin system, experiments in zebrafish have showed that the orexin system modulates MLT synthesis and sleep consolidation. For instance, the synthesis of arylalkylamine-*N*-acetyltransferase, a key enzyme of MLT synthesis, is reduced in the zebrafish hypocretin/orexin receptor mutant (hcrtr-/-) pineal gland during the dark phase, whereas the administration of hypocretin/orexin perfusion of cultured zebrafish pineal glands induces MLT release (Appelbaum et al., 2009).

1.1.6 The reticular thalamic nucleus and NREMS

Even though the neuronal mechanism and function of different brain structures in sleep are not completely understood, we know that one of the brain areas involved in the regulation of NREMS, as well as in the physiological processes linked to this stage of sleep, is the reticular thalamic (Rt) nucleus (Steriade 1999). The Rt nucleus is a thin layer that contains GABAergic

cells that encapsulate most of the anterior and lateral thalamus. It has been proven that Rt neurons express rhythmic patterns of activity alternated with shifts in sleep/wake state (Marks and Roffwarg 1993). During NREMS, Rt neurons display spike-burst activity in long lasting trains followed by pauses or tonic activity. Interestingly, the Rt nucleus is the main pacemaker that generates EEG spindle activities, a landmark of sleep onset. In fact, there is a positive correlation between Rt spike-burst activities with frequency oscillations (7-14 Hz) corresponding to the spindle activity range in the EEG signal, whereas the tonic activity or pauses are associated with low frequency oscillations (0.3-0.2 Hz).

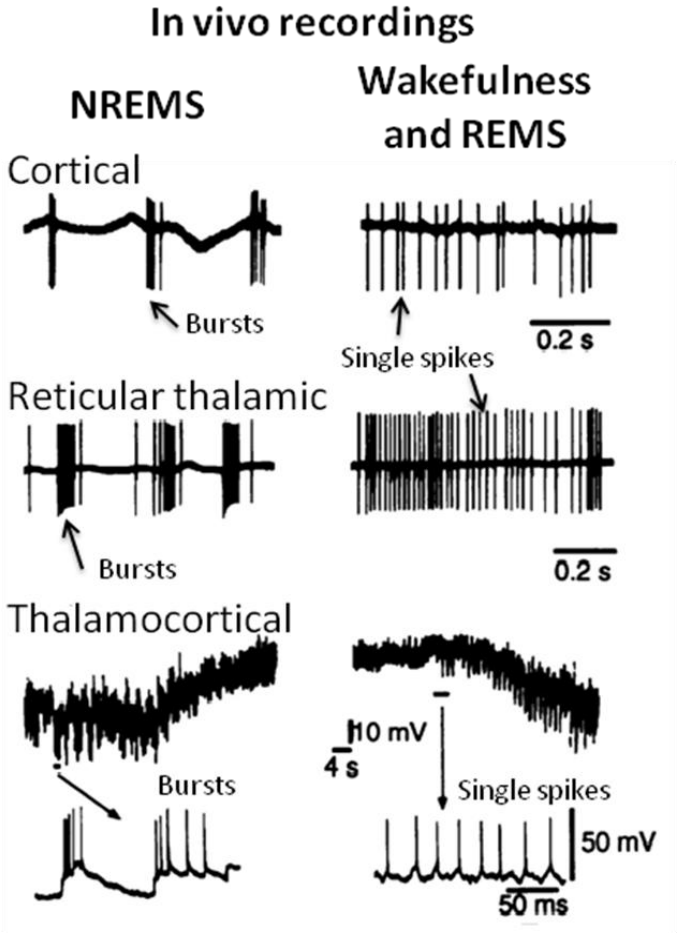

Figure 2. Changes in the activity patterns generated by cortical neurons, reticular thalamic nucleus and thalamic relay neurons. These neurons change their activity from rhythmic spike bursts during natural NREMS to firing of single spikes during wakefulness and REMS (Modified from Steriade et al., 1993b).

In addition, the pattern activity of Rt neurons showed burst activity with no pauses during REMS, whereas during wakefulness, the pattern displayed a continuous single-spike mode (Marks and Roffwarg 1993)(see figure 2). The Rt nucleus plays an important role in the communication between the thalamus and the cortex, regulating the interaction of thalamocortical and corticothalamic circuits (Gilleri et al., 1998). Rt neurons receive projections from collaterals of both corticothalamic and thalamocortical (interconnected) neurons (Lam et al., 2006). These connections provide a glutamatergic, excitatory input to Rt neurons. Rt neurons are characterized by an intrinsic low-threshold spike that is mainly localized in the dendrites of these neurons. During sleep, fast cortical discharges from corticothalamic neurons, produce the high frequency spike burst pattern in Rt neurons. Rt neurons, in turn, innervates thalamocortical neurons (TC) and release the inhibitory neurotransmitter GABA which produces a long-lasting inhibition on TC neurons by acting in $GABA_A$ and $GABA_B$ receptors in TC neurons in the dorsal thalamus (Steriade 2005). As a result, the thalamocortical transmission is modified and such an effect is responsible for sleep rhythms, spindle generation and synaptic plasticity during NREMS (Steriade and Timofeev 2003), as well as the inhibition of external signals leading to unconsciousness (White and Alkire 2003).

Other than its role in sleep regulation, the Rt nucleus participates in the mechanism of action of hypnotic drugs. In particular, BZs analogs, eszopiclone and zolpidem, modulate the synaptic $GABA_A$ receptors expressed in Rt neurons, an effect associated with hypnotic effects (Jia et al., 2009). In agreement, the bilateral lesion of the Rt nucleus in freely-moving rats increases the latency to sleep onset, diminishes the total time of NREMS, and gradually eliminates delta rhythm (Marini et al., 2000). In addition, a lesion of the Rt nucleus has been shown to impair the memory processes associated with NREMS in rats, such as spatial memory in the radial and T mazes (M'Harzi et al.1991; Collery et al., 1992).

1.1.7 Insomnia

Several psychological and physiological factors can dramatically influence the balance of the sleep-wake pattern, thereby resulting in sleep impairments commonly called insomnia (Basta et al., 2007). Insomnia is a very common disorder experienced by almost everybody, with a higher risk rate at older ages, and becomes chronic in roughly 10% of the population. Insomnia is defined by any of the following symptoms: difficulty falling asleep, difficulty maintaining sleep,

waking up too early and not being able to go back to sleep, and waking up feeling unrefreshed (Gershell 2006; DSM-5). Insomnia is associated with significant occupational, social and behavioural impairment, as well as other areas of functioning (DSM-5). The main causes leading to insomnia are psychiatric disorders, including mood disorders, major depression disorder, bipolar disorder, substance abuse disorders and most anxiety disorders. In addition, the prevalence of insomnia is also associated with factors such as stressful events, advanced age related sleep homeostasis impairments and bad sleep habits (Ohayon 2002; Basta et al., 2007).

Insomnia is a complex disorder characterized by the imbalance of different neurotransmitters (Thase 1998). For instance, the levels of 5-HT, a neurotransmitter widely implicated in sleep regulation, are lower in patients with insomnia, whereas elevating 5-HT levels back to normal restore proper sleep (Vashadze 2007). Moreover, the 5-HT transporter length polymorphism has been associated with insomnia (Deuschle et al., 2010). Studies in ACh neurons have shown the activity of pontine cholinergic cells to be increased in insomnia. Studies in NE neurons revealed an overactivity of the NE system in sleep disorders (Thase 1998). Thus, such overactivity of the NE system induces not only a sympathotonic state, but also hyperarousal accompanied by insomnia, likely by inappropriate LC activity (Berridge and Waterhouse 2003).

Furthermore, sleep disorders and insomnia are characterized by changes in the secretion pattern of different hormones. For instance, mood disorders that promote insomnia, such as depression and anxiety, decrease the secretion of GH with a concomitant reduction of NREMS, whereas the activity of the hypothalamo-pituitary-adrenocortical (HPA) axis and levels of cortisol are increased along with an increment in wakefulness (Thase 1998).

BZs are one of the most prescribed classes of pharmacological sleep aids. BZs act on the GABAergic system with one of their most important actions being sedation (Lancel 1999). Several reports have shown that BZs improve insomnia by reducing the latency to sleep, the amount of REMS and the nocturnal awakening episodes (Woodward 1999). In particular, BZs are prescribed for insomnias related to acute and transient external stress. However, when used chronically, in cases of long-term insomnia, the treatment with BZs can lead to tolerance, dependency, next-day cognitive impairments and abuse liability (Wiegand 2008). Additionally, the quality and the physiological sleep architecture are also considerably affected by BZs (Lancel 1999). Subsequently, these alterations in sleep architecture contribute to other side effects that

affect daily performance like mood impairment, weakness, tiredness, cognitive functioning and, memory impairments, notably associated with significant social and financial cost (Durmer and Dinges 2005).

Antidepressants constitute another pharmacological option used to treat sleep disorders and insomnia. Antidepressants are usually prescribed for the short-term treatment of insomnia and, the best results are obtained in patients with symptoms of depression (Wiegand 2008). In particular, tricyclic antidepressants, 5-HT antagonists, and reuptake inhibitors, have been shown to have a sedative effect accompanied by an improvement in the quantity and quality of sleep (Haria et al 1994; Mayers and Baldwin 2005). For instance, lower doses of doxepin, a tricyclic antidepressant recently approved by the US Food and Drug Administration (FDA) for the treatment of depression, anxiety disorders and insomnia, facilitates the initiation and maintenance of sleep in patients with insomnia. Besides blocking the 5-HT transporter, the sleep-enhancing effects of doxepin are associated with its selective antagonistic actions on the histamine H_1 receptor (Weber et al., 2012). Nevertheless, the effects on sleep depend on the class of antidepressant used; in fact, other types of antidepressants show little or no effect on NREMS along with diverse impairments in the quality of sleep and REMS density (Mayers and Baldwin 2005). In particular, the early therapy with selective 5-HT re-uptake inhibitors (SSRIs) such as fluoxetine has been related to problems falling sleep, suppressed REMS and an increased number of episodes of wakefulness that therefore reduce the quality of sleep (Wilson and Argyropoulos 2005). Thus, the therapeutic effects and advantages of antidepressants to treat insomnia in comparison to BZs are still being debated (Wiegand 2008). In this context, developing new effective hypnotic drugs that selectively increase NREMS without altering the whole sleep architecture remains a scientific and medical challenge; therefore, there is a growing interest in exploring novel pharmacotherapeutic targets for the treatment of insomnia and sleep disorders. Among these emerging targets, the melatonergic system has gained considerable attention (Ochoa-Sanchez et al., 2011).

1.1.8 MLT and non-selective MT_1/MT_2 agonists as treatment for insomnia

Notably, the circadian production of MLT has been associated with the regulation of natural sleep patterns and it is considered as sleep facilitator (Cajochen et al., 2003). In fact, alterations in the production of MLT have been involved in the etiology of sleep disorders (De

Leersnyder et al., 2003), consequently, it has been proposed that the use of MLT or MLT analogs may be useful in the treatment of insomnia and sleep disorders.

Basically, two major effects of MLT have been reported; sleep induction and phase-shifting the circadian clock (Cajochen et al., 2003). Exogenous MLT phase shifts the circadian clock not only in rodents (Redman et al., 1983), but also in humans. For instance, in humans, exogenous oral MLT for 3 days administered at different times of the day, morning or afternoon, phase shifts the cycle with a maximal advance effect when it is taken in the afternoon (reset the circadian clock to an earlier time) and maximal delay when it is taken in the morning (reset the circadian clock to a later time) (Burgess et al., 2010).

In addition, there is a large body of research demonstrating the hypnotic effects of MLT in clinical studies (Zhdanova et al., 1995; Nave et al., 1996; Zhdanova 2005; Brzezinski et al., 2005; Buscemi et al., 2006; Luthringer et al., 2009). However, the clinical efficacy is still unclear (Mendelson 1997) and some studies have failed to find significant effects (James et al., 1987; Singer et al., 2003). Similarly, different studies in animal models have shown little or no effect in sleep maintenance. For instance, in cats, MLT (0.01-1 mg/kg) increases NREMS, an effect detected only during the first 2 h (Miyamoto et al., 2004). In rats, MLT reduces the time to sleep onset and increases both NREMS and REMS (Holmes and Sugden 1982; Wang et al., 2003b) while these mentioned effects are blocked by the $GABA_A$ antagonists flumazenil and picrotoxin (Wang et al., 2003a). Others suggest that MLT is only involved in the control of REMS since the lesion of the pineal gland or inhibition of MLT synthesis using a beta-adrenergic antagonist (propanolol), decreases REMS during light and dark phases (Mouret et al., 1974; Mendelson et al., 1980). In addition, MLT (2.5 and 5 mg/kg) increased the number of sleep cycles and the total duration of REMS (Maillet et al., 2001). On the other hand, also in rats, MLT was not effective in modifying sleep and circadian rest-activity rhythm (Tobler et al., 1994). Besides, MLT at a dose of 0.3 mg/kg, but not at 1 and 3 mg/kg, decreases sleep latency while having no effects on NREMS and REMS durations in monkeys (Yukuhiro et al., 2004).

Factors that limit the effectiveness and use of MLT in sleep disorders and other medical conditions are the short half-life (< 1 h), the high first-pass metabolism and the binding of multiple receptors (lack of selectivity). In addition, the effects of MLT depend on the time of administration and the phase of the day (Turek and Gillette 2004). For example, the hypnotic

effect of MLT in humans varies depending on the time of administration, consistent with the circadian phase-resetting properties of MLT. The tendency to sleep is more evident when MLT is administered at noon than when it is administered at night, thereby suggesting a time-dependant hypnotic effect and suggests that endogenous MLT participates in such effects (Tzischinsky and Lavie 1994). In addition, as mentioned above, the time of administration of MLT can either advance or delay the circadian clock; for instance, the maximal phase shift of exogenous MLT occurs when exogenous levels of MLT are low, and phase shift is reduced when endogenous MLT rises (Burgess et al., 2010). Two different solutions to this problem have been proposed: developing prolonged-release formulations of the natural hormone and developing melatonergic drugs with a longer half-life.

Recently, circadin, a prolonged release MLT tablet, was approved in Europe for the treatment of insomnia in elderly patients (> 55 years old). Indeed, prolonged release MLT (2 mg) in elderly patients with insomnia improves sleep quality, without withdrawal and rebound symptoms upon discontinuation as is seen with BZs (Hardeland 2009; Lemoine et al., 2011). However, circadin's efficacy was significantly lower than that shown by regular hypnotics and side effects, such as somnolence, have been reported (Hardeland 2009). In relation to MLT ligands, many other synthetic non-selective MLT agonists with different affinities toward MT_1 and MT_2 receptors and longer half-lives than MLT have been developed. For instance, the non-selective MT_1/MT_2 receptor agonist ramelteon (TAK-375, Takeda, Japan), was approved by the FDA for the treatment of insomnia characterized by sleep onset impairment (McGechan 2005; Mini et al., 2007). Meanwhile, the European Medicines Agency (EMEA) did not approve this compound for insomnia due to limited evidence of therapeutic effects. Ramelteon has been shown to reduce the latency to sleep and increases the total sleep time in patients with insomnia (Roth et al., 2005a). Nevertheless, in spite of the long-acting agonistic effects of ramelteon, the long-term enhancement of total sleep time is very mild (Mini et al., 2007). Similarly, the novel antidepressant agomelatine (Servier laboratories), an MT_1/MT_2 agonist and $5\text{-}HT_{2C}$ antagonist (Millan et al., 2005; de Bodinat et al., 2010), improves sleep efficiency and increases NREMS in patients with depression (Quera-Salva et al., 2010). In brief, the use of MLT and non-selective MT_1/MT_2 agonists clearly reduce the latency to sleep but the effects on sleep maintenance are mild or controversial.

Since MLT and non-selective MLT ligands display high affinity for both MT_1 and MT_2 receptors, it is difficult to determine the single role of either MT_1 or MT_2 receptor subtypes in sleep regulation and other physiological actions. Therefore, the functional characterization of MT_1 and MT_2 receptors in target tissues is still controversial and hampered by paucity of selective MT_1 or MT_2 receptor agonists.

Fortunately, over the last few years, structural determinants that confer MT_1 or MT_2 binding selectivity have been elucidated, and today we have selective ligands (Mor et al., 2010; Rivara et al., 2012). In particular, selective MT_2 receptor ligands were designed and synthesized, allowing researchers to explore the single role of MT_2 receptors in brain functions. Indeed, since structure-activity relationships for the binding at the MT_2 receptor are quite consolidated (Rivara et al., 2007), several MT_2 receptor ligands belonging to different chemical classes have been developed. For instance, the MT_2 full agonist IIK7 (Fisher and Sugden 2009) and the MT_2 antagonist 4-phenyl-2-propionamidotetralin (4P-PDOT)(Dubocovich et al., 1998) have been tested through preclinical psychopharmacology tests and in neurobiological studies aimed at dissecting the role of MT_2 receptors in brain function. In this dissertation, we tested new compounds that belong to a novel series of selective MT_2 receptor agonists, see section 1.1.12. On the other hand, the synthesis of selective MT_1 receptor ligands is still a challenge and to the best of our knowledge, only one MT_1 selective agonist (Rivara et al., 2012) has been synthesized with the first experiments in process in our lab.

1.1.9 Anxiety and current treatments for anxiety

Anxiety is a normal emotion that constitutes an adaptive response to potential danger, a threat to the psychological or the physiological integrity (Gutierrez-Garcia and Contreras 2013). Consequently, anxiety prepares the organism to cope with the environment and is typically involved in survival strategies (DSM-IV, 1994). Physiological anxiety is regulated by the limbic system, which is activated under normal circumstances as a protective mechanism in the presence of danger or possible threat. In fact, the limbic system is also known as the brain's anxiety "switch" because it modulates the way we code, remember, and respond emotionally to sensory stimuli. The limbic system is a complex set of interconnected brain regions that includes the hippocampus, the hypothalamus, the amygdala, and cingulate cortex. In particular, the hippocampus inhibits the hypothalamic stress response by sending negative feedback to the HPA

axis. The amygdala, also known as the emotional center, modulates the expression of emotions such as fear, aggression, defensive behaviour (species-specific), and retrieval of emotional fear-related memories. Thus, the amygdala interconnectivity with other brain regions and its role in social behaviour are associated with the regulation of anxiety (Martin et al., 2010).

According to the DSM-IV, anxiety is a physiological and psychological state characterized by an unpleasant emotional state, fear and distressing physical symptoms in response to stressors. Hence, anxiety is a highly disabling condition characterized by emotional, cognitive and psychological impairments (DSM-IV, 1994). Anxiety states may be focused on some particular situation or may be generalized, known as general anxiety disorder (GAD), affecting the person's life (Gutierrez-Garcia and Contreras 2013), where such responses are activated by a variety of continuous and persistent stimuli (stressors) that may not represent any danger to the individual. For instance, the central feature of GAD is excessive anxiety and worry about a variety of matters, such as finances, health, safety, work, school, relationships and minor issues. Other related symptoms in anxiety include restlessness, irritability, muscle tension and sleep disorders (DSM-IV, 1994). Epidemiological studies show that anxiety disorders, as a group, are among the most common type of psychiatric disorders and represent a public health problem with a lifetime prevalence of 16.6 % (Somers et al., 2006). Economically, anxiety is associated with high medical expenditures, lost productivity, and functional impairment thereby making anxiety disorders very costly (Devane et al., 2005).

Pathological anxiety results in part from neurotransmitter, neuroendocrine, and neuroanatomical disruptions at the level of limbic system and cortical brain regions. In fact, the likelihood of such disruptions can be increased by environmental experiences and underlying genetic predispositions (Martin et al., 2010). In addition, in pathological anxiety, the hippocampal volume and neurogenesis are significantly reduced, and the hippocampal negative feedback on the HPA axis is impaired (Dranovsky and Hen 2006; Revest et al., 2009; Levita et al., 2014). Also, the volume of the amygdala is increased in patients with GAD, thus, the activation of the amygdala is highly increased during negative emotional processing, an effect positively correlated with the severity of anxiety (Etkin et al., 2009). Interestingly enough, this hyperactivation in the amygdala has been negatively correlated with activity in the right ventrolateral prefrontal cortex, a region associated with cognitive control. Therefore, amygdala

hyperactivity and its effects in other regions of the limbic system and cortical regions may promote inaccurate interpretations of social behaviour in patients with anxiety (Guyer et al., 2008). The neurotransmitters involved in anxiety include GABA and 5-HT. For instance, amygdala hyperactivation may result from decreased GABA inhibitory neurotransmission and a down-regulation of $GABA_A$ receptors (Liu et al., 2014). In relation to the 5-HT system, the 5-HT transporter (SERT) density negatively correlates with symptom severity in GAD (Jennings et al., 2006). Consistent with the role of GABA and 5-HT in anxiety, the treatment with a $GABA_A$ agonist or with antidepressants (SSRIs) have been shown to reduce symptoms of anxiety (Whiting, 2006; Martin et al., 2010), in fact, both pharmacological options are among the most clinically prescribed medications for the treatment of anxiety disorders. However, both treatments have been correlated with unwanted side effects. As mentioned above, the side effects of BZs include sedation, tolerance, dependence, next-day impairments, and abuse liability (Whiting, 2006). In spite of the fact that antidepressants may constitute a safer medication than BZs, they can cause agitation, sexual dysfunction, weight gain, sleep disturbance; and the most troubling adverse effects are noticed during long-term antidepressant therapy (Ferguson 2001). Besides, many patients with anxiety disorders fail to adequately respond to existing pharmacological treatments. Therefore, safer and more effective alternative novel treatments, as well as the exploration of novel pharmaco-therapeutic targets, are needed to address anxiety treatment resistance (Stein and Seedat 2004). Among these emerging targets, the melatonergic system has gained considerable attention (Ochoa-Sanchez et al., 2012).

1.1.10 MLT, MLT receptor agonists and anxiety

Melatonin has been related to several functions, including sedative, antioxidant, anxiolytic, antidepressant, anticonvulsant and analgesic properties (Rios et al., 2010). In relation to anxiety and mood disorders, it has been shown that serum MLT levels during the night are lower in patients with major depression than in healthy volunteers (Beck-Friis et al., 1984). Moreover, the elderly are characterized by a decrement in the levels of MLT (Graham and McLachlan 2004) and GAD is also common later life, either as a primary problem or as a symptom of another disorder such as depression or dementia (Lenze et al., 2003). Contrarily, relaxing exercises and meditation, such as yoga, have been shown to increase plasma MLT, and there is a significant correlation between the maximum night time MLT levels and the well-being

score associated with lower stress and anxiety (Harinath et al., 2004). Moreover, relaxing exercises have also been found to reduce stress-induced disorders including insomnia, anxiety and depression (Harinath et al., 2004; McVicar et al., 2007). These findings suggest that levels of MLT are associated with anxiolytic effects.

In agreement, external MLT has elicited anxiolytic effects in both preclinical (Golus and King, 1981; Golombek et al., 1993, Papp et al., 2006; Crupi et al., 2010) and human studies (Srinivasan et al., 2006; Caumo et al., 2009). In particular, in animal models (mainly rodents), MLT induces anxiolytic-like effects in the elevated plus maze test (EPMT) by increasing the time spent in the open arms (Golombek et al., 1993), in the open field test (OFT) by increasing the activity in the central area (Golus and King 1981) and in the novelty suppressed feeding test (NSFT) by decreasing the latency to eat (Crupi et al., 2010). In addition to MLT, the non-selective MT_1/MT_2 receptor agonists Neu-P11, agomelatine and S23478, also displayed anxiolytic-like effects in rodents. Neu-P11 promotes anxiolytic-like effects in EPMT in mice (Tian et al., 2010). S23478 decreases anxious reactions in the free-exploratory and light/dark choice test in mice (Kopp et al., 2000). Agomelatine shows anxiolytic-like effects in the EPMT, conditioned foot-shock-induced ultrasonic vocalization (Papp et al., 2006) and punishing drinking tests in rats (Loiseau et al., 2006).

In humans, MLT has shown anxiolytic effects in patients undergoing an abdominal hysterectomy, helping to reduce morphine consumption for pain (Caumo et al., 2009). In addition, similar to the anxiolytic midazolam (BZs), the treatment with MLT reduced the preoperative anxiety in children but in the case of MLT, no side effects such as postoperative sleep disturbance were observed (Samarkandi et al., 2005). Controlled release MLT has also facilitated the discontinuation of BZs consumption and improves sleep quality in elderly patients with insomnia (Garfinkel et al., 1999). In contrast, it has been shown that MLT does not reduce anxiety more than placebo in elderly population (Capuzzo et al., 2006). Nonetheless, it has been reported that the responsiveness to MLT treatment is reduced in elderly and this effect correlated with down-sensitivity of MLT receptors (Zhdanova 2005).

Besides MLT, non-selective MT_1/MT_2 receptor agonists such as ramelteon and agomelatine, two drugs currently on the market as a hypnotic and an antidepressant, respectively, have also produced anxiolytic effects in humans (den Boer et al., 2006; Stein et al., 2008; Gross

et al., 2009). This information supports the anxiolytic properties of MLT and non-selective MLT agonists whose mechanism of action may be linked to the activation of MT_1 and/or MT_2 receptors. Nevertheless, given the lack of selectivity towards MT_1 or MT_2 receptor, MLT and its analogs do not provide information as to whether only one or both MLT receptor subtypes are involved in such anxiolytic effects. For these reasons, development of new selective MT_1 and MT_2 receptor agonists and antagonists is needed in order to elucidate which MLT receptor subtype is involved in anxiety regulation.

1.1.11 Anxiety-insomnia relationship

Anxiety and sleep disorders, both disabling and chronic, are highly co-morbid conditions that put a significant number of individuals at risk for developing secondary complications such as depressive disorders, suicidal behaviours, heart diseases or painful physical afflictions (e.g. back pain) (Ohayon 2002; Drake et al., 2003). Such co-morbidity suggests a strong relationship between these conditions. Commonly, insomnia precedes, co-occurs with, or follows the onset of a co-morbid anxiety disorder (Johnson et al., 2006). According to the DSM-IV, sleep disturbances are included in the criteria for GAD and most anxiety disorders are moderately associated with sleep disturbances in the general population (DSM-IV 1994). In particular, psychiatric disorders such as depression and anxiety are associated with most cases of insomnia (Ohayon 2002; Basta et al., 2007). People with major depression or anxiety, commonly suffer from insomnia, with alterations in sleep neurophysiology, notably decreased NREMS density, increased NREMS latency, reduced REMS latency and increased REMS density (Thase 1998; Belanger et al., 2004). Epidemiological evidence also suggests that sleep disorders appear to be more frequent among elderly people (Quan et al., 2005), a population characterized by high incidence of anxiety disorders (Salzman and Lebowitz, 1991; Schoevers et al., 2005). Therefore, not only do mood and anxiety disorders affect sleep, but sleep disorders can also affect mood and anxiety (Neckelmann et al., 2007), thus making insomnia a risk factor for mood and anxiety disorders and vice versa (Neckelmann et al., 2007; Deuschle et al., 2010).

The physiological and neurobiological relationship between anxiety and insomnia remain one of medicine's mysteries. One possibility is that insomnia and anxiety originate from different neurobiological abnormalities showing a different course of illness, medical complications and treatment response. Another possibility could be that insomnia and anxiety originate from

independent environmental, neurobiological or genetic factors that independently affect each disorder (Uhde et al., 2009).

The other possibility is that anxiety and insomnia represent the same disorder with a common underlying neurobiological impairment leading to the same type of symptoms, onset of illness, course of illness and response to treatments (Uhde et al., 2009). In this context, sleep disturbances exacerbate anxiety disorders, leading to worse anxiety symptoms and more impaired functioning (Ramsawh et al., 2009), while anxiety interferes with the ability and quality of sleep leading to sleep disorders and insomnia. For instance, people with GAD show increased sleep latency, increased number of wakefulness episodes after sleep onset and have early morning awakenings (Uhde et al., 2009). In agreement with this hypothesis, neurotransmitters such as 5-HT, NE and GABA, are associated with the pathology and pharmacological treatment of anxiety and insomnia (Uhde et al., 2009; Ravindran and Stein 2010). In fact, the pharmacological intervention to relieve sleep disorders also helps to improve the symptoms in anxiety disorders (Staner 2003). For instance, BZs are still considered by many clinicians as a good treatment option for both disorders (Cloos and Ferreira 2009). For example, BZs, like alprazolan, diazepam (DZ) and clonazepam are often prescribed for short-term use in both conditions (Kales et al., 1991; Lader 1999). Normally the promotion of anti-anxiety effects or hypnotic effects depends on the dosage, low doses or higher doses, respectively (Short and Chui 1991; Sun et al., 2008).

As mentioned above, MLT has been associated with the regulation of both anxiety and sleep. In this context, lower levels of MLT have been detected in patients with sleep and mood impairments, mainly in elderly (Beck-Friis et al., 1984; Zhdanova et al., 2001), whereas the treatment with MLT or MLT receptor agonists improve sleep and anxiety disorders in the elderly (Garzon et al., 2009), an effect associated with discontinuation of conventional hypnotic therapy along with a reduction of side effects (Garfinkel et al., 1999; Garzon et al., 2009). For these reasons, we hypothesized that the melatonergic system constitutes a common neurobiological substrate not only for the etiology of both anxiety and sleep disorders, but in the treatment of these medical conditions as well.

1.1.12 Development of novel MT_2-selective receptor agonists

One of the research tools used to study the single role of MT_1 and MT_2 receptors include experiments with selective agonists and antagonists for the MT_1 or MT_2 receptor. Consequently, novel selective MT_1 and selective MT_2 ligands have been developed. In particular, there are several MT_2 selective ligands with different chemical structures, but only a few have been tested in preclinical studies to understand the role of MT_2 receptors in brain function. For instance, the selective MT_2 full agonist IIK7, a 6H-Isoindolo[2,1-a]indoles derivative, exerts hypnotic effects and involves the MT_2 receptor in the regulation of sleep (Fisher and Sudgen 2009). Moreover, selective MT_2 antagonists such as 4P-PDOT (4-phenyl-2-propionamidotetralin), a tetralin derivative and K-185 a 6,7-dihydro-5H-benzo[c]azepino[2,1-a]indoles derivative were used to demonstrate the role of MT_2 receptor in pain modulation (see Srinivasan et al., 2012).

In this thesis, we studied the effects of a novel class of MLT ligands named *N*-(anilinoethyl)amides in sleep and anxiety modulation. Notably, the chemical structure of these ligands can be modulated, thereby improving metabolic stability while maintaining the same receptor profile. For instance, specific structure modifications on the aniline nitrogen, on the benzene ring, and/or on the amide chain allow us to obtain different compounds with different MT_1 and MT_2 binding affinity and different intrinsic activity profiles (Rivara et al., 2009). In particular, one of these synthesized compounds is the MT_2-selective partial agonist UCM765 (*N*-{2-[(3-methoxyphenyl)phenylamino]ethyl}acetamide), which exhibits greater affinity for MT_2 receptors (pKi = 10.18) than for MT_1 receptors (pKi = 8.38), and showing even greater affinity for the MT_2 receptor than MLT itself (pKi = 9.62) (Rivara et al., 2007). Biochemical experiments have shown that UCM765 does not display significant affinity for other receptors, ion channels, and transporters, including those known for their involvement in sleep and mood regulation, such as $GABA_A$, $5\text{-}HT_{2A}$, and histamine receptors (data are from Cerep, Celle L'Evescault, France; data not shown). Based on the stimulation of basal [^{35}S]GTPγS binding, the relative intrinsic activity values of UCM765 toward MT_1 and MT_2 receptors have been estimated at 0.8 and 0.6, respectively (Rivara et al., 2007). Since physiological concentrations of MLT induce a desensitization and internalization of MT_2 receptors (Gerdin et al., 2003), we hypothesized that such a partial agonist might represent a therapeutic advantage for obtaining submaximal receptor activation without desensitization. Moreover, UCM765 (i.v.) has

acceptable pharmacokinetic properties (area under the curve = 68,954 min * ng/ml; Cmax = 2562 ng/ml), half-life (T1/2 = 44 min) and optimal hydrophilic–lipophilic balance (LogP of 2.64) for hypnotic and anxiolytic drug development (Ochoa-Sanchez et al., 2011). This unique pharmacological profile prompted us to examine its effects *in vivo*.

Considering that metabolic instability plays a negative role for MLT ligands available at the moment, it will be interesting to study the effects of the MT_2-selective partial agonist UCM924 (*N*-{2-[(3-bromophenyl)(4-fluorophenyl)amino]ethyl}acetamide), a class congener of UCM765, whose chemical structure was modified to make UCM924 more metabolically stable than UCM765. In particular, UCM924 has the free phenyl ring of UCM765 protected by the introduction of a fluorine atom and the methoxy group bioisosterically replaced with bromine (Rivara et al., 2009). Consequently, the half-life displayed in the presence of rat liver S9 fraction by UCM924 is much longer than that of UCM765 (40 min vs 8 min) (Rivara et al., 2009). Despite these differences, UCM924, similar to UCM765, also exhibits greater affinity for MT_2 receptors (pKi = 9.27) than for MT_1 receptors (pKi = 6.76) (Rivara et al., 2007, Rivara et al., 2009).

1.2 Goals and objectives

1.2.1 Role of MT_2 receptors in sleep regulation

The first goal in this section was to characterize the single role of the MT_2 receptor in the sleep-wake cycle of rats. We questioned whether a selective MT_2 partial agonist UCM765 would promote sleep (Chapter II). Therefore, we explored the effects of UCM765 on sleep in rats by using 24 h EEG/EMG recordings. First, we tested the dose-response of UCM765 across the 24 h light-dark cycle in rats and compared the effects of the effective dose to those of the clinically effective DZ. The selective MT_2 antagonist 4P-PDOT was mainly used to inhibit and confirm the role of MT_2 receptors in sleep behaviour.

Additionally, to further confirm that the effects of UCM765 were linked to the activation of MT_2 receptors but not MT_1 receptors, we tested whether UCM765 was active in mice lacking MT_1 receptors but not in mice lacking MT_2 receptor.

Later, we questioned whether the sleep promoting effects of UCM765 were related to its class properties [*N*-(substituted-anilinoethyl)amides], or to its selective partial agonistic activity toward MT_2 receptors, but not the dual activation of MT_1/MT_2 receptors. Consequently, we tested and compared the effects of UCM765 with those of the non-selective MT_1/MT_2 agonist UCM793. Moreover, in chapter IV, we compared the sleep actions of another MT_2 receptor partial agonist UCM924 (UCM765's analog) with those of UCM793 and MLT which activate both MT_1/MT_2 receptors.

The second goal was to characterize the sleep profile of MT_2KO mice by employing EEG/EMG; and to compare it to those sleep profiles of WT, MT_1KO and MT_1/MT_2KO mice in order to confirm the involvement of the MT_2 receptor and determine which of the receptor subtypes is involved in the regulation of sleep (Chapter II and III). Therefore, we assessed whether a MT_1, MT_2 or double MT_1/MT_2 genetic deletion leads to impaired NREMS, REMS and wakefulness parameters. Later, to further demonstrate that the melatonergic, but no other sleep-related neurotransmitter system, is impaired in MT_2KO mice, we explored whether DZ is still active in this strain.

The third goal was to identify the brain localization of MT_2 receptors by carrying out immunohistochemical experiments in rat tissue with selective polyclonal anti-MT_2 antibodies and light microscopy.

Given the presence of MT_2 receptors in the Rt nucleus, a brain area associated with the induction of NREMS (Steriade, 1999), the fourth goal of this project was to explore the role of MT_2 receptors expressed in the Rt nucleus in sleep regulation as well as its role in the mechanism of action of UCM765. We assessed the effects of systemic administration of UCM765 over the electrical activity (firing rate) of Rt neurons by performing single-unit extracellular recordings in anesthetized rats. Then, to confirm that the effects of UCM765 in Rt neurons was indeed associated with the promotion of NREMS, we evaluated the effects of a bilateral microinfusion of UCM765 into the Rt nucleus on sleep parameters in freely moving rats. In order to either confirm or discard the involvement of other regions expressing MT_2 receptors, we also microinfused UCM765 into the substantia nigra and assessed the effects on sleep parameters. We verified whether 4P-PDOT inhibits the electrophysiological and behavioural effects of UCM765 in order to confirm that these effects are mediated by MT_2 receptors expressed in the Rt neurons.

1.2.2 Role of MT_2 receptors in anxiety regulation

Here the main goal was to characterize the single role of MT_2 receptors in the regulation of anxiety by exploring the effects of the selective MT_2 partial agonist UCM765 in animal models of anxiety. First, we assessed the dose-response effects of UCM765 in the EPMT and NSFT in rats. Second, the effects of the anxiolytic dose of UCM765 were compared to those of MLT and the anxiolytic drug DZ in the EPMT, NSFT and OFT. Third, to further confirm that the anxiolytic-like effects of MLT in the EPMT and NSFT are due to the single activation of the MT_2 receptor; we selectively blocked the MT_2 or both the MT_1/MT_2 receptors by 4P-PDOT and luzindole, respectively.

Foreword to Chapter II

Promotion of non-rapid eye movement sleep and activation of reticular thalamic neurons by a novel MT_2 melatonin receptor ligand

The physiological actions of MLT in the brain are mediated by two high-affinity G-protein-coupled receptors, MT_1 and MT_2 (Dubocovich and Markowska 2005), whose respective roles in sleep are still to be defined. This is in part because of the lack of selective MT_1 and MT_2 ligands and also because more research in mice lacking MT_1 (MT_1KO) or MT_2 receptors (MT_2KO) needs to be done. Here, we investigated the role of the MT_2 receptor in sleep, studying the pharmacological effects of a novel MT_2-selective partial agonist UCM765 in the sleep-wake cycle of rats and the sleep profile of MT_2KO mice.

It was reasonable to begin our sleep studies with attempts to carry out a dose-response experiment to detect the putative hypnotic effects of UCM765 (s.c.) across the 24 h light-dark cycle using EEG/EMG in freely moving rats. The analysis of sleep by phase of day (light and dark) and the 1 h intervals allowed us to identify the hypnotic dose of UCM765 and demonstrate that it is significant (increased NREMS) only during the light phase, particularly at the end of the light phase. To better characterize the hypnotic effects of UCM765, we compared such effects to those of the $GABA_A$ agonist DZ, an effective anxiolytic. The analysis includes latency, total duration, number of episodes and single episode length for each state, NREMS, REMS and wakefulness. In addition, power spectrum analysis for NREMS and REMS was also assessed.

The next question to address was whether the effects of UCM765 were indeed mediated by the MT_2 receptor. Therefore, MT_2 receptors were blocked by the pre-treatment with 4P-PDOT, a selective MT_2 antagonist. We also questioned whether the effects of UCM765 were associated with its class properties [*N*-(substituted-anilinoethyl)amides], or with its selective partial agonistic activity toward MT_2 receptors. Thus, we compared the effects of UCM765 on sleep to those of its class congener UCM793, a non-selective MT_1/MT_2 agonist, in rats.

Then, to further characterize the role of MLT receptors in sleep, in particular the MT_2 receptor, we studied the 24 h sleep-wake cycle of MT_2KO mice to compare them to MT_1KO and wild-type (WT) mice. The experiments in KO mice confirmed the role of MT_2 receptors in the regulation of NREMS. Consequently, we tested whether the NREMS promoting effects of

UCM765 were cancelled by the genetic deletion of MT_2 receptors in MT_2KO mice. Since UCM765 also displays a low affinity for the MT_1 receptor, we questioned whether this affinity for the MT_1 receptor might be associated with the hypnotic effects of UCM765. Therefore, we tested to see if UCM765 was still active in MT_1KO. Additionally, MT_2KO mice, a strain with reduced NREMS, were treated with DZ to further discard a possible impairment in other sleep-related neurotransmitter systems in this strain, such as the GABAergic system.

We were also interested in the localization of MT_2 receptors in the brain. To achieve this goal, immunohistochemical experiments using selective polyclonal anti-MT_2 antibodies were carried out in rat brain tissue. Having found MT_2 receptors to be abundant in the reticular thalamic (Rt) nucleus, a region widely involved in sleep regulation, we hypothesized that MT_2 receptors located in the Rt nucleus are responsible for the sleep promoting effects of UCM765. Therefore, we studied the effect of UCM765 (i.v.) on the firing activity of Rt neurons in anesthetized rats. Then, in order to confirm that the effect of UCM765 on Rt neurons was modulated by MT_2 receptors expressed in the Rt nucleus, pre-treatment of the antagonist 4P-PDOT was administered into the Rt nucleus. In addition, we combined bilateral intrathalamic microinfusion of UCM765 into Rt nucleus and EEG/EMG recording in freely moving rats, to determine whether UCM765 induces NREMS by modulating the electrical activity of Rt neurons. Finally, since MT_2 receptors are located in different brain regions, similar experiments were done in the substantia nigra pars reticulate to confirm if the effect of UCM765 on sleep is selectively modulated by MT_2 receptors expressed in the Rt nucleus or in different regions.

Notably, this chapter demonstrates that the MT_2 receptor is involved in the regulation of NREMS and in the mechanism of action of UCM765. In particular, the selective activation of MT_2 receptors reduces the latency to sleep and increases the amount of NREMS during the light phase, whereas the genetic blockage of MT_2 receptors impairs NREMS.

Chapter II

Promotion of non-rapid eye movement sleep and activation of reticular thalamic neurons by a novel MT_2 melatonin receptor ligand

2.1 Research paper

Promotion of Non-Rapid Eye Movement Sleep and Activation of Reticular Thalamic Neurons by a Novel MT_2 Melatonin Receptor Ligand

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Abstract

Melatonin activates two brain G-protein coupled receptors, MT_1 and MT_2, whose differential roles in the sleep–wake cycle remain to be defined. The novel MT_2 receptor partial agonist, *N*-{2-[(3-methoxyphenyl) phenylamino] ethyl} acetamide (UCM765), is here shown to selectively promote non-rapid eye movement sleep (NREMS) in rats and mice. The enhancement of NREMS by UCM765 is nullified by the pharmacological blockade or genetic deletion of MT_2 receptors. MT_2, but not MT_1, knock-out mice show a decrease in NREMS compared to the wild strain. Immunohistochemical labeling reveals that MT_2 receptors are localized in sleep-related brain regions, and notably the reticular thalamic nucleus (Rt). Microinfusion of UCM765 in the Rt promotes NREMS, and its systemic administration induces an increase in firing and rhythmic burst activity of Rt neurons, which is blocked by the MT_2 antagonist 4-phenyl-2-propionamidotetralin. Since developing hypnotics that increase NREMS without altering sleep architecture remains a medical challenge, MT_2 receptors may represent a novel target for the treatment of sleep disorders.

Introduction

Insomnia is a common public health problem, with a prevalence ranging from 11 to 16% (Leland, 2006; Morin et al., 2006). In mammals, normal sleep is characterized by an orderly progression from wakefulness to non-rapid eye movement sleep (NREMS), also referred to as slow-wave sleep (SWS), and then to paradoxical sleep or rapid eye movement sleep (REMS). SWS is also known as "restorative sleep" since it is involved in memory consolidation (Stickgold, 2005; Marshall et al., 2006) and metabolic regulation (Tasali et al., 2008).

Currently used hypnotic drugs, such as BZs and derivates (i.e., zopiclone), act on the GABAergic system, affecting both NREMS and REMS, thus altering sleep architecture (Lancel, 1999). Most of them induce next-day cognitive impairments and abuse liability. Antidepressants also alter REMS density, but with little or no effect on SWS (Mayers and Baldwin, 2005). Developing new effective hypnotic drugs selectively increasing NREMS without altering the whole sleep architecture therefore remains a scientific and medical challenge.

Melatonin (*N*-acetyl-5-methoxytryptamine; MLT), synthesized by the pineal gland, has been reported to have hypnotic effects in humans, although results are still controversial (Brzezinski et al., 2005; Buscemi et al., 2006). Animal studies have demonstrated that MLT reduces time to sleep onset and increases both NREMS and REMS (Holmes and Sugden, 1982; Mirmiran and Pevet, 1986), with both effects being blocked by the $GABA_A$ receptor antagonists flumazenil and picrotoxin (Wang et al., 2003a). Other studies showed little or no effect (Mailliet et al., 2001; Wang et al., 2003b).

The physiological actions of MLT in brain are mediated by two high-affinity G-protein-coupled receptors, MT_1 and MT_2, whose respective roles in sleep remain to be defined. Three novel nonselective MT_1/MT_2 agonists, TIK-301 (Zemlan et al., 2005), ramelteon (Mini et al., 2007), and tasimelteon (Rajaratnam et al., 2009), have been tested in humans for the treatment of insomnia; all three significantly reduced the latency to sleep, but were less potent in potentiating the long-term enhancement of total sleep time and without selectivity toward NREMS or REMS. The nonselective antidepressant MT_1/MT_2 agonist agomelatine improves sleep after 6 weeks of treatment (Quera Salva et al., 2007), but its mechanisms of action remain to be elucidated, as it also binds to serotonergic $5\text{-}HT_{2B}$ and $5\text{-}HT_{2C}$ receptors, already known to be involved in sleep (Den Boer et al., 2006). Considering their lack of selectivity, none of the above compounds

allow for a selective pharmacological investigation of the role of brain MT_1 and MT_2 melatonin receptors.

In the present study, we examine the effects of the partial MT_2 receptor agonist *N*-{2-[(3-methoxyphenyl) phenylamino] ethyl} acetamide (UCM765) (Rivara et al., 2007) on the sleep–wake cycle of rats and that of mice lacking MT_1 or MT_2 receptors, disclosing a selective NREMS promoting effect of UCM765. In addition, having found MT_2 receptors to be abundant in the reticular thalamic nucleus (Rt), we combined extracellular recording in anesthetized rats, as well as bilateral intrathalamic microinfusion and EEG/EMG recording in freely moving rats, to demonstrate that UCM765 induces NREMS by modulating the electrical activity of Rt.

Materials and Methods

All experimental procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care, and the protocols were approved by the Animal Care Committee at McGill University.

Monitoring of the sleep–wake cycle.

These experiments were performed in adult male Sprague Dawley rats (225–340 g; Charles River Laboratories), adult male C3H/He wild-type (WT) mice (28–35 g; Charles River Laboratories), and mice of the same genetic background lacking MT_1 (Liu et al., 1997) or MT_2 receptors (Jin et al., 2003), kindly provided by Drs. Weaver and Reppert (University of Massachusetts, Worcester, MA). The knock-out (KO) mice were originally derived from a 129/sv KO mouse bred to a C3H female; heterozygous males from each generation were backcrossed exclusively to C3H females (10 generations and more of backcrossing), and male and female heterozygous were interbred to produce C3H WT and KO mice on the C3H background (Jin et al., 2003).

All animals were housed in small groups at 22°C, with *ad libitum* access to food and water, and maintained under a 12 h light/dark cycle (lights on at 7:30 AM; lights off at 7:30 P.M.). They were housed in separate cages after surgery.

Surgery.

Rats and mice were deeply anesthetized with equithesin (1 ml per 300 g, i.p.) and placed in a stereotaxic frame. For EEG monitoring, three stainless-steel epidural electrodes were positioned through 1.5 mm burr holes: one over the parietal cortex on each side, and the third (as a reference) in the right parietal cortex. In rats, their respective locations relative to bregma were −2 mm anteroposterior (AP) and −3 mm lateral (L), −7 mm AP and −3 mm L, and −4.5 mm AP and +3 mm L, according to Paxinos and Watson (2006). In mice, their respective locations relative to bregma were −0.95 mm AP and −2 mm L, −2.85 mm AP and −2 mm L, and −1.9 mm and +2 mm L, according to Franklin and Paxinos (1997). To monitor EMG signals, three flexible stainless-steel wire electrodes, isolated except for the last 3–4 mm, were implanted into the neck muscles (two bilaterally and one in the middle). Wires and connector were fixed to the skull with dental acrylic (Coltene/Whaledent).

After 24 h of recovery and during the next 5 d, the rats or mice were placed in a recording chamber and connected to a flexible cable for several hours daily, to habituate before the recording of sleep parameters.

EEG and EMG data analysis.

EEG/EMG signals were amplified at a total gain of 10.000 and filtered locally (EEG, low filter, 1 Hz; high, 1 kHz; EMG, low filter, 30 Hz; high, 3 kHz; Grass, P55), digitized using a CED power 1401 converter and Spike 2 software (CED) (Urbain et al., 2006), stored with a resolution of 128 Hz, and displayed on a PC monitor. Consecutive 2 s epochs were subjected to a fast Fourier transform (FFT), and EEG power spectrum density was computed in the frequency range of 0–64 Hz.

The three classical vigilance states as described in the rat were discriminated on the basis of the cortical EEG and neck EMG (Jones, 2005). Wakefulness was identified by a low-amplitude and desynchronized EEG, with sustained EMG activity. NREMS was clearly distinguished by high-voltage delta waves (1–4 Hz) and spindles associated with a weak EMG activity. REMS was characterized by a low-amplitude EEG with a pronounced theta rhythm (6–11 Hz) and a complete loss of nuchal muscle tone. To avoid transitional periods such as

drowsiness, only periods of typical stationary EEG and EMG lasting at least 10 s were considered for further analyses of wakefulness, NREMS, and REMS.

The latency to NREMS was defined as the time from the first injection (6:00 P.M.) of vehicle, UCM765, *N*-{2-[(3-methoxyphenyl)methylamino]ethyl} acetamide (UCM793), or diazepam (DZ) to the first NREMS episode longer than 2 min. The latency to REMS was the time from the first injection to the first episode of REMS lasting >10 s. Power spectra of the corresponding EEGs were then calculated by using the FFT of each 10 s epoch. Power in the 0–25 Hz range of artifact-free epoch was averaged, and the mean values were plotted in 0.5 Hz bins.

To determine the variations in power spectra induced by the pharmacological treatment or MLT receptor genetic deletion, the power densities were summed over the frequency band of 0–25 Hz (total power). The data were then standardized by expressing all power spectral densities at the different 0.5 Hz bin frequency ranges (delta, theta, sigma, and beta) as a percentage relative to the total power of the same epoch [modified from the study by Parmentier et al. (2002)]. Frequency ranges for sigma and beta bands were 11–15 and 15–18 Hz, respectively. Spindle-wave episodes (group of rhythmic waves characterized by a progressive increasing then decreasing amplitude, with activity between 10 and 14 Hz) were readily detected after bandpass filtering of the EEG at frequencies between 10 and 15 Hz (Espinosa et al., 2008). Spindles were counted manually in 1 s epochs within all NREMS episodes lasting >2 min, using Spike2 software.

Pharmacological treatments.

For experiments in which the EEG and EMG were recorded from 6:00 to 9:00 P.M., vehicle (DMSO 70% and 30% saline, 0.2 ml for rats, 0.1 ml for mice), DZ (2 mg/kg), UCM765 (40 mg/kg), UCM793 (40, 60, or 80 mg/kg), or the MT_2 antagonist 4-phenyl-2-propionamidotetralin (4P-PDOT; 10 mg/kg) were injected subcutaneously at the beginning of the recording period. For 24 h experiments with UCM765 at different doses, 20, 40, or 60 mg/kg was injected subcutaneously every 4 h, starting at 6:00 P.M. For comparison with DZ, 40 mg/kg UCM765 was injected subcutaneously every 4 h starting at 6:00 P.M., whereas a sleep promoting dose of DZ [2 mg/kg (Gottesmann et al., 1998)], was injected subcutaneously at 6:00

P.M. and 6:00 A.M., given the long, 6–8 h half-life of DZ. Vehicle was then injected at 10:00 P.M., 2:00 A.M., 10:00 A.M., and 2:00 P.M. to mimic the sequence of UCM765 injection.

Immunolabeling of MT_2 receptors.

Polyclonal anti-MT2 antibodies were produced as described previously (Angeloni et al., 2000) and affinity purified. In preliminary immunohistochemical experiments on brain tissue from wild-type and MT2 KO mice, only ubiquitous, nonspecific staining of neurons and glial cells was observed (results not shown), precluding the use of these antibodies in this species. Sequence alignment between human and rat MT2 proteins was suggestive of interspecies conservation of an immunogenic epitope (see Fig. 5, top left). In Western blots of rat brain regions (Angeloni et al., 2000), the antibodies reacted with a band of ~40 kDa, corresponding to the predicted molecular weight of the native MT2 receptor protein (see Fig. 5, top right).

Adult male Sprague Dawley rats (250 ± 50 g; n = 5) were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused through the heart with 200 ml of fixative solution [4% paraformaldehyde (PFA) and 0.25% acrolein in 0.1 mM phosphate buffer (PB), pH 7.4] followed by 500 ml of 4% PFA in PB. The brain was removed, postfixed in 4% PFA (60 min, 4°C), and washed in PBS (0.9% NaCl in 50 mM PB, pH 7.4). Sections (50 μm thick) were cut with a vibratome and processed free floating for immunoperoxidase labeling as follows. Sections were immersed in 3% hydrogen peroxide (H_2O_2) for 30 min, rinsed in PBS, immersed in 0.1% sodium borohydride in PBS for 30 min, rinsed in PBS, and preincubated for 2 h in a blocking solution (BS) of PBS containing 10% normal goat serum (Vector Laboratories), 0.5% gelatin, 0.01% BSA, and 0.3% Triton X-100. Sections were then washed in PBS, incubated overnight with MT_2 antibody (1:250 in BS), rinsed in PBS, incubated for 2 h with biotinylated goat anti-rabbit IgGs 1:1000 in BS (Jackson ImmunoResearch), and incubated for 1 h in a 1:1000 dilution of horseradish peroxidase-conjugated streptavidin (Jackson ImmunoResearch). After washes in 0.05 mM Tris-buffered saline (TBS), pH 7.4, MT_2 labeling was revealed 2–5 min in TBS containing 0.05% 3,3-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2. The reaction was stopped in TBS. Sections were then air dried on gelatin-coated slides, dehydrated in ethanol, cleared in toluene, and mounted with DPX (Fluka; Sigma-Aldrich). Immunohistochemical controls included omission of the primary antibody (n = 2) and preincubation of the anti-MT_2

antibody with a 100-fold excess of its blocking peptide (n = 2), which both completely prevented any immunostaining (data not shown).

In four other rats, sections across the thalamus, collected at 50 μm intervals, were similarly processed for MT2 immunohistochemistry and counterstained with cresyl violet, and the Rt nucleus carefully examined by light microscopy at relatively high magnification (40× objective).

Extracellular recording of reticular thalamic neurons.

Each adult male Sprague Dawley rat, anesthetized with urethane (1 g/kg, i.p.), was placed in a stereotaxic frame (David Kopf Instruments) and a catheter was inserted into a tail vein for systemic drug administration. Single-barreled glass micropipettes (R&D Scientific Glass; tip diameter, 2–3 μm) filled with 2% pontamine sky blue dye in sodium acetate (2 M, pH 7.5; electrode impedance, 5–10 MΩ) were used for extracellular recording. A burr hole was drilled and the micropipette lowered into the Rt at the following stereotaxic coordinates: −2.56 mm AP, −3.5 mm L, and 5.0–6.5 mm ventral relative to bregma (Paxinos and Watson, 2006).

Filtered (AC, 0.2–2 kHz) electrode signal was amplified (Bak Electronics Model RP-I) and fed to an oscilloscope (BK Precision; 20 MHz, 1522) and an audio monitor. Spike shapes were digitalized by a CED 1401 interface system, processed on-line, and analyzed off-line by Spike 2 software, in parallel with analog-to-digital samplings of amplified (Grass, P55) polygraphic signals (EEG; sampling rate, 100 or 200 Hz). Single-unit activity was isolated with an amplitude spike discriminator. Rt neurons were recognized by their long burst (>50 ms) and *accelerando-decelerando* bursting firing pattern (Fuentealba et al., 2004), followed by tonic tail (Domich et al., 1986). Firing and burst neuronal activity was analyzed with Spike 2 software for 3 min periods for each treatment: basal, vehicle [propylene glycol (PG), saline 50%, 0.1 ml], and UCM765 (20 mg/kg). The burst of Rt cells consisted of a discharge of at least four spikes (Contreras et al., 1993), with an onset defined by a maximum interspike interval ≤20 ms and a preburst and postburst interval >100 ms (Domich et al., 1986). The longest interval allowed within a burst was 70 ms to include the tonic tail, which is merely the end of a spike barrage (Domich et al., 1986).

Combined extracellular recording and intrathalamic microinfusion.

In six rats anesthetized with urethane (1 g/kg, i.p.), a Hamilton syringe (24 gauge) was lowered at an angle of 29° from the vertical axis into the Rt, and 5.5 mm ventral from the brain surface. Vehicle (PG–saline 50%; 10 μl) was slowly infused, and immediately after, an electrode was positioned into the Rt for *in vivo* electrophysiological recording of Rt neurons. Once a stable firing Rt neuron was found, baseline activity was recorded for 1–5 min, and then vehicle (0.1 ml, i.v.) was injected and the neuronal activity recorded for an additional 3–5 min. Afterward, UCM765 (20 mg/kg, i.v) was injected, and the firing activity recorded for 3–5 min. The entire procedure did not last longer than 30 min.

In six other rats, 4P-PDOT (10 μg in 10 μl) was directly injected into Rt with the Hamilton syringe, and the baseline activity of a single Rt neuron as well as its activity after vehicle and UCM765 injection were recorded as above.

Bilateral intrathalamic microinfusion and EEG/EMG recording in freely moving rats.

Rats were deeply anesthetized with equithesin (1 ml per 300 g, i.p.) and placed in a stereotaxic frame. Two stainless-steel guide cannulas (23 gauge, 12 mm) were implanted bilaterally into the Rt at stereotaxic coordinates −2.56 mm AP, +3.5/−3.5 mm L, and 5 mm ventral relative to bregma (Paxinos and Watson, 2006). Then, EEG/EMG electrodes were positioned as above for the monitoring of the sleep–wake cycle. EEG electrodes and cannulas were secured with dental acrylic. Animals were habituated for 5 d as described above.

EEG and EMG were then recorded from 6:00 to 7:00 P.M., immediately after microinfusion of 2.5 μl of vehicle (PG–saline 50%; $n = 5$) or UCM765 (10 μg in 2.5 μl; $n = 5$) in each cannula.

Bilateral substantia nigra microinfusion and EEG/EMG recording in freely moving rats.

A similar experimental protocol was also used in eight rats to test for eventual effects of UCM765 on sleep, if microinfused in another brain region also displaying MT_2 immunoreactive neurons, i.e., the substantia nigra, pars reticulata. Vehicle or UCM765 was infused at the following stereotaxic coordinates: −5.4 mm AP, +2.75/−2.75 mm L, and 7.2 mm ventral relative to bregma (Paxinos and Watson, 2006).

Histological verification.

At the end of extracellular recording and/or microinfusion experiments, the brain was fixed by immersion in a 20% formaldehyde solution and embedded in paraffin, and 10-μm-thick sections collected at 50 μm intervals across the whole thalamus or substantia nigra were stained with cresyl violet and examined by light microscopy to identify the site of recording or microinfusion.

Statistical analyses.

SigmaStat (version 3.5) and SPSS software (version 13) were used to analyze data. Results are expressed as the mean ± SEM. Since assumptions of normality and variance homogeneity were satisfied from all data, one-way, one-way repeated-measures, or two-way mixed-design ANOVA (with factors as indicated in Results), followed by the Student–Newman–Keuls (SNK) or Fisher test for *post hoc* comparison, was used to determine differences. Student's *t* test was used to compare the effect of UCM765 with DZ in MT_2 KO mice, and with vehicle in the bilateral microinfusion experiments. Calculation of ED_{50} values was performed using Microcal Software Origin (version 7). Relative changes in the power of frequency bands (delta, theta, beta) as a percentage of control (vehicle or WT mice, respectively) were analyzed by one-way ANOVA followed by Bonferroni's test for *post hoc* comparison. Statistical values reaching $p \leq 0.05$ were considered significant.

Results

Pharmacological properties of UCM765

UCM765 is a novel partial agonist exhibiting greater affinity for MT_2 (pK_i = 10.18) than for MT_1 receptors (pK_i = 8.38), and even greater MT_2 affinity than MLT itself (pK_i = 9.62) (Rivara et al., 2007). UCM765 does not display significant affinity for other receptors, ion channels, and transporters, including those known for their involvement in sleep–wake regulation, such as $GABA_A$, 5-$HT2_A$, and histamine receptors (data are from Cerep, Celle L'Evescault, France; data not shown).

Based on the stimulation of basal [^{35}S]GTPγS binding, the relative intrinsic activity values of UCM765 toward MT_1 and MT_2 receptors have been estimated at 0.8 and 0.6, respectively (Rivara et al., 2007). Since physiological concentrations of MLT induce a

desensitization and internalization of MT_2 receptors (Gerdin et al., 2003), we hypothesized that such a partial agonism might represent a therapeutic advantage for obtaining submaximal receptor activation without desensitization. Moreover, UCM765 (5 mg/kg, i.v.) has acceptable pharmacokinetic properties (area under the curve = 68,954 min * ng/ml; C_{max} = 2562 ng/ml) and half-life ($T_{1/2}$ = 44 min) for hypnotic drug development, as well as optimal hydrophilic–lipophilic balance (LogP of 2.64). This unique pharmacological profile prompted us to examine its effects *in vivo*.

Pharmacological modulation of the sleep–wake cycle

With EEG/EMG measurements in freely moving rats, we first queried whether a selective MT_2 ligand might promote sleep. Since MLT and its receptors have shown circadian variations across the 24 h light/dark cycle, we recorded EEG/EMG activity during 24 h in four groups of rats, one injected with vehicle (n = 5) and the others with 20 mg/kg (n = 6), 40 mg/kg (n = 5) or 60 mg/kg (n = 6) UCM765 every 4 h (first injection at 6:00 P.M. for all groups). This protocol was chosen on the basis of the pharmacokinetic properties of UCM765. As shown in Figure 1*A–D*, the latency of the first long (>2 min) episode of NREMS was significantly affected by the dose of UCM765 ($F_{(3,18)}$ = 4.2; p = 0.01): 40 and 60 mg/kg respectively decreased latency by 59% and 49% ($p < 0.05$).

Two-way ANOVA on total time in NREMS indicated a significant main effect of doses ($F_{(3,18)}$ = 4.80; p = 0.013) and phase of day ($F_{(1,18)}$ = 89.27; $p < 0.001$), with no interaction. In particular, UCM765 prolonged the total amount of NREMS during the inactive/light phase by 48% ($p < 0.01$) and 33% ($p < 0.05$) at doses of 40 and 60 mg/kg, respectively. No differences were observed between 40 and 60 mg/kg, suggesting that a plateau was reached at these doses. The ED_{50} was 30.3 mg/kg for the NREMS total time in the inactive phase. No significant effects of UCM765 were reported on the total time of REMS (Fig. 1*C*).

UCM765 decreased the time of wakefulness in the inactive/light phase (effect of doses, $F_{(3,18)}$ = 6.24, p = 0.004; phase of day, $F_{(1,18)}$ = 75.11, $p < 0.001$, with no interaction). In particular, 40 and 60 mg/kg UCM765 decreased the total time of wakefulness during the inactive/light phase by 37% ($p < 0.001$) and 26% ($p < 0.005$), respectively (Fig. 1*D*). When the effect of UCM765 (40 mg/kg) on NREMS total time was compared to vehicle by 1 h intervals over 24 h, a main effect of treatment and time was observed (effect of treatment, $F_{(1,8)}$ = 8.32, p =

0.02; effect of time, $F_{(23,184)} = 6.35$, $p < 0.001$, with no interaction). The effect of UCM765 became statistically significant mostly at the end of the day (4:00–7:00 P.M.) (Fig. 1*E*), when the expression of MT_2 receptors is maximal (Witt-Enderby et al., 2003). These results indicated that UCM765 activated MT_2 receptors mostly during the inactive/light phase, without altering the 24 h sleep–wake cycle. No significant modifications of REMS were detected (Fig. 1*E*). The cumulative sleep effect was significant only in the inactive light phase (Fig. 1*F*).

The effects of UCM765 on sleep were also compared to those of the clinically used hypnotic benzodiazepine (DZ; Valium). As shown in Figure 2, the group of rats treated with UCM765 (40 mg/kg every 4 h; $n = 5$) and the group treated with DZ (2 mg/kg every 12 h; $n = 6$) both exhibited shorter onset latencies of the first NREMS episode compared to the group of rats ($n = 5$) receiving vehicle every 4 h (Fig. 2*A*, left) ($F_{(2,8)} = 9.0$, $p < 0.01$; vehicle vs UCM765 or DZ, $p < 0.01$). UCM765 and DZ increased total time of NREMS during the inactive/light phase only (Fig. 2*A*) (effect of treatment, $F_{(2,13)} = 8.54$, $p = 0.004$; effect of phase of day, $F_{(1,13)} = 102.24$, $p < 0.001$; with an interaction, $F_{(2,13)} = 4.17$, $p = 0.04$). DZ, but not UCM765, markedly reduced the number of NREMS episodes (Fig. 2*A*, center) (effect of treatment, $F_{(2,13)} = 7.04$, p=0.008, phase of day, $F_{(1,13)} = 46.61$, $p < 0.001$; with an interaction, $F_{(2,13)} = 9.73$, $p = 0.003$; DZ vs vehicle or UCM765, $p < 0.001$). Only during the light phase did both UCM765 and DZ increase mean NREMS episode length (Fig. 2*A*, right) (effect of treatment, $F_{(2,13)} = 11.14$, p=0.002; phase of day, $F_{(1,13)} = 14.08$, p=0.002; with an interaction, $F_{(2,13)} = 7.52$, $p = 0.007$). Both UCM765 and DZ increased REMS latency ($F_{(2,8)} = 14.1$, $p < 0.001$), without apparent effects on REMS total time. The number of REMS episodes appeared to be affected by phase of day (Fig. 2*B*) ($F_{(1,13)} = 5.57$, $p = 0.03$), but not by treatment.

Total time of wakefulness was significantly reduced after treatment with either UCM765 or DZ during the inactive/light phase only (Fig. 2*C*, left; effect of treatment, $F_{(2,13)} = 9.28$, p=0.003; phase of day, $F_{(1,13)} = 75.15$, $p < 0.001$, without interaction). DZ, but not UCM765, considerably decreased the number of episodes of wakefulness during the light phase (effect of treatment, $F_{(2,13)} = 7.95$, $p = 0.006$; phase of day, $F_{(1,13)} = 41.97$, $p < 0.001$; with an interaction, $F_{(2,13)} = 9.17$, $p = 0.003$). DZ, but not UCM765, significantly increased the mean wakefulness episode length (Fig. 2*C*) (effect of treatment, $F_{(2,13)} = 5.92$, $p = 0.015$; phase of day, $F_{(1,13)} = 52.46$, $p < 0.001$, without interaction). Moreover, similarly to DZ, UCM765 increased the

number of spindles/min during the inactive/light phase only (Fig. 2*D*) (effect of treatment, $F_{(2,13)}$ = 24.51, $p < 0.001$; phase of the day, $F_{(1,13)}$ = 110.20, $p < 0.001$; with an interaction, $F_{(2,13)}$ = 13.33, $p < 0.001$).

The quality of sleep after UCM765 and DZ was thus compared using power spectra analysis during both light and dark phases (Fig. 3). Upon calculation of the relative EEG power (vehicle set at 100%) (Fig. 3, insets), a significant effect of treatment was observed on the delta bands of NREMS ($F_{(2,15)}$ = 9.60, p = 0.002) and REMS ($F_{(2,15)}$ = 209.43, $p < 0.001$) and on beta bands of NREMS ($F_{(2,9)}$ = 13.71, p = 0.002) and REMS ($F_{(2,9)}$ = 16.08, $p < 0.001$), but not on theta. In particular, DZ produced a significant decrease in the power of the delta band in both NREMS and REMS sleep, and an increase of the peak of beta (10–12 Hz) (Fig. 3, insets), as reported previously (Kopp et al., 2004; van Lier et al., 2004). On the contrary, UCM765 produced a slight increase by 12% in delta of NREMS during the light phase. Similar findings were also observed during the dark phase (Fig. 3, insets, bottom).

Sleep in MT_2 and MT_1 knock-out mice

To further characterize the role of MT_2 and MT_1 receptors in sleep, the sleep parameters were also measured over the 24 h light/dark cycle in mice lacking MT_2 receptors (MT_2 KO, n = 5) to compare them with wild-type mice (WT, n = 5) and with mice lacking MT_1 receptors (MT_1 KO, n = 5).

Compared to WT and MT_1 KO mice, MT_2 KO mice showed a decrease in NREMS (−16%; p = 0.017) during the inactive/light phase only (Fig. 4*A*). On the contrary, MT_1 KO mice showed an increase in NREMS (+40%; p = 0.001) during the active/dark phase (Fig. 4*A*) (effect of strain, $F_{(2,12)}$ = 10.82, p = 0.002; phase of day, $F_{(1,12)}$ = 79.40, $p < 0.001$; with an interaction, $F_{(2,12)}$ = 6.14, p = 0.015). MT_2 KO mice displayed an increase of wakefulness (+19%; p = 0.017) compared to WT and MT_1 KO mice during the light/inactive phase only, whereas MT_1 KO mice showed a decrease in wakefulness (−24%; p = 0.001) during the dark/active phase (Fig. 4*C*) (effect of strain, $F_{(2,12)}$ = 7.93, p = 0.006; phase of day, $F_{(1,12)}$ = 94.40, $p < 0.001$; with an interaction, $F_{(2,12)}$ = 7.47, p = 0.008).

In WT and MT_2 KO mice, REMS lasted longer in the inactive/light phase (+46%; p = 0.005 and +50%; $p < 0.001$, respectively) than in the active/dark phase; in contrast, MT_1 KO

mice spent equal time in REMS during both the light and dark phases (p = 0.69), indicating a disruption of the light/dark REMS cycle (Fig. 4*B*) (effect of phase of day, $F_{(1,12)}$ = 16.84, p = 0.001; no strain effect and interaction).

Altogether, these results suggested that MT_2 receptors are mostly involved in the maintenance of NREMS during the inactive phase and not at all in REMS. Interestingly, the power spectra analyses (Fig. 4*D*,*E*) during the light and the dark phases also indicated a modified spectral power mostly in MT_1 KO mice. Upon calculating the relative EEG power (WT set at 100%) during the light phase (Fig. 4*D*, insets), a significant effect of strain was observed on the delta band of NREMS ($F_{(2,15)}$ = 8.85, p = 0.003), on the theta bands of NREMS ($F_{(2,27)}$ = 4.60, p = 0.019) and REMS ($F_{(2,27)}$ = 5.98, p=0.007), on sigma band of NREMS ($F_{(2,21)}$ = 131.22, p < 0.001), and on beta bands of NREMS ($F_{(2,9)}$ = 828.75, p < 0.001) and REMS ($F_{(2,9)}$ = 4.98, p = 0.035). For all the bands, there were significant differences mainly between MT_1 KO and WT mice (p < 0.05). Similar findings were obtained after the analysis of NREMS and REMS power spectra during the dark phase (Fig. 4*E*).

Selectivity of UCM765 for MT_2 receptors

We then questioned whether the effects of UCM765 were linked to its class properties [*N*-(substituted-anilinoethyl)amides], or to its selective partial agonistic activity toward MT_2 receptors. For this purpose, we compared the effects of UCM765 to those of its class congener UCM793 (Rivara et al., 2007), which exhibits a nonselective MT_1/MT_2 profile of agonistic activity (MT_1, p*K*i = 9.09; MT_2, p*K*i = 9.19). In these experiments, sleep parameters were measured between 6:00 and 9:00 P.M., a period during which several NREMS and REMS episodes occur (Ruigt et al., 1989).

As shown in Table 1 for Treatment Group A, UCM793 did not modify the total time in NREMS and REMS. In much the same way that the MT_1/MT_2 agonist ramelteon acts in rats (Fisher et al., 2008) and humans (Erman et al., 2006), UCM793 at the dose of 40 mg/kg (n = 7) slightly reduced (−15%) the latency of the first long episode (>2 min) of NREMS (vehicle, 26.4 ± 2.8 min; UCM793, 22.5 ± 3.7 min). Thus, the effect of UCM765 on NREMS appeared to be imputable to its selectivity toward MT_2 receptors.

The MT_2 antagonist 4P-PDOT is a reference compound exhibiting good binding affinity for the cloned human MT_2 receptor ($pK_i = 8.8$) and a selectivity of at least 100-fold for MT_2 over the MT_1 subtype (Boutin et al., 2005). To verify that the promotion of NREMS by UCM765 is MT_2 mediated, UCM765 (40 mg/kg) was administered 10 min after the pretreatment with vehicle or 4P-PDOT (10 mg/kg, $n = 7$). As shown in Table 1*B*, 4P-PDOT blocked the effects of UCM765 on NREMS and wakefulness total time, strongly suggesting that the maintenance of NREMS was mediated by MT_2 receptors. Indeed, two-way ANOVA showed a significant interaction between treatment and pretreatment on NREMS total time ($F_{(1,20)} = 4.6$, $p = 0.04$) and total time of wakefulness ($F_{(1,20)} = 4.4$, $p = 0.04$). SNK *post hoc* test indicated a significance ($p < 0.05$) of comparing 4P-PDOT plus UCM765 and vehicle plus UCM765.

Even though UCM765 is selective for MT_2 receptors, it does display a low affinity for MT_1 receptors. To further confirm the involvement of MT_2 and determine which of the receptor subtypes is involved in the NREMS promoting effect, we tested UCM765 in MT_2 KO ($n = 6$), MT_1 KO ($n = 6$), and WT ($n = 8$) mice (Table 1, Group C). UCM765 decreased the latency to sleep in WT (−53%; $p < 0.001$) and MT_1 KO (−53%; $p < 0.001$), but not in MT_2 KO mice (effect of treatment, $F_{(1,34)} = 27.8$, $p < 0.001$; strain, $F_{(2,34)} = 3.9$; $p < 0.02$, with no interaction). However, MT_1 KO mice showed a long latency to the first onset of sleep compared to WT (+46%; $p < 0.01$) after the injection of vehicle. UCM765 increased NREMS total time in MT_1 KO (+32%; $p < 0.05$), but not in MT_2 KO mice (effect of treatment, $F_{(1,34)} = 12.0$, $p < 0.001$; strain, $F_{(2,34)} = 19.2$, $p < 0.001$; with a significant interaction, $F_{(2,34)} = 4.5$, $p < 0.01$), indicating that the presence of MT_2, but not of MT_1, receptors is essential and necessary for the restoration of NREMS (Table 1, Group C). REMS latency was not modified in MT_1 KO and MT_2 KO mice, and UCM765 had no effect on latency and total time in REMS. UCM765 decreased the total time of wakefulness in WT (−23%; $p < 0.001$) and MT_1 KOs (−12%; $p < 0.05$), but not in MT_2 KO mice (Table 1, Group C) (effect of treatment, $F_{(1,34)} = 10.4$, $p = 0.003$; strain, $F_{(2,34)} = 13.9$, $p < 0.001$; with a significant interaction, $F_{(2,34)} = 3.6$, $p = 0.03$).

Overall, the lack of effect in MT_2 KO mice concurred with the prior evidence that the NREMS promoting effect of UCM765 was selectively mediated by MT_2 receptors. To determine whether the reduction in NREMS observed in MT_2 KO mice was due to the lack of MT_2 receptors rather than to impairment in other sleep-related neurotransmission systems, MT_2 KO

mice were treated with the $GABA_A$ agonist DZ (Table 1, Group D). As in rats, DZ in MT_2 KO mice ($n = 4$) decreased the latency of the first episode of sleep (−64%; $p < 0.05$, Student's *t* test), enhanced NREMS total time (+49%; $p < 0.002$), and tended to decrease REMS total time (−21%) and time in wakefulness (−20%; $p < 0.003$), thus suggesting that the GABAergic regulation of sleep was intact in these mice.

Immunohistochemical localization of MT_2 receptors

MT_2 receptor immunoreactivity was detected in numerous anatomical regions across rat brain (Fig. 5). Strong labeling of neuronal cell bodies and proximal dendrites was consistently observed in Rt (Fig. 5*A–E*), septum, CA3 of dorsal hippocampus (Fig. 5*F*), substantia nigra reticulata and red nucleus (Fig. 5*G*), supraoptic nucleus (Fig. 5*H*), oculomotor nuclei, and ventral tegmental nucleus. In high-resolution micrographs obtained after counterstaining with cresyl violet (Fig. 5*E*), it was apparent that all neurons in Rt were immunoreactive (arrows). Neuronal labeling was moderate in the ventral pallidum, globus pallidus, other sectors of hippocampus (e.g., the dentate gyrus), paraventricular nucleus of the hypothalamus, and inferior colliculus. In hippocampus, the sparse distribution of labeled neurons was suggestive of interneurons. There was no somatodendritic labeling in suprachiasmatic nucleus (Fig. 5*I*), but this nucleus appeared slightly darker than surrounding areas, perhaps due to punctate (axon terminal) labeling. No MT_2 labeling was observed in the white matter and numerous other gray matter structures, notably in cerebral cortex, striatum, lateral hypothalamic area, habenular nuclei, substantia nigra pars compacta, periaqueductal gray, and raphe nuclei.

UCM765 activates neurons of the reticular thalamic nucleus

Given the presence of MT_2 receptors in the Rt nucleus (Fig. 5*A–D*) and the importance of this nucleus in the induction of NREMS (Steriade, 1999), we first examined the effects of systemically administered UCM765 (20 mg/kg, i.v.) on the firing activity of these neurons. As reported in Table 2, UCM765 ($n = 8$), but not vehicle ($n = 8$), elicited an increase in firing activity (+91%, $p < 0.001$), percentage of spikes in burst (+134%; $p = 0.002$), and mean spike per burst (+50%; $p = 0.02$) in all neurons tested. Figure 6*A* illustrates an integrate histogram of a single-unit recording displaying the classical pattern of a typical Rt neuron before and after the UCM765 intravenous injection. A sustained increase in firing (second trace) and burst (third trace) activities was observed immediately after the injection of UCM765. On top of Figure 6

(insets), the typical *accelerando-decelerando* pattern of Rt neurons is shown at higher magnification. Figure 6*B* is the plot of mean interspike intervals against the number of spikes per burst in the basal condition and after vehicle and UCM765 injections for all Rt neurons tested (n = 8). UCM765 increases the number of spikes within bursts, but does not change the pattern of interspike interval distribution.

To confirm that the firing and burst activation of Rt neurons was due to a direct activation of MT_2 receptors localized within the nucleus, the MT_2 antagonist 4P-PDOT (10 μg/10 μl; n = 7) was locally infused in the Rt before the intravenous injection of UCM765. The increase of firing and burst activation of Rt neurons after UCM765 injection was prevented by the local infusion of 4P-PDOT, confirming that this effect was mediated by MT_2 receptors present within the nucleus [two-way ANOVA analysis for factors infusion (vehicle or 4P-PDOT) and treatment (basal, vehicle or UCM765), $F_{(2,10)} = 10.23$, $p = 0.004$] (Fig. 7*A*–*D*).

In all brains in which the recording sites could be localized histologically, the electrode tract ended in the dorsal Rt, either in its anterior or in its middle third, rostrocaudally (Fig. 7*B*). In a single case in which the Rt was missed, the electrode tract was found immediately adjacent, but lateral, to the internal capsule.

Microinfusion of UCM765 in Rt but not in substantia nigra induces NREMS

To further confirm that UCM765 promoted NREMS by acting on Rt neurons, the compound was bilaterally infused into the Rt nuclei in nonanesthetized freely moving rats (n = 5), in which EEG/EMG activity was simultaneously recorded for 1 h (6:00–7:00 P.M.). UCM765 (10 μg bilaterally) significantly decreased NREMS latency compared to vehicle (−49%, 9.2 ± 2.4 min vs 18.2 ± 3.0; t = 2.31; 8 df; p = 0.04). Moreover, UCM765 increased NREMS total time (+93%; t = −2.81; 8 df; p = 0.02) and decreased wakefulness (−31%; t = 2.49; 8 df; p = 0.03) without affecting REMS (Fig. 8*A*–*C*). In three brains examined histologically after bilateral microinfusion, the lesions produced by the cannulas and deposits of pontamine sky blue encroached on the dorsal Rt, again in its rostral or middle third, rostrocaudally (Fig. 8*B*).

When UCM765 was bilaterally microinfused into the substantia nigra pars reticulata (n = 8), another area rich in MT_2 receptor (Fig. 5*G*), no changes in the sleep–wake parameters were

displayed (Fig. 8*D–F*) compared to vehicle, indicating that activation of MT_2 receptors located into Rt are necessary for UCM765 to promote sleep.

Discussion

This is the first demonstration that activation of MT_2 receptors by the novel selective partial agonist UCM765 induces and promotes NREMS. This effect of UCM765 is dependent on MT_2 receptors, since it was nullified by the pharmacological blockade or genetic deletion of these receptors.

The promotion of NREMS is likely the result of the burst activation of Rt neurons. Indeed, the increase of Rt neural activity induced by systemic administration of UCM765 paralleled the NREMS enhancement, and its local microinfusion into Rt was sufficient to produce this effect. When UCM765 was microinfused into the substantia nigra, another area rich in MT_2 receptor, but with less impact on sleep, there were no apparent changes in sleep–wake parameters.

MT_2 receptors have not been implicated previously in the regulation of sleep, except in a recent study suggesting that the MT_2 agonist IIK7 may transiently perturb the sleep–wake cycle (Fisher and Sugden, 2009). Thus far, MT_2 receptors had been mostly implicated in the regulation of circadian rhythm, even though controversial results had been obtained by *in vitro* and *in vivo* techniques. For example, in brain slices from WT and MT_2 KO mice, but not MT_1 KO mice, MLT was found to inhibit neuronal firing in the suprachiasmatic nucleus, whereas the phase shift of neuronal firing rhythms induced by the MT_1/MT_2 agonist 2-iodoMLT was of smaller magnitude in suprachiasmatic nucleus (SCN) brain slices from MT_1 KO mice than WTs (Liu et al., 1997). Moreover, *in vivo* studies have demonstrated that MLT induces phase shifts in WT and MT_2 KO mice (Hudson et al., 2005), but not in MT_1 KO mice (Dubocovich et al., 2005), whereas circadian rhythms appear to be unaltered in MT_2 KO mice (Jin et al., 2003). These observations altogether suggest that MT_1 but not MT_2 receptors are directly involved in circadian regulation. Indeed, prior studies having hypothesized a role for MT_2 receptors in phase shift presented several pitfalls; the experiments *in vivo* had been performed with relatively low doses of melatonin receptor ligands (Dubocovich et al., 1998), and those using SCN slices had inevitably eliminated the whole brain circuitry (Hunt et al., 2001). Nevertheless, although sleep and circadian rhythms are coupled, they are presumably generated by different neuronal

mechanisms, in keeping with the notion that the time course of their ontogenic development is not consistently comparable (Mirmiran et al., 2003).

The bulk of our results suggest that MT_2 receptors play a specific role in NREMS regulation during the inactive/light phase, whereas MT_1 receptors are likely involved in the 24 h sleep–wake cycle, sleep regulation in the active phase, and overall spectral activity. Indeed, MT_1 KO mice exhibited increased NREMS associated with a modified 24 h sleep–wake cycle. Conversely, the presence of MT_1 receptors in MT_2 KO mice left the 24 h sleep–wake cycle intact with a decrease in NREMS total time. In keeping with this view, two functions associated with NREMS (Marshall and Born, 2007), long-term potentiation in the hippocampus and memory consolidation (Larson et al., 2006), have been shown to be impaired in MT_2 KO mice.

Our polysomnographic recordings suggest that the effects of UCM765 on sleep are different from those of nonselective, MT_1/MT_2, class congeners. In particular, the congener UCM793 slightly decreased sleep onset, but without effect on NREMS maintenance, similar to ramelteon, suggesting that the affinity of these compounds for both MLT receptors accounts for their effect on sleep onset, whereas selectivity toward MT_2 receptors results in a more incisive effect on sleep onset as well as NREMS maintenance. As two receptors for the same neurotransmitter may have divergent functions, selectivity for a single receptor can provide a therapeutic advantage. For example, in hippocampal slices, MT_1 and MT_2 receptor activation appears to differentially modulate $GABA_A$ receptor function, suggesting that MLT, through activation of different receptor subtypes, may exert opposite effects in the same brain area (Wan et al., 1999). Similarly, MT_1 and MT_2 receptors have also been shown to act in an opposite manner on the vascular system, producing vasoconstriction and vasodilatation, respectively (Doolen et al., 1998). Our data also suggest differential functions in sleep; indeed NREMS is decreased in MT_2 KO mice during the inactive/light phase and increased in MT_1 KO mice during the active/dark phase.

As visualized by light microscopic immunohistochemistry, many regions of rat brain, more or less tightly implicated in different aspects of sleep, contain neurons bearing MT_2 receptors. The focus of the present study was on Rt, because in rodents, Rt neurons are known to be all GABAergic and to provide inhibitory inputs to thalamic relay cells (Jones, 2007). Rt is of great importance for sleep regulation (Steriade, 1999), innervating thalamocortical relay neurons

with a feedback inhibition that influences thalamocortical rhythm generation (Deleuze and Huguenard, 2006). During episodes of NREMS, Rt neurons discharge in a slow, rhythmic burst-firing mode that is transmitted to thalamic relay nuclei and modulated by corticothalamic inputs, resulting in a widespread synchronization across neuronal assemblies (Steriade et al., 1991) and a modification of consciousness (Tononi and Massimini, 2008).

Another remarkable finding of the present study was the induction by UCM765 of the burst activity of Rt neurons. This activity pattern was MT_2-mediated since it was abolished by the intrathalamic microinfusion of a selective MT_2 receptor antagonist. Since this burst activity promotes NREMS, MT_2 receptors may thus be viewed as a key component in sleep regulation. The presence of MT_2 receptors on Rt GABA neurons reinforces the evidence for an allosteric interaction between MT_2 and $GABA_A$ receptors (Wan et al., 1999). The hypnotic effects of MLT are indeed blocked by BZ-$GABA_A$ antagonists in experimental animals (Wang et al., 2003a). However, the fact that MT_2 ligands have a distinct profile from BZs, and that BZs are still active in MT_2KOs, suggest that MT_2 ligands might modulate GABAergic activity in a differential manner compared to BZ-$GABA_A$ receptors, using, for example, a different intracellular pathway.

The signaling cascade through which UCM765 exerts its hypnotic effects remains to be elucidated, as well as the subregions of Rt involved in the promotion of NREMS mediated by MT_2 receptors. Moreover, the potential effects of UCM765 in other brain structures known to be implicated in sleep, such as the basal forebrain (which has projections to Rt), the ventrolateral preoptic nucleus, and the suprachiasmatic nucleus, await some clarification. Nevertheless, the selective promotion of NREMS through the activation of Rt neurons designates MT_2 receptors as a promising novel target for the treatment of insomnia and sleep disorders.

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G.G., G.T., G.S., A.B., M.M., S.R., and F.F. are inventors of Patent WO/2007/079593 (International Application No. PCT/CA2007/000055; publication date, July 19, 2007). The patent is owned by McGill University and Universities of Urbino, Parma, and Milan. G.G. is the founder of CosmasTherapeutics Development Inc.

Tables and figures

Table 1

Table 1. Selective effects of MT_2 agonist UCM765 on standard sleep parameters

Treatment group	NREM sleep latency (min)	NREM sleep total time (min)	REM sleep latency (min)	REM sleep total time (min)	Wakefulness total time (min)
A (rat)					
Vehicle (n = 7)	24.9 ± 2.8	60.9 ± 3.2	44.4 ± 3.0	11.2 ± 2.0	107.9 ± 4.7
UCM793 40 mg (n = 7)	22.5 ± 3.6	55.3 ± 5.6	61.6 ± 15.0	10.9 ± 2.2	113.2 ± 6.4
UCM793 60 mg (n = 6)	27.6 ± 3.7	58.8 ± 5.7	57.5 ± 10.2	9.3 ± 1.1	111.9 ± 5.7
UCM793 80 mg (n = 6)	22.8 ± 30.9	48.3 ± 5.2	56.4 ± 9.2	8.3 ± 0.4	123.4 ± 5.9
B (rat)					
Vehicle + vehicle (n = 6)	31.3 ± 4.2	53.7 ± 4.1	51.8 ± 6.0	7.3 ± 0.5	119.0 ± 4.6
Vehicle + UCM765 40 mg (n = 6)	19.5 ± 2.7*	72.6 ± 5.0$^{*,\#}$	75.3 ± 11.2	7.8 ± 1.2	99.6 ± 5.8$^{*,\#}$
4P-PDOT 10 mg + vehicle (n = 6)	25.8 ± 3.4	60.5 ± 5.2	75.6 ± 9.0	8.2 ± 1.5	111.3 ± 6.3
4P-PDOT 10 mg + UCM765 40 mg (n = 6)	20.7 ± 3.2	57.4 ± 5.7	65.8 ± 11.4	6.9 ± 0.8	115.7 ± 6.2
C (mouse)					
WT + vehicle (n = 8)	26.3 ± 1.9	64.4 ± 4.4	52.5 ± 6.2	6.4 ± 1.3	109.2 ± 5.7
WT + UCM765 40 mg (n = 8)	12.4 ± 1.6**	89.3 ± 5.6***	71.6 ± 10.0	6.1 ± 1.0	84.6 ± 5.7***
MT_1 KO + vehicle (n = 6)	38.5 ± 4.5	50.5 ± 3.9	63.3 ± 5.0	4.0 ± 0.5	125.6 ± 4.0
MT_1 KO + UCM765 40 mg (n = 6)	18.0 ± 2.6***	66.8 ± 3.8*	69.1 ± 11.7	3.5 ± 0.5	109.7 ± 3.0*
MT_2 KO + vehicle (n = 6)	27.9 ± 2.6	51.0 ± 4.0	63.8 ± 9.5	6.3 ± 0.8	122.7 ± 4.2
MT_2 KO + UCM765 40 mg (n = 6)	20.0 ± 1.4	48.8 ± 4.1	65.5 ± 11.1	5.8 ± 1.4	125.4 ± 5.8
D (mouse)					
MT_2 KO + vehicle (n = 6)	27.9 ± 2.6	51.0 ± 4.0	63.8 ± 9.5	6.3 ± 0.8	122.7 ± 4.2
MT_2 KO + DZ 2 mg (n = 4)	10.1 ± 0.7*	76.0 ± 2.8**	62.1 ± 12.3	5.1 ± 1.0	98.9 ± 2.3**

Table 1. EEG/EMG recordings are from 6:00 to 9:00 P.M. (lights off at 7:30 P.M.). Data are expressed as mean ± SEM; the number of animals per group is indicated in brackets. Group A, Lack of effect of increasing doses of the nonselective MT_1/MT_2 agonist class congener UCM793 (40, 60, and 80 mg/kg, s.c.) in rats. Group B, Blockade of UCM765 effects by the MT_2 antagonist 4P-PDOT in rats. Treatment with vehicle or UCM765 (40 mg/kg, s.c.) was administered 10 min after the pretreatment with vehicle or 4P-PDOT (10 mg/kg, s.c.). $*p < 0.05$, vehicle + UCM765 versus vehicle + vehicle; $^{\#}p < 0.05$, 4P-PDOT + UCM765 versus vehicle + UCM765, by SNK *post hoc* test. No differences were observed in latency and total time in REMS. Group C, Effects of UCM765 (40 mg/kg, s.c.) on sleep parameters in MT_2 and MT_1 receptor KO and WT mice. UCM765 decreased the latency to sleep in WT and MT_1 KO, but not in MT_2 KO mice. UCM765 increased NREMS total time in MT_1 KO, but not in MT_2 KO mice. REMS latency was not modified in MT_1 KO and MT_2 KO mice, and UCM765 had no effect on latency and total time in REMS. UCM765 decreased the total time of wakefulness in WT and MT_1 KO, but not in MT_2 KO mice. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ by Fisher's PLSD test comparing vehicle versus UCM765 treatment. Group D, Diazepam (2 mg/kg, s.c.) was still active in MT_2 KO mice. $*p < 0.05$; $**p < 0.01$ by Student's *t* test.

Table 2

Table 2. Firing and burst activity parameters of reticular thalamic neurons recorded *in vivo* in the basal condition and after administration of vehicle and UCM765 (10 mg/kg, i.v.)

	Spontaneous firing rate activity				**Spontaneous burst activity**				
	Firing rate (Hz)	Interspike interval (s)	Coefficient of variation (%)	Number of bursts (3 min)	Spikes in bursts (%)	Spikes per burst	Burst interspike interval (ms)	Burst length (ms)	Intraburst firing rate (Hz)
Treatment groups									
Basal (n = 8)	2.3 ± 0.7	1.2 ± 0.4	72.0 ± 21.0	23.4 ± 8.0	16.2 ± 3.1	6.6 ± 1.2	13.0 ± 2.2	99.0 ± 31.8	118.5 ± 36.5
Vehicle (n = 8)	2.2 ± 0.8	1.2 ± 0.3	72.8 ± 15.0	19.1 ± 6.3	21.8 ± 7.2	6.5 ± 1.2	11.1 ± 1.7	74.5 ± 17.1	121.8 ± 31.0
UCM765 20 mg (n = 8)	4.4 ± 0.9***	0.3 ± 0.1*	68.6 ± 13.4	55.0 ± 8.0***	37.9 ± 8.0**	9.9 ± 1.5*	16.9 ± 2.8	164.6 ± 32.1	67.4 ± 7.4
Statistics	$F_{(2,14)}$=12.8	$F_{(2,14)}$=4.0	$F_{(2,14)}$=0.04	$F_{(2,14)}$=14.3	$F_{(2,14)}$=9.9	$F_{(2,14)}$=4.7	$F_{(2,14)}$=1.2	$F_{(2,14)}$=2.3	$F_{(2,14)}$=1.7
	***p* < 0.001**	***p* = 0.04**	p = 0.9	***p* < 0.001**	***p* = 0.002**	***p* = 0.02**	p = 0.3	p = 0.1	p = 0.2

Data are expressed as mean ± SEM from eight neurons (1 neuron per rat).

*p < 0.05; **p < 0.01; ***p < 0.001, by SNK *post hoc* test.

Figure 1

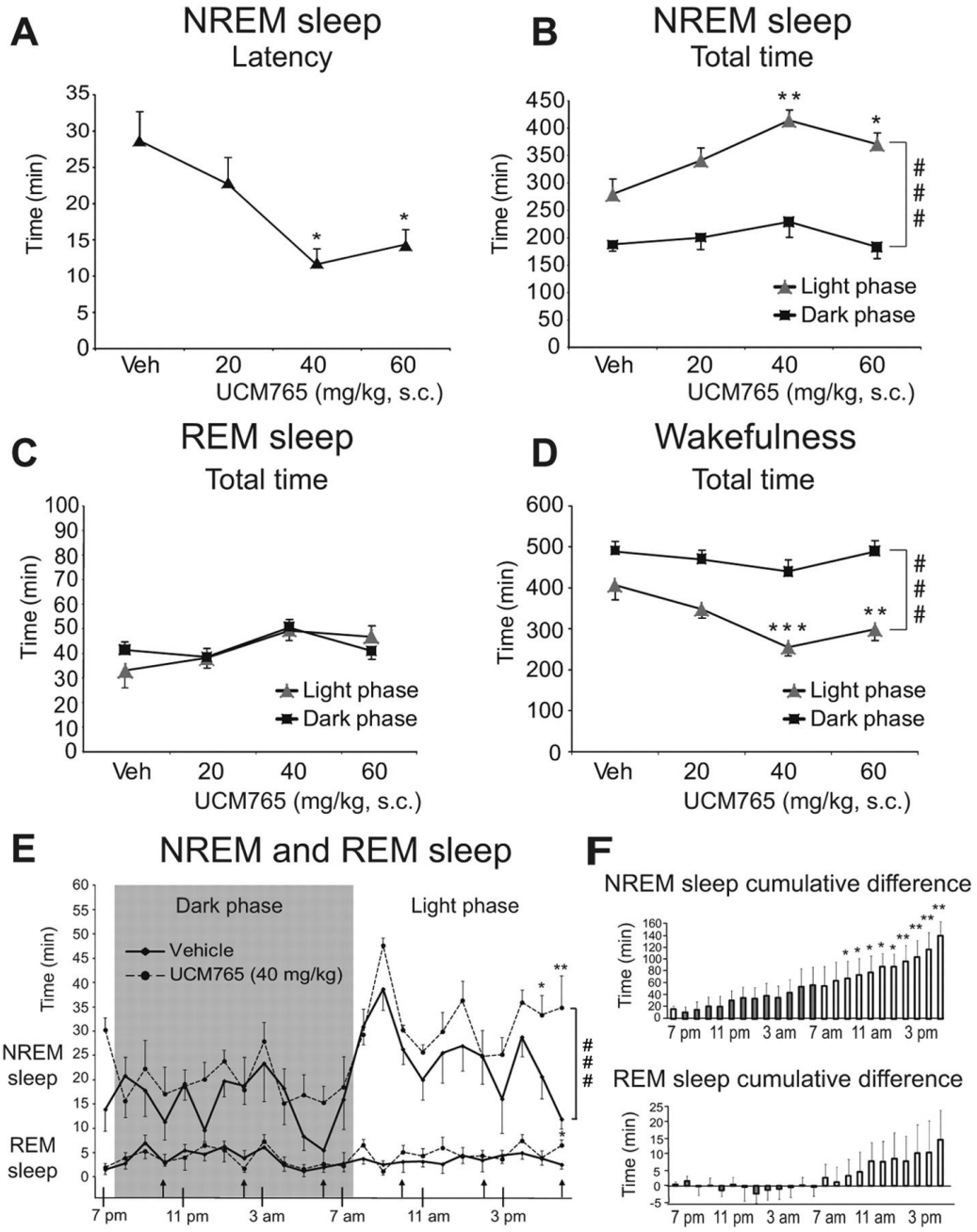
A
NREM sleep
Latency
Time (min)
Veh
20
40
60
UCM765 (mg/kg, s.c.)
B
NREM sleep
Total time
Light phase
Dark phase
C
REM sleep
Total time
D
Wakefulness
Total time
E
NREM and REM sleep
Dark phase
Light phase
Vehicle
UCM765 (40 mg/kg)
NREM sleep
REM sleep
7 pm
11 pm
3 am
7 am
11 am
3 pm
F
NREM sleep cumulative difference
REM sleep cumulative difference

Figure 1. Promoting effect of UCM765 on sleep parameters (24 h recordings; light on at 7:30 A.M. and light off at 7:30 P.M.). Four different groups of rats were used: vehicle (n = 5) and UCM765 at doses of 20 mg/kg (n = 6), 40 mg/kg (n = 5), and 60 mg/kg (n = 6), injected subcutaneously every 4 h (first injection at 6:00 P.M.). The means ± SEM are shown. A, UCM765 (40 and 60 mg/kg) decreased the latency of the first NREMS episode. B, UCM765 increased NREMS total time. C, UCM765 had no effect on total time in REMS. D, UCM765 decreased wakefulness total time. ###$p < 0.001$, light versus dark phase, by SNK post hoc test. E, Effects of UCM765 on NREMS and REMS total time: 1 h interval analysis during 24 h in 5 rats. Top, UCM765 (40 mg/kg) increased the amount of NREMS during the inactive/light phase (white background), but not during the active/dark phase (gray background), with a concomitant reduction in wakefulness total time. Bottom, REMS was not modified by UCM765 (40 mg/kg). ###$p < 0.001$, vehicle versus UCM765 treatment, by SNK post hoc test. F, Cumulative differences between UCM765 and vehicle over 24 h EEG/EMG recording. Top, NREMS. Bottom, REMS. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus vehicle by SNK post hoc test.

Figure 2

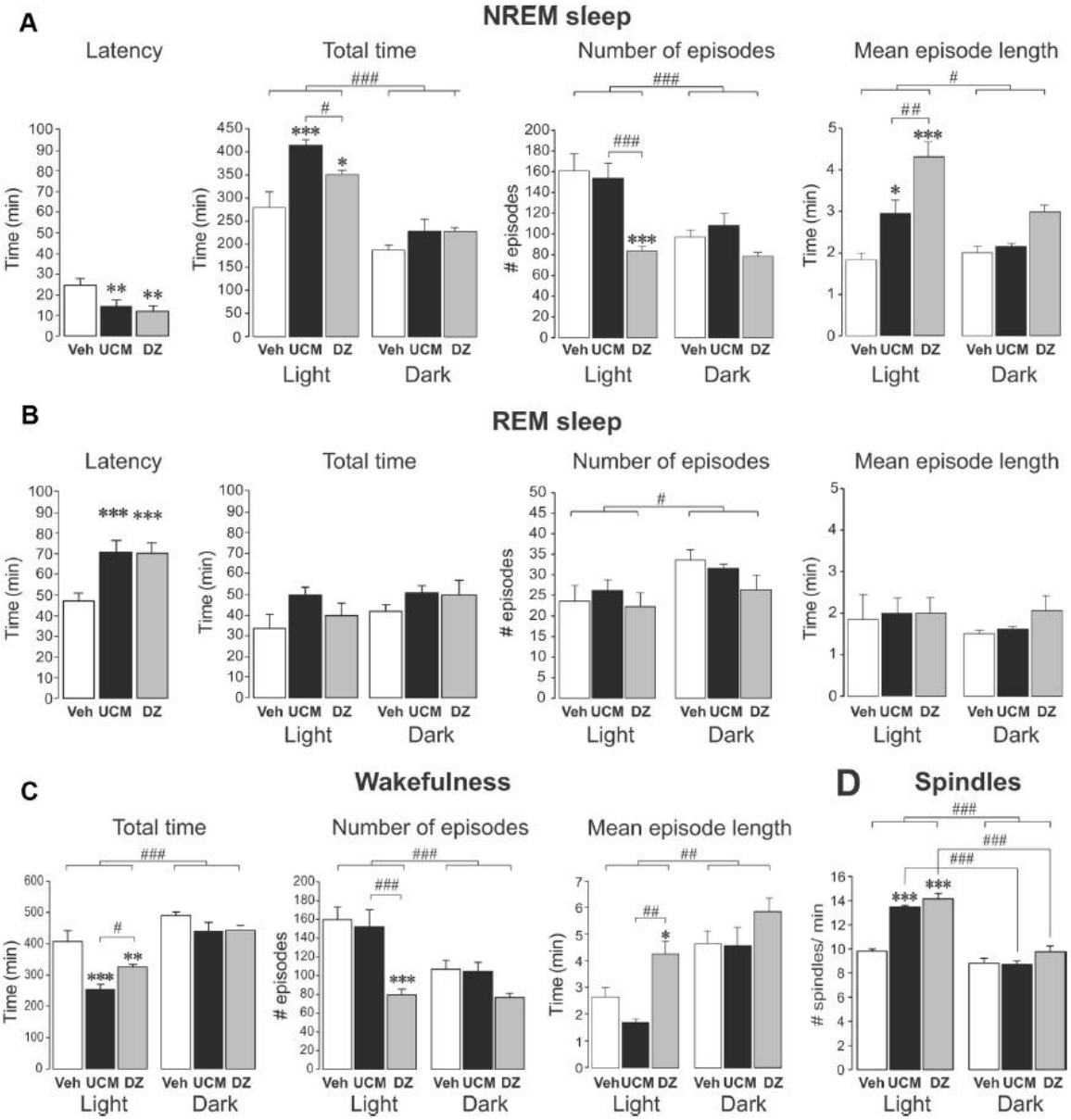
A
NREM sleep
Latency
Total time
Number of episodes
Mean episode length
Time (min)
episodes
Veh UCM DZ
Light
Dark
B
REM sleep
C
Wakefulness
D
Spindles
spindles/ min

Figure 2. UCM765 (UCM) promotes NREMS like DZ [24 h recordings; light on at 7:30 AM, light off at 7:30 P.M.; UCM, 40 mg/kg, s.c., every 4 h (n = 5); DZ, 2 mg/kg, s.c., every 12 h (n = 6); first injection at 6:00 P.M.]. The means ± SEM are shown. A, Like DZ, UCM765 decreased the latency for NREMS. UCM765 and DZ increased NREMS during the inactive/light phase only (left). DZ, but not UCM765, markedly reduced the number of NREMS episodes (center). UCM765 and DZ increased mean NREMS episode length during the light phase only (right). B, Both UCM765 and DZ increased REMS latency, without apparent effect on REMS total time. The number of REMS episodes appeared to be affected by phase of day, but not treatment. The mean REMS episode length was not affected by the treatment and phase of day. C, Both UCM765 and DZ decreased the total time of wakefulness during the inactive/light phase (left). DZ, but not UCM765, considerably decreased the number of episodes of wakefulness during the light phase (center). DZ, but not UCM765, significantly increased the mean wakefulness episode length (right). D, Number of spindles. UCM765 and DZ increased the number of spindles per minute during the light phase. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus vehicle; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, DZ versus UCM765 or light versus dark phase, by SNK post hoc test.

Figure 3

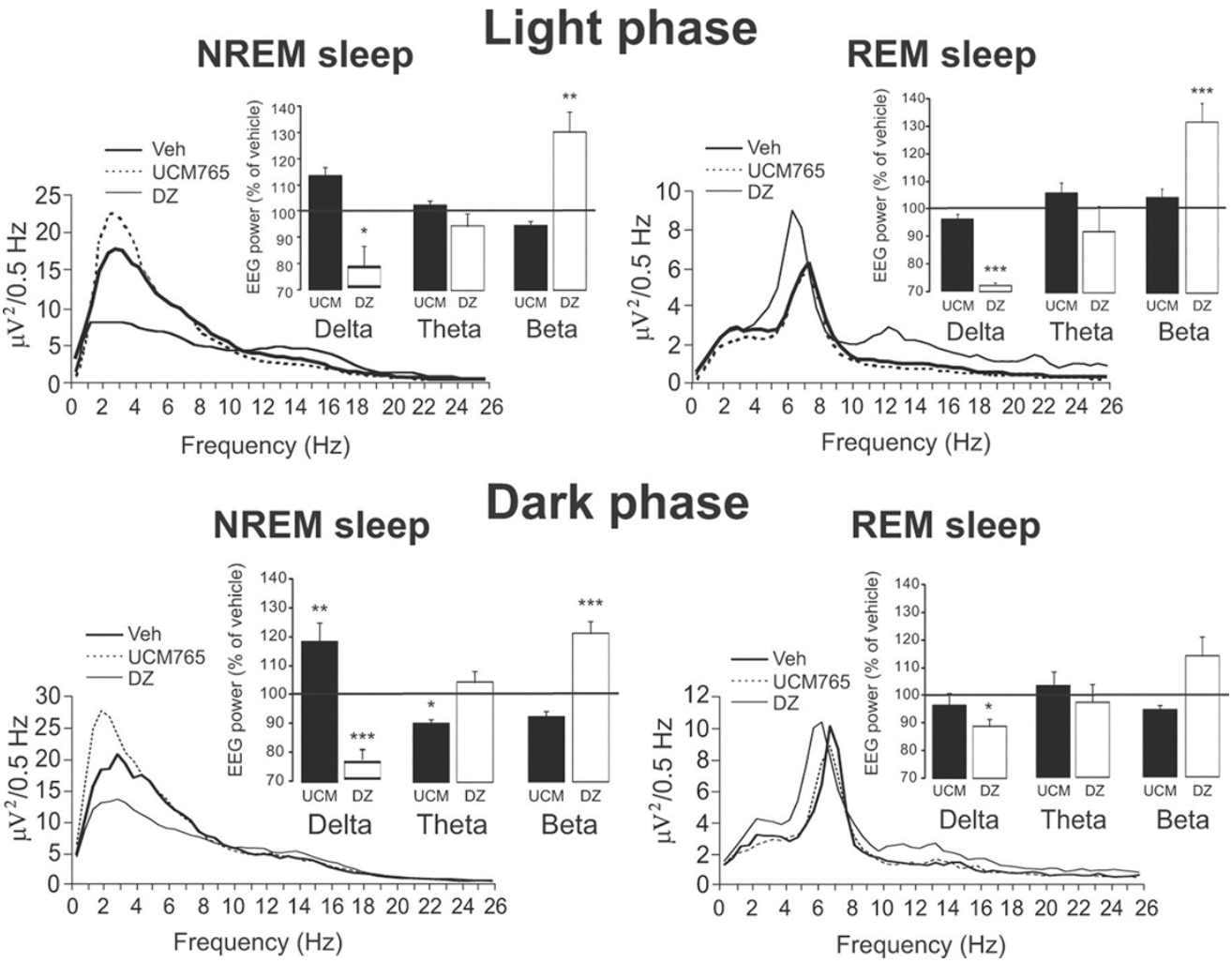

Figure 3. Power spectra of NREMS (left traces) and REMS (right traces) after treatment with vehicle (bold lines), UCM765 (dotted lines), and DZ (thin lines) during the light (top) and dark (bottom) phases. Insets, EEG power spectra after UCM765 (UCM) and DZ treatments relative to those of vehicle (100%, horizontal line) expressed as the mean percentage change (±SEM). The mean percentage power density was calculated as the mean power (in square microvolts) in each 0.5 Hz frequency bin divided by the total power (0–25 Hz) in the same epoch. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, by Bonferroni's post hoc test versus control (vehicle).

Figure 4

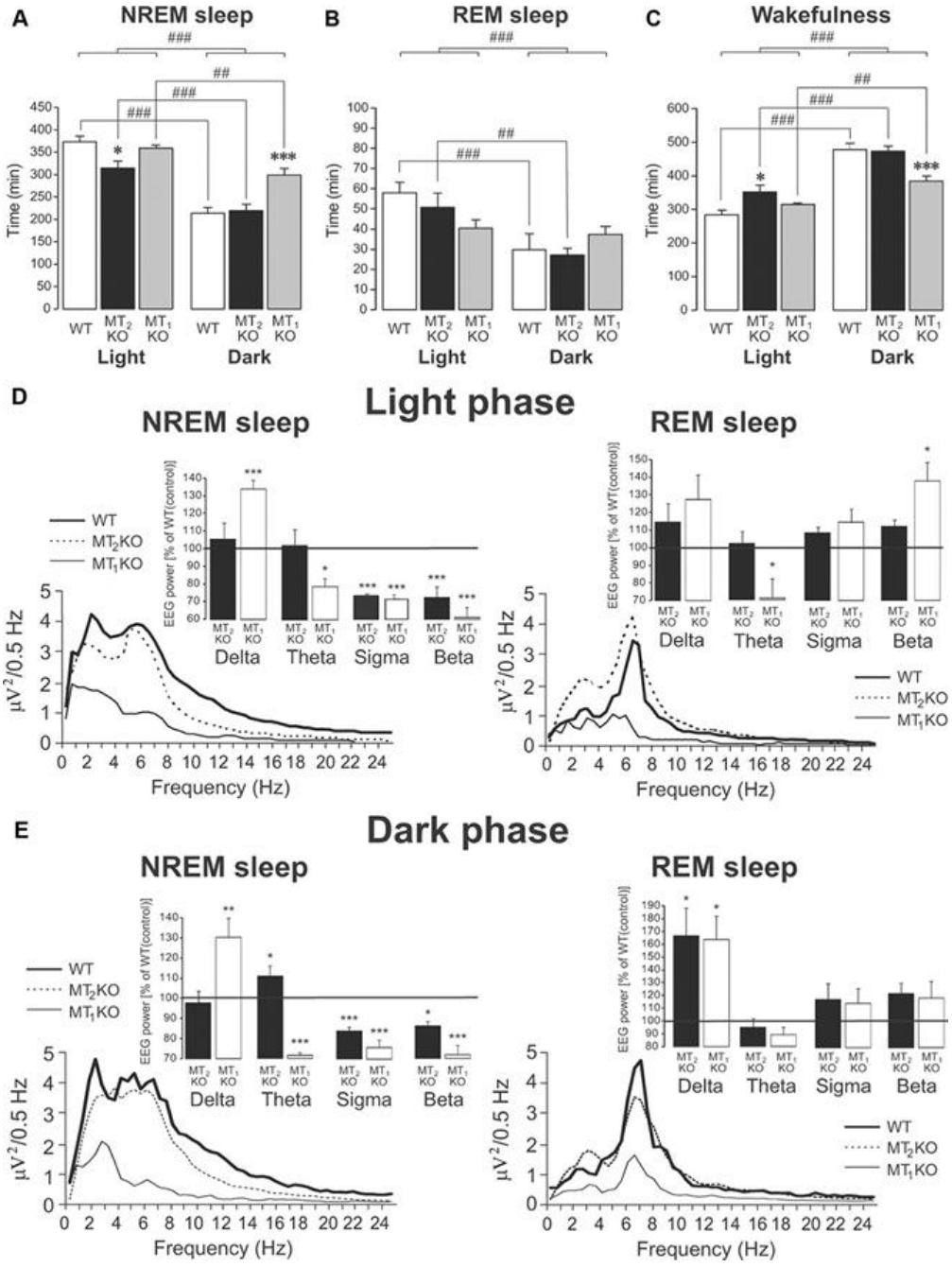
A
NREM sleep
B
REM sleep
C
Wakefulness
Time (min)
WT
MT2 KO
MT1 KO
Light
Dark
D
Light phase
NREM sleep
REM sleep
WT
MT2KO
MT1KO
EEG power [% of WT(control)]
Delta
Theta
Sigma
Beta
μV²/0.5 Hz
Frequency (Hz)
E
Dark phase
NREM sleep
REM sleep

Figure 4. Twenty-four hour sleep recordings in WT (n = 5), MT_2 KO (n = 5) and MT_1 KO (n = 5) mice (light on at 7:30 AM and off at 7:30 P.M.). The recordings started at 6:00 P.M. The means ± SEM are shown. A, NREMS during the light and dark phases. Compared to WT and MT_1 KO mice, MT_2 KO mice showed a decrease in NREMS during the inactive/light phase only. MT_1 KO mice showed an increase in NREMS during the active/dark phase. B, REMS during the light and dark phases. Both WT and MT_2 KO mice displayed longer REMS during the light/inactive phase compared to the active/dark phase. This difference was blunted in MT_1 KO mice. C, Wakefulness during the light and dark phases. MT_2 KO mice showed an increase of wakefulness compared to WT and MT_1 KO mice, during the light/inactive phase only. MT_1 KO mice showed a decreased in wakefulness during the dark/active phase. Note that wakefulness in the dark phase differed between MT_1 KO and MT_2 KO mice ($p < 0.001$; data not shown). *$p < 0.05$, ***$p < 0.001$ versus vehicle; ##$p < 0.01$, ###$p < 0.001$ light versus dark phase by SNK post hoc test. D, E, Power spectra of NREMS (left traces) and REMS (right traces) in WT (bold lines), MT_2 KO (dotted lines) and MT_1 KO (thin lines) mice during the light (D) and dark (E) phases. Insets, EEG power spectra in MT_1 and MT_2 KO mice, expressed as a mean percentage (±SEM) change relative to those of control (WT; 100%; horizontal line). The mean percentage power density was calculated as the mean power (in square microvolts) in each 0.5 Hz frequency bin divided by the total power (0–25 Hz) in the same epoch. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, by Bonferroni's post hoc test versus control (WT mice).

Figure 5

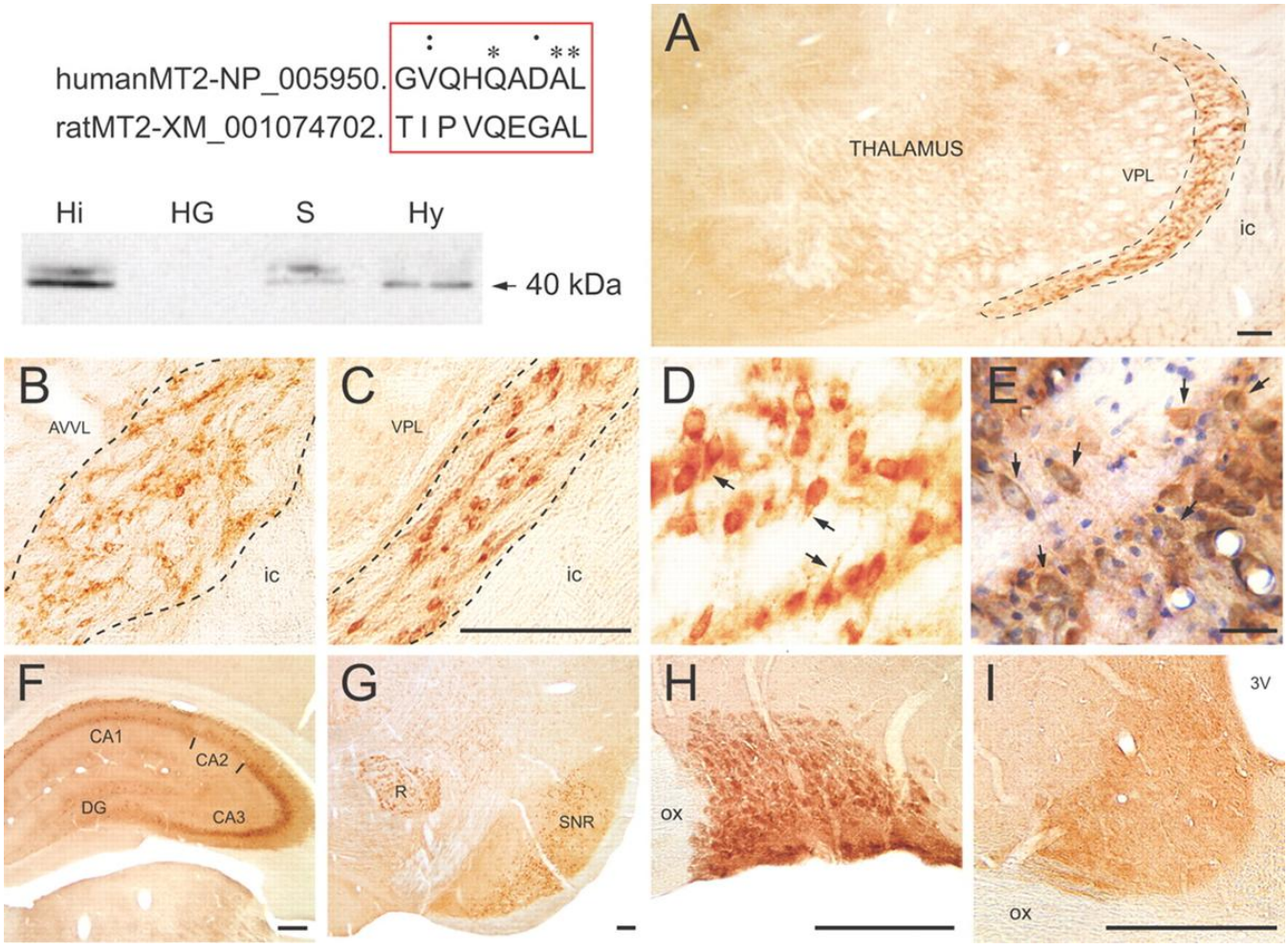
humanMT2-NP_005950. GVQHQADAL
ratMT2-XM_001074702. T I P VQEGAL
Hi
HG
S
Hy
40 kDa
A
THALAMUS
VPL
ic
B
AVVL
ic
C
VPL
ic
D
E
F
CA1
CA2
DG
CA3
G
R
SNR
H
ox
I
3V
ox

Figure 5. Light microscopic visualization of MT_2 receptor immunoreactivity. Top left, Alignment between human and rat MT_2 protein sequences, and Western blots performed on homogenates from hippocampus (Hi), Harderian gland (HG; negative control), septum (S), and hypothalamus (Hy). Polyclonal anti-MT_2 receptor antibodies decorate a doublet of ~40 kDa corresponding to the MT_2 receptor. A–I, Particular aspects of the anatomical distribution of the MT_2 receptor immunoreactivity in adult rat brain. A–E, Low- (A) and higher-magnification pictures (B–E) of the somatodendritic MT_2 receptor labeling in Rt. A corresponds to the middle rostrocaudal level across the nucleus (outlined) at which electrophysiological recordings and microinjections were made. Note the lack of immunoreactivity in adjacent thalamic nuclei, and notably in the ventral posterolateral thalamic nucleus (VPL). B, C, The neuronal MT_2 labeling in the rostral (B) and the caudal (C) thirds of Rt (outlined). D is within the middle third of Rt and illustrates the somatodendritic nature of MT_2 immunoreactivity. E is from a similar area as D in a section counterstained with cresyl violet. As exemplified at arrows, all Rt neurons are MT_2 immunoreactive. F–I, Other brain regions displaying MT_2 immunoreactivity: dorsal hippocampus (F), red nucleus (R) and substantia nigra reticulata (SNR) (G), supraoptic nucleus (H), and suprachiasmatic nucleus (I). Scale bars: A, C, F–I, 250 μm; D, E, 50 μm. 3V, Third ventricle; AVVL, anteroventral, ventrolateral thalamic nucleus; CA1, CA2, CA3, Ammon's horn sectors; DG, dentate gyrus; ic, internal capsule; ox, optic chiasma.

Figure 6

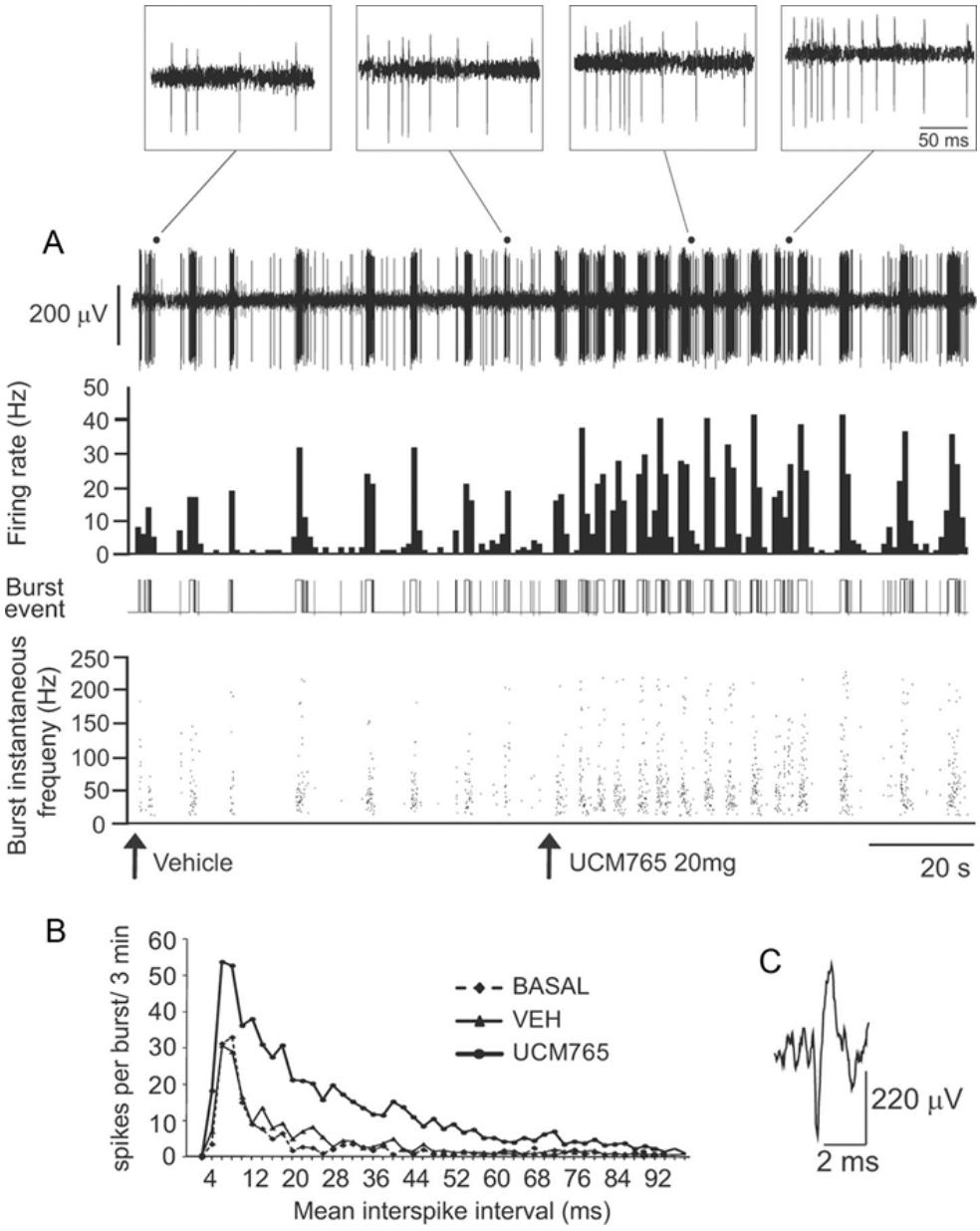
50 ms
A
200 μV
Firing rate (Hz)
50
40
30
20
10
0
Burst event
Burst instantaneous frequeny (Hz)
250
200
150
100
50
0
Vehicle
UCM765 20mg
20 s
B
spikes per burst/ 3 min
60
50
40
30
20
10
0
BASAL
VEH
UCM765
4 12 20 28 36 44 52 60 68 76 84 92
Mean interspike interval (ms)
C
220 μV
2 ms

Figure 6. UCM765 in vivo enhanced the firing rate and rhythmic burst activity of Rt neurons. A, Single-neuron activity recording (first trace), with integrated histogram of spontaneous firing rate (second trace), burst event (third trace), and burst instantaneous frequency (fourth trace) after injection of vehicle or UCM765 (20 mg/kg, i.v.). Arrows indicate the start of injection. The time scale (20 s) applies to all four traces. B, Plot of mean interspike intervals (in milliseconds) against the number of spikes per burst, for 3 min in the basal condition and immediately after the intravenous injection of vehicle or UCM765. The mean from eight neurons (1 neuron per rat) is shown. C, Typical waveform of Rt neurons. Top insets, Amplification of recordings after vehicle and UCM765 injections, visualizing the typical accelerando-decelerando pattern of Rt neurons characterized by an initial part with frequency acceleration (200–250 Hz) and deceleration, followed by long-lasting tonic barrage of spikes of ~100 Hz (tail). Note that after UCM765 injection, the number of spikes per burst increases. Time scale, 50 ms.

Figure 7

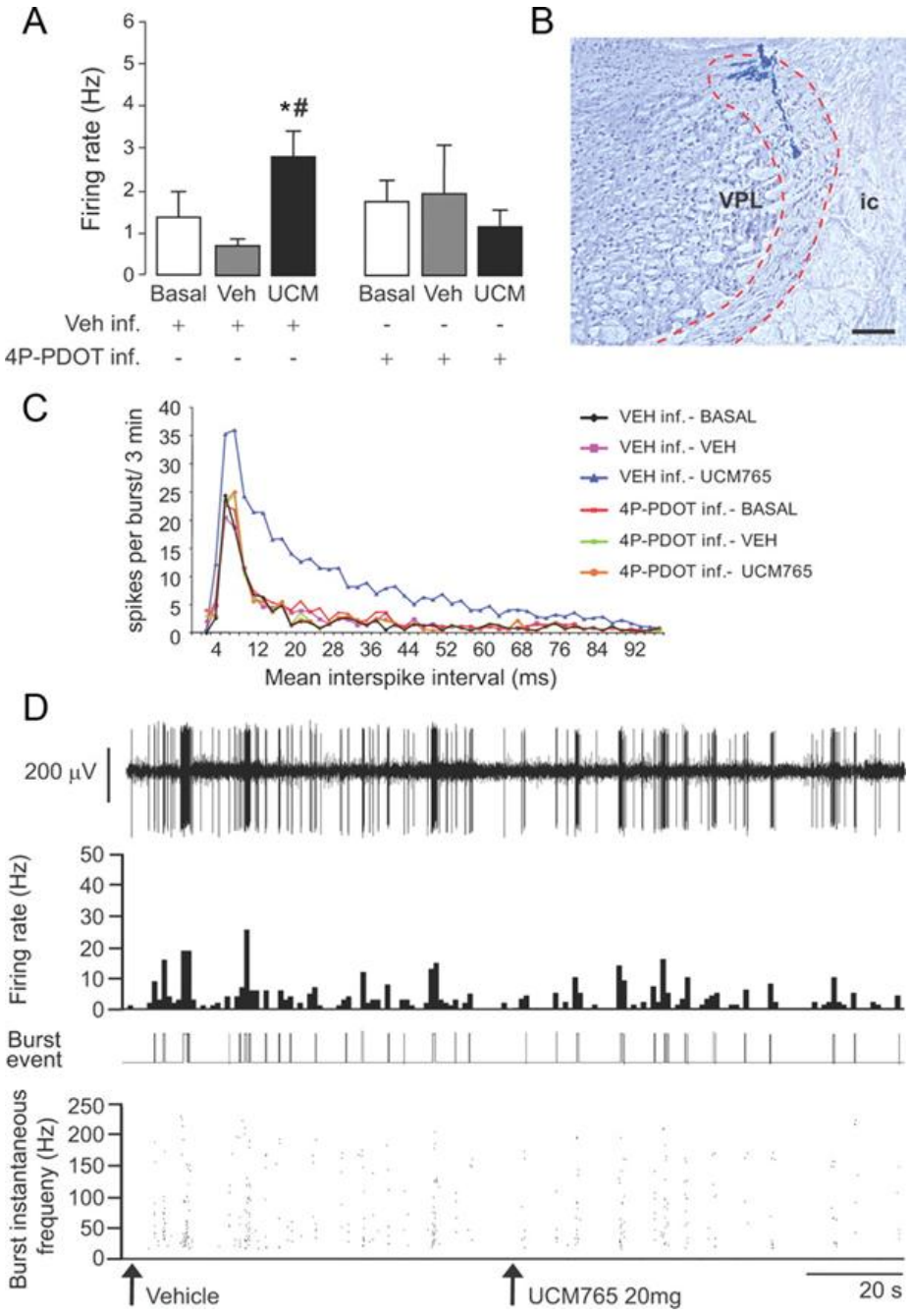
A
Firing rate (Hz)
*#
Basal Veh UCM Basal Veh UCM
Veh inf. + + + - - -
4P-PDOT inf. - - - + + +
B
VPL
ic
C
spikes per burst/ 3 min
Mean interspike interval (ms)
VEH inf.- BASAL
VEH inf.- VEH
VEH inf.- UCM765
4P-PDOT inf.- BASAL
4P-PDOT inf.- VEH
4P-PDOT inf.- UCM765
D
200 µV
Firing rate (Hz)
Burst event
Burst instantaneous frequeny (Hz)
Vehicle
UCM765 20mg
20 s

Figure 7. The effects of UCM765 on reticular thalamic neurons are mediated by MT_2 receptors. Microinfusion of the MT_2 antagonist 4P-PDOT into rat Rt prevents the firing rate and burst neuronal activation induced by UCM765 (20 mg/kg, i.v.; n = 7) A, Spontaneous firing rate (in hertz) of Rt neurons after either microinfusion of vehicle (Veh) into Rt followed by intravenous injection of vehicle or UCM765 (UCM), or microinfusion of 4P-PDOT (10 μg) followed by intravenous injection of vehicle or UCM765 (20 mg/kg). *$p < 0.05$, Veh inf. plus UCM765 versus Veh inf. plus Veh and versus Veh inf. plus basal; #$p < 0.05$, Veh.inf. plus UCM765 versus 4P-PDOT inf. plus UCM765, by SNK post hoc test. The means ± SEM from seven neurons (1 neuron per rat) are shown. B, Histological control of a recording site in Rt. The electrode track may be traced into the inner aspect of the dorsal Rt, at a middle rostrocaudal level across the nucleus. VPL, Ventral posterolateral thalamic nucleus; ic, internal capsule. Scale bar, 250 μm. C, Plot of mean interspike intervals (ms) against the number of spikes per burst for 3 min. The mean from seven neurons (1 neuron per rat) is shown. D, Single-neuron activity recording example (first trace), with integrated histogram of spontaneous firing rate (second trace), burst event (third trace), and burst instantaneous frequency (fourth trace); 4P-PDOT (10 μg) was microinfused into Rt prior the intravenous injections of vehicle and UCM765 (20 mg/kg). The time scale (20 s) applies to all four traces. Arrows indicate the start of intravenous injection.

Figure 8

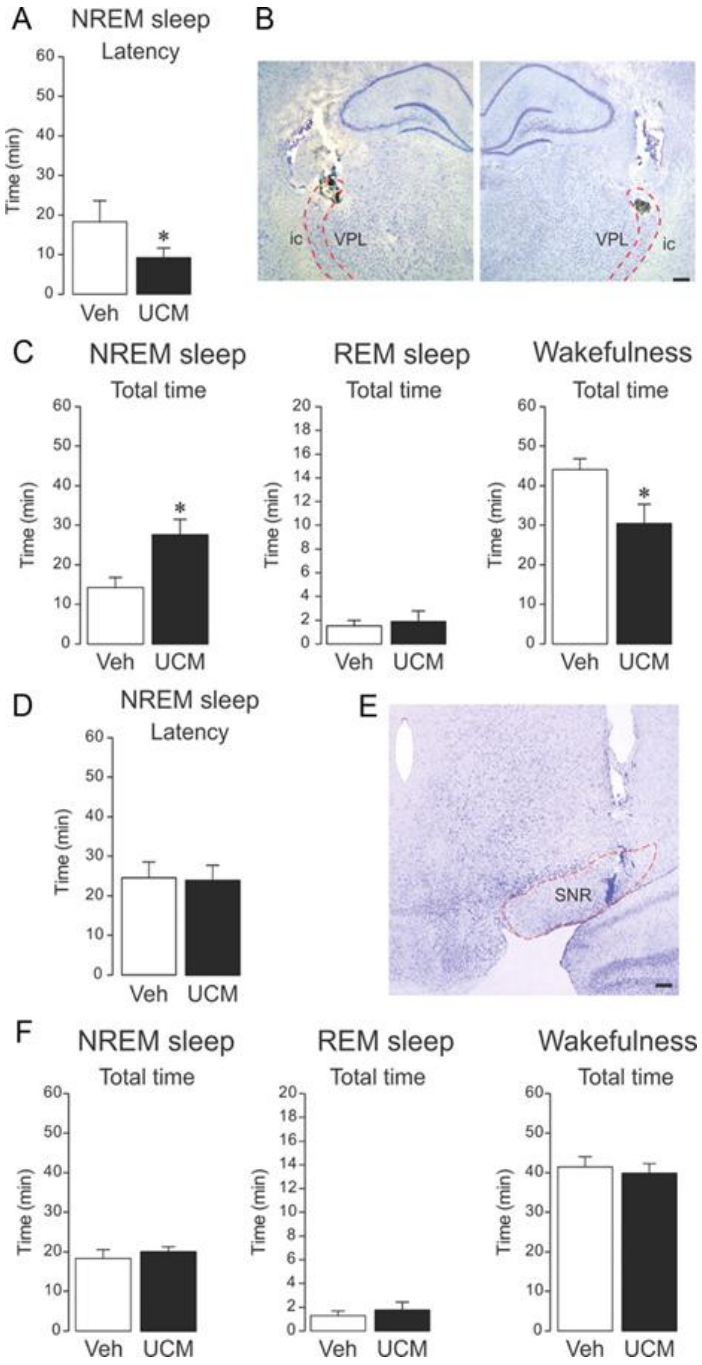
A
NREM sleep
Latency
Time (min)
Veh
UCM
B
ic
VPL
VPL
ic
C
NREM sleep
Total time
REM sleep
Total time
Wakefulness
Total time
D
NREM sleep
Latency
E
SNR
F
NREM sleep
Total time
REM sleep
Total time
Wakefulness
Total time

Figure 8. Local microinfusion of UCM765 into reticular thalamus promotes NREMS, whereas microinfusion into substantia nigra pars reticulata (SNR) does not affect sleep–wake parameters in freely moving non-anesthetized rats. A–C, Effects of bilateral microinfusion of UCM765 (10 μg) into the Rt and concomitant EEG/EMG recordings from 6:00 to 7:00 P.M. The means ± SEM from five rats are shown. A, UCM765 decreased the latency of the first episode of sleep. *$p < 0.05$. B, Histological control of bilateral microinfusion sites. On both sides, the lesion and deposit of pontamine sky blue is located in the middle third of the dorsal Rt. Abbreviations are as in Figure 7B. C, UCM765 increased the total time of NREMS and reduced wakefulness. *$p < 0.05$, t test. D–F, Effects of bilateral microinfusion of UCM765 (10 μg) into SNR and concomitant EEG/EMG recordings from 6:00 to 7:00 P.M. The means ± SEM from eight rats are shown. D, Latency of the first NREMS episode. E, Histological control of microinfusion site: the pontamine sky blue deposit is located within the SNR. F, Total time of NREMS, REMS, and wakefulness. Scale bars: 250 μm.

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2.2 Interim discussion

Here, we demonstrated that the MT_2 receptors expressed in Rt neurons are involved in sleep regulation and modulate the sleep promoting effects of UCM765. The UCM765 dose-response pattern was a threshold model with low doses being inert while high doses were active, reaching a plateau effect after 40 mg/Kg. Notably, the effects of UCM765 were observed during the light but not the dark phase, mostly at the end of the day. These effects are likely explained by internalization and desensitization of MT_2 receptors during the night, when higher levels of MLT desensitize MT_2 receptors, whereas the expression and sensitivity of MT_2 receptors is not decreased during the light phase when levels of MLT are low and below the threshold to induce desensitization. We hypothesized that sub-maximal activation of MT_2 receptors by the partial agonist UCM765 may represent an advantage over MLT itself (full agonist) because of its lower or absence of desensitization of MT_2 receptors.

Then, we confirmed that the sleep-promoting effects of UCM765 are mediated by MT_2 receptors since such effects are inhibited by the MT_2 antagonist 4P-PDOT and by the genetic deletion of these receptors. In addition, the negative results obtained with the non-selective MT_1/MT_2 agonist UCM793 also indicate that selective activation of MT_2 receptors is needed to induce and maintain sleep.

Our immunohistochemical experiments demonstrated the localization of MT_2 receptors in the Rt nucleus. This was a key finding because the GABAergic neurons located in this nucleus are widely involved in sleep regulation, in particular the bursting pattern of these neurons are associated with the generation of sleep spindles during NREMS (Steriade et al., 1993). Besides the enhancement of sleep spindles in the EEG, the systemic administration of UCM765 (20 mg/kg; i.v.) increases the firing rate and bursting activity of Rt neurons, whereas such effects are blocked by 4P-PDOT. Bilateral microinfusion of UCM765 into the Rt nucleus was sufficient to induce sleep in rats, an effect nullified by the microinfusion of 4P-PDOT. Besides, when UCM765 was microinfused into another area rich in MT_2 receptors, such as the substantia nigra, there were no apparent changes in sleep behaviour. The overall results confirm the key role of MT_2 receptors including those expressed in Rt neurons in sleep regulation as well as in the mechanism of action of UCM765.

Further experiments are needed to fully elucidate the single role of MT_2 receptors in sleep and the mechanism of action of UCM765. We hypothesized that other brain regions implicated in sleep regulation that express MT_2 receptors may be involved. Nevertheless, the MT_2 receptors expressed in the Rt nucleus and other brain regions should be considered a new target for the treatment of sleep disorders.

Foreword to Chapter III

Sleep–wake characterization of double MT_1/MT_2 receptor knockout mice and comparison with MT_1 and MT_2 receptor knockout mice

MLT is a neurohormone associated with the regulation of sleep and circadian rhythms. Most of the physiological actions of MLT in the brain are mediated by MT_1 and MT_2 receptors (Dubocovich and Markowska 2005). However, the mechanism by which MLT regulates sleep and the single role of MT_2 and MT_1 receptors in the regulation of the different sleep stages is not fully understood. One reason for this is due to the limited number of selective agonists and antagonists for MT_1 or MT_2 receptors. In addition, there is a lack of sleep studies in MT_2KO and MT_1KO mice; and double MT_1/MT_2KO mice.

Previously, we demonstrated the single role of the MT_2 receptor in the sleep-wake cycle by using a selective MT_2 agonist and experiments in MT_2KO and MT_1KO mice. In particular, we demonstrated that the MT_2 receptor regulates NREMS only during the light phase, whereas MT_1 receptors are involved in changes during the dark phase, including increased NREMS and overall changes in power spectra. Interestingly, the 24 h sleep-wake cycle in MT_1KO mice was disrupted; an effect that might explain the NREMS changes at night. In keeping with this view, the sleep-wake cycle in MT_2KO mice, a strain that express MT_1 receptors, is not altered, thereby suggesting that the changes detected in MT_1KO mice are likely due to alterations in the 24 h sleep-wake cycle rather than direct changes in NREMS. However, to fully elucidate the role of the MT_2 receptor in sleep, we need to understand the interaction between MT_2 and MT_1 receptors, which may exert differential functions in sleep.

Therefore, we carried out 24 h EEG recordings in MT_1/MT_2KO mice to compare them to those of MT_1KO, MT_2KO and WT mice. Since the sleep architecture in mice is more fragmented than in rats, in this chapter we carried out a more detail analysis, for instance, the minimum episode duration used to discriminate between different vigilante states was 4 s instead of the 10 s used in chapter II. Moreover, we looked at other parameters not considered in our first study such as the 1 h interval analysis as well as the number and length of episodes for the three vigilance states in mice.

The effect of the double genetic inactivation of MT_1/MT_2 receptors was quite difficult to predict, although based on our previous study we expected the MT_1/MT_2KO mice to possibly have an impairment in all three vigilance states, in particular NREMS which is regulated by the MT_2 receptor or disruption of the 24 h sleep-wake cycle due to the lack of the MT_1 receptor.

This chapter confirms the different role of the MT_1 and MT_2 receptors in sleep: the MT_2 receptor regulates NREMS whiles the MT_1 receptor controls REMS, both effects are associated with increased wakefulness duration.

Notably, this is the first study in MT_1/MT_2KO mice and interestingly, we demonstrate that the double inactivation of MT_1/MT_2 receptors increases total wakefulness time with no significant changes in NREMS or REMS duration. It is likely that the lack of MT_1 and MT_2 receptors have minimal effects (reduction) on NREMS and REMS, respectively, across the 24 h light/dark cycle that contributes to significant changes in wakefulness total time in MT_1/MT_2KO mice.

Chapter III

Sleep–wake characterization of double MT_1/MT_2 receptor knockout mice and comparison with MT_1 and MT_2 receptor knockout mice

3.1 Research paper

Sleep–wake characterization of double MT_1/MT_2 receptor knockout mice and comparison with MT_1 and MT_2 receptor knockout mice

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Abstract

The neurohormone melatonin activates two G-protein coupled receptors, MT_1 and MT_2. Melatonin is implicated in circadian rhythms and sleep regulation, but the role of its receptors remains to be defined. We have therefore characterized the spontaneous vigilance states in wild-type (WT) mice and in three different types of transgenic mice: mice with genetic inactivation of MT_1 ($MT_1^{-/-}$), MT_2 ($MT_2^{-/-}$) and both MT_1/MT_2 ($MT_1^{-/-}/MT_2^{-/-}$) receptors. Electroencephalographic (EEG) and electromyographic sleep–wake patterns were recorded across the 24-h light–dark cycle. $MT_1^{-/-}$mice displayed a decrease (−37.3%) of the 24-h rapid eye movement sleep (REMS) time whereas $MT_2^{-/-}$mice showed a decrease (−17.3%) of the 24-h non rapid eye movement sleep (NREMS) time and an increase in wakefulness time (14.8%). These differences were the result of changes occurring in particular during the light/inactive phase. Surprisingly, $MT_1^{-/-}/MT_2^{-/-}$ mice showed only an increase (8.9%) of the time spent awake during the 24-h. These changes were correlated to a decrease of the REMS EEG theta power in $MT_1^{-/-}$mice, of the NREMS EEG delta power in $MT_2^{-/-}$mice, and an increase of the REMS and wakefulness EEG theta power in $MT_1^{-/-}/MT_2^{-/-}$ mice. Our results show that the genetic inactivation of both MT_1 and MT_2 receptors produces an increase of wakefulness, likely as a result of reduced NREMS due to the lack of MT_2 receptors, and reduced REMS induced by the lack of MT_1 receptors. Therefore, each melatonin receptor subtype differently regulates the vigilance states: MT_2 receptors mainly NREMS, whereas MT_1 receptors REMS.

1. Introduction

The neurohormone melatonin (MLT), synthesized by the pineal gland during the dark period of the light/dark cycle, acts mostly through two G protein-coupled receptors, MT1 and MT_2, with high affinity for MLT (Ki ≈ 0.1 nM). It can also act through MT_3 receptors, which are the homologue of the human quinone reductase, but with lower affinity (Kd = 3–9 nM)[1], and with receptor-independent mechanisms [2]. MLT acts at both central and peripheral levels affecting circadian rhythms, sleep, mood, cardiovascular and immune systems, metabolism, cell proliferation, and pain [3].

MLT, by acting through MT_1 and MT_2 receptors, controls the activity of the suprachiasmatic nucleus (SCN) [4], [5] and [6], the "master clock", which drives circadian manifestations of behavior and body physiology [7] and [8]. Sleep is regulated by circadian and homeostatic processes [8]; however, it is still a matter of debate whether MLT indirectly regulates sleep through the control of circadian processes such as body temperature or modulates the activity of brain nuclei directly involved in the regulation of sleep. Several human [9], [10] and [11] and animal [12] and [13] studies have shown hypnotic properties of exogenous MLT, but others have failed to demonstrate these effects [14] and [15]. Methodological (dosage, time of administration, monitoring of the wake-sleep cycle) as well as chemical properties of MLT (short half life 40 min [16]) can partially explain such contrasting findings. However, it is generally accepted that MLT can increase sleep propensity by shortening the latency to sleep, rather than by increasing total sleep time and sleep efficiency [7] and [17]; yet, the physiological and molecular mechanisms by which the melatonergic system promotes sleep and controls the sleep–wake cycle have yet to be fully elucidated. Moreover, evidence on the role played by MT_1 and MT_2 receptors in sleep regulation is scant, and there are no sleep studies in knockout (KO) mice for both MT_1 and MT_2 receptors ($MT_1^{-/-}/MT_2^{-/-}$). For these reasons, the main goal of the present study was to fill the gap in knowledge regarding the involvement of each MLT receptor in the control of sleep by conducting 24-h electroencephalographic (EEG) and electromyographic (EMG) recordings in $MT_1^{-/-}/MT_2^{-/-}$ mice.

Our recent preliminary data [18] in MT_1 ($MT_1^{-/-}$) and MT_2 ($MT_2^{-/-}$) receptor KO mice suggested complementary functions of MT_1 and MT_2 receptors. Here, by examining the 24-h

vigilance states in $MT_1^{-/-}/MT_2^{-/-}$ mice and then comparing them with those of $MT_1^{-/-}$ and $MT_2^{-/-}$ mice, we provide a better comprehension on the role of each MLT receptor subtype in the regulation of the sleep–wake cycle.

2. Materials and methods

Experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care and the Animal Care Committee of McGill University.

2.1. Animals

Adult male C3H/He wildtype (WT) mice (n = 7, 28–35 g, Charles River, St-Constant, QC, Canada), and mice of the same genetic background (back-crossed to C3H/He females for more than 20 generations) lacking MT_1 receptors ($MT_1^{-/-}$) (n = 7), MT_2 receptors ($MT_2^{-/-}$) (n = 7), or both MT_1 and MT_2 receptors ($MT_1^{-/-}/MT_2^{-/-}$) (n = 7) [5] and [6] were used in this study. KO mice were kindly provided by Drs. Weaver and Reppert (Univ. of Massachusetts, USA). The number of animals was chosen based on a power analysis from our preliminary data in $MT_1^{-/-}$and $MT_2^{-/-}$mice [18]: for the NREMS amount, the minimum detectable difference was 13.86 min and the standard deviation of residuals 13.99 min. Having 4 groups and anticipating a similar effect size, in order to obtain a power of 0.80 with an alpha of 0.01, 7 animals per group were required. All animals were housed at 22 °C under a 12:12 h light:dark cycle (light on:7 AM; light off: 7 PM), with free access to food and water.

2.2. Surgery

EEG and EMG electrodes were implanted in mice deeply anaesthetized with equithesin. For EEG monitoring, three stainless-steel epidural electrodes were positioned according to Franklin and Paxinos atlas [19] through 1.5 mm burr holes: one over the parietal cortex on each side, and the third–as a reference – in the right parietal cortex (AP – 0.95 mm and L – 2 mm, AP – 2.85 mm and L – 2 mm, AP – 1.9 mm and L + 2 mm with respect to bregma). For EMG monitoring, three flexible stainless-steel wire electrodes were implanted into the neck muscles (two bilaterally and one medially). The EMG recording was made as the difference signal between two electrodes and the third grounding electrode helped to minimize 50 or 60 Hz line

interference (for details: Grass Technologies, Astro-Med Inc., Brossard, QC, CA). All electrodes were soldered to a six-pin female connector and the entire assembly was secured to the skull with dental acrylic (Coltene/Whaledent Inc.). Twenty-four hour after surgery, mice were placed in the recording room and connected for eight hour the first two days and constantly for the following four days to the recording cable (a flexible 6-braid cable, Tensolite Co.) in a freely-moving manner to allow for habituation.

After 6 days of recovery and habituation, the spontaneous sleep–wake cycle was recorded for 24-h starting at the onset of the light phase (7 AM).

2.3. EEG and EMG data analysis

EEG/EMG signals were amplified at a total gain of 10,000 and filtered locally (EEG: low filter 1 Hz; high 1 kHz; EMG: low filter 30 Hz; high 3 kHz, Grass, P55). The signals were digitized at 128 Hz by an analogical/digital converter (CED power 1401, Cambridge Electronic Design, Cambridge, UK) connected to a PC in which data were stored and analyzed offline using the Spike 2 software (CED, Cambridge, UK). Consecutive 2 s epochs were subjected to a Fast Fourier Transform (FFT), and EEG power spectra density was computed in the frequency range of 0–64 Hz.

The three classical vigilance states were determined for 4-s epochs on the basis of the cortical EEG and neck EMG and a bout of a specific sleep state was considered as one or more consecutive epochs. Analysis was carried out using a Spike2 script kindly provided by Cambridge Electronic Design (Cambridge, UK), followed by a visual scoring on a PC screen to correct for epoch misassignments (7.4 ± 0.7% for NREMS, 13.3 ± 1.2% for REMS, and 9.5 ± 0.6% for wakefulness). Vigilance states (NREMS, REMS and wakefulness) were characterized according to previously described criteria [18]. Only periods of typical stationary EEG and EMG lasting at least 10 s were considered for the analysis of power spectra. Power in the 0–26 Hz range of artifact-free epoch was averaged (96.3 ± 1.2% of recording time), and the mean values were plotted in 0.5 Hz bins. The power in each frequency band (delta: 1–4 Hz; theta: 4–9 Hz; alpha: 8–12 Hz; sigma: 11–15 Hz) and the total power (0–26 Hz) were calculated by summing the power in 0.5 Hz bins.

2.4. Statistical analyses

Statistical analysis was carried out using SigmaPlot (version 11.0; San Jose, California). Data were expressed as mean ± S.E.M. After testing for assumptions of normality of data distribution and homogeneity of variance, differences between genotypes in the three vigilance states were computed using one-way ANOVA or two-way mixed-design ANOVA followed by the Student–Newman–Keuls (SNK) test for post hoc comparison. Spectral power differences in delta, theta, sigma and alpha bands were assessed using one-way ANOVA followed by Fisher's LSD post hoc test. Statistical values reaching $P \leq 0.05$ were considered significant.

3. Results

3.1. 24-h Vigilance states

As reported in Table 1, $MT_1^{-/-}$ mice displayed decreased 24-h REMS time compared to WT ($P = 0.038$) mice. Differently, $MT_2^{-/-}$ mice showed decreased 24-h NREMS time and increased time awake compared to WT ($P = 0.010$ and $P < 0.001$, respectively) and $MT_1^{-/-}$ ($P = 0.002$ and $P = 0.003$) mice. Surprisingly, $MT_1^{-/-}/MT_2^{-/-}$ mice did not show significant differences for daily NREMS and REMS variables (time, number of episodes and mean episode duration), but spent more time awake ($P = 0.031$) when compared to WT controls. Moreover, $MT_1^{-/-}/MT_2^{-/-}$ mice exhibited decreased NREMS time compared to $MT_1^{-/-}$ mice ($P = 0.049$). In $MT_1^{-/-}$ mice, the mean episode duration of wakefulness was decreased compared to $MT_1^{-/-}/MT_2^{-/-}$ ($P = 0.043$) and $MT_2^{-/-}$ ($P = 0.018$) mice.

3.2. Vigilance states during the light phase

The changes observed in the 24-h vigilance states in MLT receptor knockout mice are mainly the result of alterations occurring during the light/inactive phase (Table 2). A robust decrease in REMS time was observed in $MT_1^{-/-}$ mice compared to WT and $MT_1^{-/-}/MT_2^{-/-}$ mice ($P = 0.005$ and $P = 0.05$, respectively). In $MT_2^{-/-}$ mice, NREMS time and the duration of episodes were decreased ($P = 0.005$ and $P = 0.014$, respectively), while wakefulness time and duration of episodes were increased ($P = 0.002$ and $P = 0.012$, respectively) compared to WT mice. $MT_1^{-/-}/MT_2^{-/-}$ mice did not show any differences in REMS/NREMS or total wakefulness

time; they only showed shorter NREMS episodes compared to WT ($P = 0.012$). Comparing $MT_2^{-/-}$ *versus* $MT_1^{-/-}$ mice, there was a decreased NREMS time ($P = 0.006$) and an increased wakefulness time ($P = 0.044$) in the former genotype.

3.3. Vigilance states during the dark phase

As shown in Table 2, no alterations of NREMS, REMS and wakefulness variables were observed in $MT_2^{-/-}$ and $MT_1^{-/-}/MT_2^{-/-}$ mice during the dark phase. $MT_1^{-/-}$ mice instead displayed higher number of episodes of wakefulness than WT ($P = 0.013$), $MT_1^{-/-}/MT_2^{-/-}$ ($P = 0.015$) and $MT_2^{-/-}$ ($P = 0.021$) mice.

3.4. Hour-by-hour sleep–wake cycle

Analyzing hour by hour the 24-h sleep–wake cycle (Fig. 1), a significantly different NREMS profile was found between WT and $MT_2^{-/-}$ ($P = 0.041$) and $MT_1^{-/-}$ and $MT_2^{-/-}$ ($P = 0.031$) mice (Fig. 1A; genotype × time of the day: $F_{69,552} = 1.32$, $P = 0.034$; genotype: $F_{3,552} = 3.54$, $P = 0.030$; time of the day: $F_{23,552} = 7.33$, $P < 0.001$). $MT_1^{-/-}$ mice showed a different REMS profile than WT ($P = 0.013$), $MT_1^{-/-}/MT_2^{-/-}$ ($P = 0.027$) and $MT_2^{-/-}$ ($P = 0.014$) mice (Fig. 1B; genotype: $F_{3,552} = 4.49$, $P = 0.012$). A different wakefulness hour by hour profile (Fig. 1C; genotype: $F_{3,552} = 8.14$, $P < 0.001$) was observed between $MT_2^{-/-}$ and WT ($P < 0.001$) and $MT_2^{-/-}$ and $MT_1^{-/-}$ ($P = 0.005$) mice. Noteworthy, no differences between the hour by hour profiles of $MT_1^{-/-}/MT_2^{-/-}$ and WT mice were detected for the three vigilance states, meaning that the increased time of wakefulness found in $MT_1^{-/-}/MT_2^{-/-}$ mice during 24-h is due to non significant small changes in the vigilance states occurring across all the 24-h. These small changes became significant only when considered as total.

3.5. EEG power spectrum

3.5.1. EEG power spectrum during the light phase (Fig. 2, top)

The NREMS EEG power spectra (Fig. 2, top left) of WT mice was different than that of $MT_1^{-/-}/MT_2^{-/-}$ ($P = 0.022$) and $MT_1^{-/-}$ ($P < 0.001$) mice (Two-way mixed-design ANOVA, genotype: $F_{3,24} = 7.76$, $P < 0.001$; frequency: $F_{48,1151} = 67.98$, $P < 0.001$; interaction:

$F_{144,1151} = 2.18$, $P < 0.001$). Moreover, SNK *post hoc* analysis revealed a difference between NREMS EEG power spectra of $MT_1^{-/-}$ and $MT_2^{-/-}$ mice ($P = 0.029$). Analysis of the NREMS EEG total power between 0 and 26 Hz (Fig. 2, inset) confirmed a main difference due to genotype (One-way ANOVA, genotype: $F_{3,24} = 6.79$, $P = 0.002$). In particular, $MT_1^{-/-}$ and $MT_2^{-/-}$ mice displayed decreased EEG spectral power in the frequency range 0–26 Hz ($P < 0.001$ and $P = 0.026$, respectively) whereas in $MT_1^{-/-}/MT_2^{-/-}$ mice there was a similar decrease but only closed to significance ($P = 0.067$). REMS EEG power spectra (Fig. 2, top center) was also deeply modified after genetic inactivation of MLT receptors (genotype: $F_{3,24} = 4.80$, $P = 0.009$; frequency: $F_{48,1151} = 64.57$, $P < 0.001$; interaction: $F_{144,1151} = 5.89$, $P < 0.001$). $MT_1^{-/-}/MT_2^{-/-}$ mice exhibited a significantly different power spectra compared to WT ($P = 0.014$), but also to $MT_1^{-/-}$ ($P = 0.008$) and $MT_2^{-/-}$ ($P = 0.016$) mice. When the REMS total power (0–26 Hz) was compared between genotypes, no significant effect was found ($F_{3,24} = 1.27$, $P = 0.30$), meaning that differences between mice were limited to only specific frequency bands. EEG power spectra of wakefulness (Fig. 2, top right) of $MT_1^{-/-}/MT_2^{-/-}$ mice was significantly different than that of WT ($P = 0.044$) and $MT_2^{-/-}$ ($P = 0.002$) mice (genotype: $F_{3,24} = 5.71$, $P = 0.004$; frequency: $F_{48,1151} = 47.00$, $P < 0.001$; interaction: $F_{144,1151} = 2.47$, $P < 0.001$). Wakefulness EEG total power (Fig. 2, inset) was affected by genotype ($F_{3,24} = 6.30$, $P = 0.003$) and in particular, $MT_1^{-/-}/MT_2^{-/-}$ mice displayed higher EEG total power than WT ($P = 0.047$) while $MT_2^{-/-}$ mice lower ($P = 0.034$). For the specific frequency bins in the power spectra in which a significant difference between WT and MLT receptor KO mice was found, please refer to the bars underneath the spectra.

3.5.2. EEG power spectrum during the dark phase (Fig. 2, bottom)

Similarly to the light phase, EEG power spectra during the dark phase were deeply altered after genetic inactivation of MLT receptors (Fig. 2, bottom). Significant effects due to genotype ($F_{3,24} = 3.64$, $P = 0.027$) and frequency ($F_{48,1151} = 42.07$, $P < 0.001$) and an interaction genotype × frequency ($F_{144,1151} = 3.22$, $P < 0.001$) was found for NREMS EEG power spectra (Fig. 2, bottom left). SNK *post hoc* analysis showed that when comparing the power spectra of $MT_1^{-/-}$ with that of WT and $MT_1^{-/-}/MT_2^{-/-}$ mice, the differences were very closed to significance ($P = 0.053$ and $P = 0.051$, respectively). NREMS EEG total power between 0 and 26 Hz (genotype: $F_{3,24} = 7.14$, $P = 0.001$) was also significantly reduced in $MT_1^{-/-}/MT_2^{-/-}$ ($P = 0.017$),

$MT_1^{-/-}$ ($P < 0.001$) and $MT_2^{-/-}$ ($P = 0.003$) mice (Fig. 2 bottom, inset). No differences between genotypes were detected for the REMS EEG power spectra (genotype: $F_{3,24} = 2.66$, $P = 0.071$; frequency: $F_{48,1151} = 56.31$, $P < 0.001$; interaction: $F_{144,1151} = 4.26$, $P < 0.001$; Fig. 2, bottom center). However, when considering REMS EEG total power (0–26 Hz), an effect due to genotype was found ($F_{3,24} = 3.01$, $P = 0.05$). The EEG power spectra of wakefulness was also significantly modified after removal of MLT receptors (genotype: $F_{3,24} = 2.94$, $P = 0.054$; frequency: $F_{48,1151} = 35.74$, $P < 0.001$; interaction: $F_{144,1151} = 1.23$, $P = 0.043$). Interestingly, the wakefulness EEG power spectra of $MT_1^{-/-}/MT_2^{-/-}$ mice was significantly different than that of $MT_2^{-/-}$ mice ($P = 0.032$). Accordingly, total wake EEG spectral power (0–26 Hz) was lower in $MT_2^{-/-}$ than in WT ($P = 0.011$), $MT_1^{-/-}/MT_2^{-/-}$ ($P = 0.016$) and $MT_1^{-/-}$ ($P = 0.022$) mice ($F_{3,24} = 3.40$, $P = 0.034$; (Fig. 2, bottom right, inset).

For the specific frequency bins in the power spectra in which a significant difference between WT and MLT receptor KO mice was found, please refer to the bars underneath the spectra.

3.6. Effects of MLT receptors on the EEG power of delta, theta, sigma and alpha bands

Since we found several modifications of the EEG power spectra in MLT receptor KO mice for all three vigilance states, in order to quantify the magnitude of these spectral changes, we separately analyzed the powers of delta, theta, sigma and alpha bands (Fig. 3).

3.6.1. EEG spectral power during the light phase (Fig. 3, left)

3.6.1.1. NREMS

$MT_1^{-/-}/MT_2^{-/-}$ mice displayed a significant decrease of NREMS EEG spectral power in the sigma band compared to WT mice ($P = 0.027$; effect of genotype: $F_{3,24} = 3.39$, $P = 0.034$), and in the delta band compared to $MT_1^{-/-}$ mice ($P = 0.14$; $F_{3,24} = 5.23$, $P = 0.006$). $MT_1^{-/-}$ mice showed reduced delta ($P = 0.006$), theta ($P = 0.028$; $F_{3,24} = 3.07$, $P = 0.047$) and sigma ($P = 0.043$) EEG powers compared to WT. $MT_2^{-/-}$ mice exhibited lower sigma EEG power than WT ($P = 0.047$).

3.6.1.2. REMS

Genetic inactivation of one or both MLT receptors affected REMS theta band only ($F_{3,24}$ = 3.23, P = 0.040). Importantly, $MT_1^{-/-}/MT_2^{-/-}$ mice showed higher EEG theta power than WT (P = 0.05), $MT_1^{-/-}$ (P = 0.006), and $MT_2^{-/-}$ (P < 0.049) mice.

3.6.1.3. Wakefulness

Delta band of wakefulness was not significantly affected by removal of one or both MLT receptors, but $MT_1^{-/-}/MT_2^{-/-}$ mice showed increased EEG power in the theta band compared to WT (P = 0.017; $F_{3,24}$ = 6.86, P = 0.002). $MT_1^{-/-}/MT_2^{-/-}$ mice also displayed higher EEG theta power than $MT_1^{-/-}$ and $MT_2^{-/-}$ mice (P = 0.015 and P = 0.001, respectively) and higher EEG alpha power than $MT_2^{-/-}$ mice (P = 0.031; $F_{3,24}$ = 3.03, P = 0.049).

3.6.2. EEG spectral power during the dark phase (Fig. 3, right)

3.6.2.1. NREMS

During the dark phase, no significant effect due to the genetic inactivation of both MLT receptors was observed in the different spectral bands of NREMS. Conversely, removal of MT_1 receptors led to a decrease in delta (P = 0.004; $F_{3,24}$ = 3.77, P = 0.024), theta (P = 0.006; $F_{3,24}$ = 3.10, P = 0.046) and sigma (P = 0.008, $F_{3,24}$ = 3.27, P = 0.038) EEG powers, and of MT_2 receptors in delta and sigma EEG powers (P = 0.032 and P = 0.021, respectively).

3.6.2.2. REMS

REMS delta and sigma EEG powers were significantly lower than controls in $MT_1^{-/-}/MT_2^{-/-}$ mice (delta: P = 0.05; $F_{3,24}$ = 3.28, P = 0.038; sigma: P = 0.043; $F_{3,24}$ = 3.03, P = 0.049). Moreover, EEG delta power was lower in $MT_1^{-/-}/MT_2^{-/-}$ than in $MT_2^{-/-}$ mice (P = 0.045) and EEG theta power was higher in $MT_1^{-/-}/MT_2^{-/-}$ than in $MT_1^{-/-}$ mice (P = 0.033; $F_{3,24}$ = 3.59, P = 0.028). Compared to WT, EEG theta power was reduced in $MT_1^{-/-}$ and $MT_2^{-/-}$ mice (P = 0.006 and P = 0.05, respectively) and sigma EEG power was lower in $MT_1^{-/-}$ mice (P = 0.008).

3.6.2.3. Wakefulness

No significant changes in delta, theta and alpha EEG powers were observed in $MT_1^{-/-}/MT_2^{-/-}$ mice. However, EEG delta raw spectral power was affected by genotype ($F_{3,24} = 3.43$, $P = 0.033$). Indeed, EEG delta power was reduced in $MT_2^{-/-}$ mice ($P = 0.041$) compared to WT. Even though wakefulness EEG theta power in $MT_2^{-/-}$ mice seemed to be lower than in WT, the effect of genotype on the EEG power of this frequency band was only close to significance ($F_{3,24} = 2.90$, $P = 0.056$).

4. Discussion

The characterization of the 24-h sleep–wake cycle in mice lacking both MLT receptors is here reported for the first time in comparison with mice lacking MT_1 or MT_2 receptors. Our data show that $MT_1^{-/-}/MT_2^{-/-}$ mice spent more time awake during 24-h and surprisingly, no significant effects were observed for NREMS and REMS times. Since $MT_1^{-/-}$ mice exhibited a decrease in REMS time and $MT_2^{-/-}$ mice a decrease in NREMS time paralleled by an increase in the time awake, the increase of wakefulness in $MT_1^{-/-}/MT_2^{-/-}$ can be interpreted as the result of reduced NREMS due to the lack of the MT_2 receptors and reduced REMS induced by the lack of MT_1 receptors. Indeed $MT_1^{-/-}/MT_2^{-/-}$ mice show a slight non-significant decrease in both NREMS ($P = 0.102$) and REMS ($P = 0.142$ during the light phase) duration, and a significant increase in wakefulness. The increase of the 24-h amount of wakefulness in $MT_1^{-/-}/MT_2^{-/-}$ mice was associated with modifications of the power spectra of the three vigilance states. In particular, there was an increase in the EEG theta power of REMS and wakefulness especially during the light/inactive phase of the 12-h/12-h light/dark cycle.

Consequently, a question arises: why does the lack of MT_1 or MT_2 receptors alone produce a decrease of REMS and NREMS times respectively, while the lack of both receptors led to increased wakefulness only? Differential or opposite effects produced by the activation of MLT receptors have been already reported for other physiological functions. At the level of the vascular system, MT_1 and MT_2 receptors act in the opposite manner by producing vasoconstriction or vasodilatation, respectively [20]. At the level of the SCN, conflicting effects are reported. *In vitro* experiments using SCN slices showed that MT_1 receptors control the

neuronal firing rate and MT_2 receptors the phase shift-circadian rhythm of the neuronal firing [4]. *In vivo*, a MLT injection phase-shifted the SCN activity onset of WT but not of $MT_1^{-/-}$ mice and also accelerated the entrainment to a new light-dark cycle in WT but not $MT_1^{-/-}$ mice [4] and [5]. Regarding sleep, the considerable effects we observed in $MT_1^{-/-}$ and $MT_2^{-/-}$ mice likely not only derive from the lack of one of the two receptors, but also may be a result of complementary effects due to the presence of the other MLT receptor subtype. Conversely, when both MLT receptors are lacking, the reducing effects of MT_1 and MT_2 receptors on REMS and NREMS times become minimal but sufficient to significantly increase the time spent awake only if we consider the whole 24-h sleep–wake cycle. These minimal changes over the 24-h period are confirmed by the hour by hour analysis of wakefulness in which no differences between WT controls and $MT_1^{-/-}/MT_2^{-/-}$ mice were found. This hypothesis explains animal as well as human studies which were not able to demonstrate robust effects of MLT or analogs on sleep.

Clinical studies conducted with MLT or other non selective MT_1/MT_2 receptor agonists such as ramelteon [21] and tasimelteon [22] showed only limited effects on sleep (mainly in shortening sleep latency), thus confirming that the dual activation of MT_1/MT_2 receptors produced only modest or null effects on sleep. Interestingly, these human studies were mainly focused on sleep without analyzing potential effects of MLT or non-selective MT_1/MT_2 receptor agonists on the waking state variables; consequently, further research should test whether the observed marginal sleep effects are paralleled by changes in wakefulness. In this direction, a study by Wehr et al. [23] showed that MLT has a relative rather than an absolute effect on sleep and sleepiness, and that there is a link between the onset and/or offset of MLT secretion and the wake EEG theta power.

In our preliminary study using $MT_1^{-/-}$ and $MT_2^{-/-}$ mice [18], we found that the NREMS time was higher in $MT_1^{-/-}$ than in WT mice (+40%) during the dark phase, while here, this difference is marginal and not significant (+10%). However, analyzing the sleep–wake cycle of $MT_1^{-/-}$ mice hour by hour, we found that at the middle of the dark phase (from 11 PM to 1 AM), NREMS time was increased compared to WT mice (11–12 PM, +61.5%, $P = 0.042$; 12 PM–1 AM, +76.8%, $P = 0.049$; Fig. 1). This apparent discrepancy derives from the different kind of EEG analysis employed in the two studies. In the previous study [18], being interested in the analysis of the time of the three vigilance states in mice in comparison to pharmacological data

in rats, we scored the sleep stages by 10 s epochs as used in several other sleep investigations in mice [24], [25] and [26]. Here, we aimed to better characterize the sleep microarchitecture of MLT receptor KO mice; consequently, we decided to employ a 4 s epoch analysis. Comparing the two analyses in $MT_1^{-/-}$ mice, we observed that in this genotype there were several 4 s short episodes of wakefulness during the NREMS that were not evident in the 10 s analysis. Remarkably, following the results by McShane et al. [27], who mathematically compared sleep analyses by 10 s *vs.* 4 s epochs, these findings indicate that NREMS microarchitecture in $MT_1^{-/-}$ mice is highly fragmented and impaired. Particularly, this fragmented NREMS microarchitecture is paralleled by significant reductions of NREMS delta, theta and sigma EEG powers, suggesting that the lack of MT_1 receptors significantly impairs NREMS.

Importantly, our data also demonstrate that MT_1 and MT_2 receptors differentially, and sometimes in complementary ways, modulate the vigilance states. MT_2 receptors seem selectively related to NREMS regulation since $MT_2^{-/-}$ mice exhibit a significant decrease of NREMS time. This selective role of MT_2 receptors on NREMS was further confirmed by our recent pharmacological data in rats and mice in which the MT_2 receptor partial agonist UCM765 produced an increase in NREMS time, an effect that was blocked by the MT_2 receptor antagonist 4P-PDOT [18]. In addition, the selective MT_2 receptor full agonist IIK7 also showed sleep-promoting properties in rats by increasing NREMS time without affecting REMS time [28]. We have also demonstrated that the MT_2 selective partial agonists UCM765 was more potent than its non selective MT_1/MT_2 conger UCM793 in sleep promoting effect [18], thus confirming the hypothesis that the modulation of one single receptor is more effective than the modulation of both of them. Conversely, MT_1 receptors modulate REMS and also arousal by controlling the number of episodes of wakefulness during the dark/active phase. The effect on REMS time observed in $MT_1^{-/-}$ mice is likely the result of the decrease in the REMS EEG theta power, while the effect on NREMS observed in $MT_2^{-/-}$ mice is due to the decrease in the NREMS EEG delta and sigma powers.

The expression of MLT receptors in CHO or NIH3T3 cells varies during the day due to the physiological circadian oscillations in the levels of circulating MLT [29]. No studies have yet investigated a possible circadian oscillation in the expression of MLT receptors in brain areas involved in sleep. Interestingly, most of the effects on the vigilance states we observed in the

MLT receptor KO mice occurred during the light/inactive phase when the levels of MLT are lower, and the expression of its receptors likely higher. Similarly, we found that the pharmacological activation of MT_2 receptors by UCM765 produced significant effects on NREMS, especially during the last hours of the light phase [18]. Altogether these results suggest that MT_2 receptors play a role in NREMS, even when the circulating MLT is at picomolar levels and independent from the peak of the indoleamine that occurs from 2 to 4 AM [7]. However, more studies are required to understand the interaction between the MT_1–MT_2 receptor function and expression, and the circadian oscillation of MLT levels; in addition, a possible involvement of other non-receptorial mechanisms cannot be ruled out [2].

$MT_1^{-/-}/MT_2^{-/-}$, but also $MT_1^{-/-}$ and $MT_2^{-/-}$ mice show a significant decrease in the power of sigma band of NREMS. Remarkably, in humans, the administration of MLT (5 mg) enhanced the NREMS EEG power density of the frequency band related to sleep spindles (13.75–14.0 Hz) [30]. Therefore, we can speculate that the spindle-promoting properties of MLT derive from the activation of both MLT receptors acting in a synergistic manner. On the other hand, MLT does not affect NREMS delta power [30] and [31]. In agreement, NREMS delta power is not altered in $MT_1^{-/-}/MT_2^{-/-}$ mice, but only in $MT_1^{-/-}$ and $MT_2^{-/-}$ mice. Cajochen et al. [32] showed that the same dosage of MLT (5 mg) also increased theta/alpha power of the waking EEG spectra mainly during the day, and in $MT_1^{-/-}/MT_2^{-/-}$ mice, we observed that the EEG power of these two frequency bands was increased during the light phase only. Interestingly, MLT receptor KO mice all displayed an overall decrease of the total NREMS EEG power, thus meaning that both MLT receptors are necessary to maintain a good sleep quality. In particular, $MT_2^{-/-}$ mice have a global reduction of the EEG power of the three vigilance states during both light and dark phases, even though for REMS this decrease is only close to significance ($P = 0.067$).

However, further studies employing sleep deprivation protocols will be essential to better clarify the meaning of the changes in the EEG power spectra between genotypes, and thus to assess how the two MLT receptor subtypes may modulate the EEG activities during the sleep–wake cycle. Moreover, these studies will allow delineation of whether MLT receptors are also implicated in the mechanism of sleep recovery.

Until now, MLT receptor KO mice have been mainly employed to study the involvement of MLT and its receptors in the control of circadian rhythms that are largely driven by the "master clock" in the SCN [4], [5], [6] and [33], which is also considered an important structure in the control of sleep and wakefulness [4] and [34]. The suppression of SCN neuronal activity by MLT represents a likely mechanism by which MLT contributes to the regulation of sleep and wakefulness in diurnal species [35]. Moreover, Hardeland [36] hypothesized that MLT, through the activation of MT_1 receptors in the SCN, promotes sleep *via* the hypothalamus, a pivotal brain region in the neural circuitries that regulates sleep and produces wakefulness [8].

While these previous hypotheses have yet to be empirically evaluated, our study indicates that MT_1 and MT_2 receptors can control sleep through the specific modulation of sleep stages. In a recent study [37], pinealectomized rats, in which MLT synthesis is suppressed, showed no impaired sleep–wake cycle but only a reduction in power in the theta band of REMS during both light and dark phases. Accordingly, in mice in which both MT_1 and MT_2 receptors are absent, similar findings would be expected since these mice are lacking the effectors (the receptors) instead of the ligand (MLT). Differently, we found that the genetic deletion of both $MT_1^{-/-}$/$MT_2^{-/-}$ receptors led to increased time of wakefulness and increased REMS and wakefulness EEG theta power. Some hypotheses that need further investigations may explain such different findings. First, data from rats may not always generalize to mice [38]. Second, in MLT receptor KO mice, MLT is still produced and consequently, a membrane receptor-independent hypnotic action of MLT [2] may not be excluded. Third, a MLT potentiating effect of endogenous GABA [39] might be also considered. Finally, developmental compensatory mechanisms might occur in mice lacking MLT receptors.

In conclusion, this study provides new insights on the role of MLT and its receptors in the regulation of the sleep/wake cycle and, in particular, suggests that dual inactivation of MT_1 and MT_2 receptors may influence wakefulness rather than sleep. Conversely, genetic inactivation of a single MLT receptor subtype appears to differentially affect the different sleep stages. Since the MT_2 receptor is predominantly involved in the modulation of NREMS, this receptor may represent a novel target for the treatment of sleep disorders. As well, since the MT_1 receptor is mainly implicated in REMS, it may deserve further investigations for its involvement in the etiology of REMS disorders.

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Tables and figures

Table 1

Table 1. Vigilance states parameters (mean ± SEM) during the 24-h light/dark cycle in WT, $MT_1^{-/-}$, $MT_2^{-/-}$, and $MT_1^{-/-}/MT_2^{-/-}$ mice (n = 7 for all genotypes). TT-NREMS, total time of NREMS; E-NREMS, number of NREMS episodes; ED-NREMS, duration of NREMS episodes; TT-REMS, total time of REMS; E-REMS, number of REMS episodes; ED-REMS, duration of REMS episodes; TT-W, total time of wakefulness; E-W, number of wakefulness episodes; ED-W, duration of wakefulness episodes.

24-h	**TT-NREMS min**	**E-NREMS no.**	**ED-NREMS min**	**TT-REMS min**	**E-REMS no.**	**ED-REMS min**	**TT-W min**	**E-W no.**	**ED-W min**
WT	582.37 ± 25.30	525.28 ± 44.95	1.36 ± 0.13	143.24 ± 14.81	406.57 ± 46.71	0.36 ± 0.03	714.59 ± 20.19	351.85 ± 16.19	2.18 ± 0.14
$MT_1^{-/-}/MT_2^{-/-}$	534.68 ± 24.85 †	533.28 ± 42.25	1.09 ± 0.13	126.96 ± 12.03	375.67 ± 42.92	0.36 ± 0.02	778.13 ± 19.09 *	352.59 ± 28.83	2.49 ± 0.23 †
$MT_1^{-/-}$	613.64 ± 15.08 ##	516.57 ± 32.73	1.16 ± 0.04	89.74 ± 10.96 *	373.86 ± 38.29	0.27 ± 0.04	733.04 ± 13.50 ##	412.43 ± 32.50	1.85 ± 0.11 #
$MT_2^{-/-}$	481.45 ± 22.41 *	447.28 ± 41.44	1.17 ± 0.13	128.71 ± 14.22	334.57 ± 51.34	0.41 ± 0.04	820.41 ± 11.7 ***	330.86 ± 20.72	2.65 ± 0.20
Statistics	$F_{3,24}$ = 6.70 ***P* = 0.002**	$F_{3,24}$ = 0.95 *P* = 0.434	$F_{3,24}$ = 0.83 *P* = 0.492	$F_{3,24}$ = 3.02 ***P* = 0.049**	$F_{3,24}$ = 0.43 *P* = 0.734	$F_{3,24}$ = 0.32 *P* = 0.807	$F_{3,24}$ = 8.24 ***P* < 0.001**	$F_{3,24}$ = 1.91 *P* = 0.154	$F_{3,24}$ = 4.06 ***P* = 0.018**

Bold was used to highlight parameters that show a significant variation between groups.

*P < 0.05 vs. WT, one-way ANOVA followed by Student–Newman–Keuls test for post hoc comparison. ***P < 0.001 vs. WT, one-way ANOVA followed by Student–Newman–Keuls test for post hoc comparison. †P < 0.05 $MT_1^{-/-}/MT_2^{-/-}$vs. $MT_1^{-/-}$ mice, one-way ANOVA followed by Student–Newman–Keuls test for post hoc comparison. #P < 0.05 $MT_1^{-/-}$ vs. $MT_2^{-/-}$ mice, one-way ANOVA followed by Student–Newman–Keuls test for post hoc comparison. ##P < 0.01 $MT_1^{-/-}$ vs. $MT_2^{-/-}$ mice, one-way ANOVA followed by Student–Newman–Keuls test for post hoc comparison.

Table 2

Table 2. Vigilance states parameters (mean ± SEM) during the 12-h light and 12-h dark phases in WT, $MT_1^{-/-}$, $MT_2^{-/-}$, and $MT_1^{-/-}/MT_2^{-/-}$ mice (n = 7 for all genotypes). TT-NREMS, total time of NREMS; E-NREMS, number of NREMS episodes; ED-NREMS, duration of NREMS episodes; TT-REMS, total time of REMS; E-REMS, number of REMS episodes; ED-REMS, duration of REMS episodes; TT-W, total time of wakefulness; E-W, number of wakefulness episodes; ED-W, duration of wakefulness episodes.

	TT-NREMS min	**E-NREMS no.**	**ED-NREMS min**	**TT-REMS min**	**E-REMS no.**	**ED-REMS min**	**TT-W min**	**E-W no.**	**ED-W min**
12-h light									
WT	338.55 ± 15.64	301.14 ± 25.07	1.50 ± 0.12	89.98 ± 8.26	239.29 ± 23.48	0.38 ± 0.01	293.29 ± 14.68	197.85 ± 8.67	1.50 ± 0.12
$MT_1^{-/-}/MT_2^{-/-}$	302.31 ± 19.59	319.43 ± 21.71	0.99 ± 0.11*	75.11 ± 6.74†	234.00 ± 28.62	0.34 ± 0.03	340.53 ± 18.58	206.43 ± 16.53	1.72 ± 0.17‡
$MT_1^{-/-}$	343.02 ± 16.51$^{\#\#}$	262.43 ± 16.34	1.21 ± 0.03	48.23 ± 5.63**	206.28 ± 24.84	0.27 ± 0.05	325.18 ± 15.26$^{\#}$	199.86 ± 9.49	1.64 ± 0.08$^{\#}$
$MT_2^{-/-}$	257.07 ± 13.67**	258.57 ± 18.40	1.04 ± 0.11*	73.85 ± 9.72	187.28 ± 27.32	0.41 ± 0.04	377.54 ± 6.43**	186.86 ± 7.97	2.06 ± 0.05*
Statistics	$F_{3,24}$ = 5.84 ***P* = 0.004**	$F_{3,24}$ = 2.07 *P* = 0.130	$F_{3,24}$ = 4.71 ***P* = 0.010**	$F_{3,24}$ = 5.05 ***P* = 0.008**	$F_{3,24}$ = 0.87 *P* = 0.471	$F_{3,24}$ = 2.31 *P* = 0.102	$F_{3,24}$ = 5.87 ***P* = 0.004**	$F_{3,24}$ = 0.53 *P* = 0.668	$F_{3,24}$ = 4.20 ***P* = 0.016**
12-h dark									
WT	243.82 ± 14.75	224.14 ± 24.37	1.22 ± 0.22	53.25 ± 7.76	167.28 ± 28.93	0.34 ± 0.05	421.30 ± 10.31	154.00 ± 11.78	2.85 ± 0.27
$MT_1^{-/-}/MT_2^{-/-}$	232.37 ± 17.11	213.86 ± 28.69	1.19 ± 0.16	51.85 ± 6.63	141.67 ± 21.87	0.38 ± 0.02	437.59 ± 15.23	146.16 ± 16.49†	3.26 ± 0.44
$MT_1^{-/-}$	270.63 ± 4.63	254.14 ± 24.51	1.11 ± 0.08	41.50 ± 5.99	167.57 ± 17.21	0.26 ± 0.04	407.87 ± 5.38	212.57 ± 19.17*	2.06 ± 0.18
$MT_2^{-/-}$	224.38 ± 15.21	188.71 ± 23.63	1.29 ± 0.16	54.85 ± 6.22	147.29 ± 24.64	0.40 ± 0.04	442.87 ± 15.91	144.00 ± 12.97$^{\#}$	3.26 ± 0.36
Statistics	$F_{3,24}$ = 2.13 *P* = 0.122	$F_{3,24}$ = 1.13 *P* = 0.354	$F_{3,24}$ = 0.21 *P* = 0.885	$F_{3,24}$ = 0.66 *P* = 0.608	$F_{3,24}$ = 0.87 *P* = 0.471	$F_{3,24}$ = 0.32 *P* = 0.807	$F_{3,24}$ = 1.64 *P* = 0.207	$F_{3,24}$ = 4.47 ***P* = 0.012**	$F_{3,24}$ = 2.93 *P* = 0.054

Bold was used to highlight parameters that show a significant variation between groups.

*P < 0.05 vs. WT, one-way ANOVA followed by Student–Newman–Keuls test for post hoc comparison. **P < 0.01 vs. WT, one-way ANOVA followed by Student–Newman–Keuls test for post hoc comparison. †P < 0.05 $MT_1^{-/-}$/$MT_2^{-/-}$vs. $MT_1^{-/-}$ mice, one-way ANOVA followed by Student–Newman–Keuls test for post hoc comparison. ‡P < 0.05 $MT_1^{-/-}$/$MT_2^{-/-}$ vs. $MT_2^{-/-}$ mice, one-way ANOVA followed by Student–Newman–Keuls test for post hoc comparison. #P < 0.05 $MT_1^{-/-}$ vs. $MT_2^{-/-}$ mice, one-way ANOVA followed by Student–Newman–Keuls test for post hoc comparison. ##P < 0.01 $MT_1^{-/-}$ vs. $MT_2^{-/-}$ mice, one-way ANOVA followed by Student–Newman–Keuls test for post hoc comparison.

Figure 1

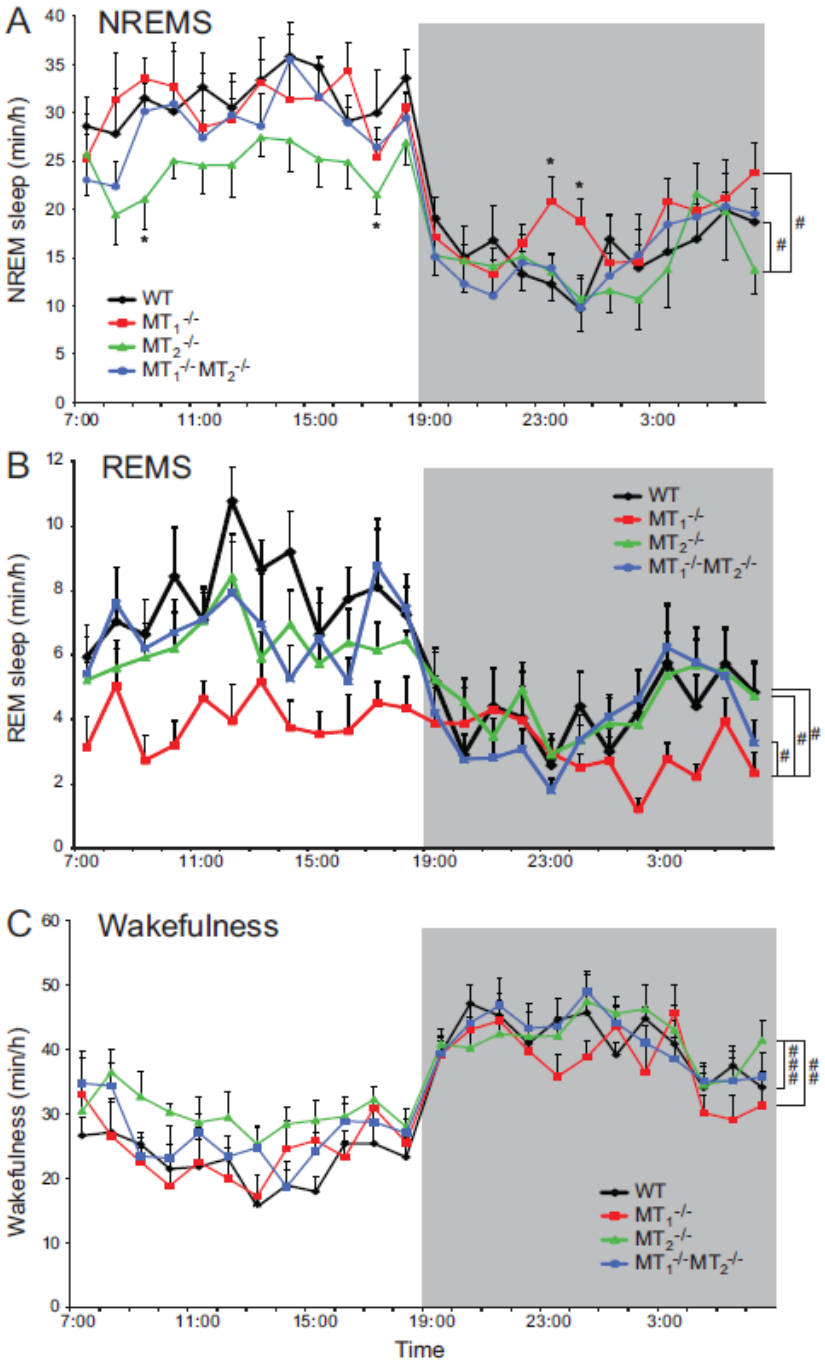

Fig. 1. Time course of NREMS, REMS, and wakefulness during the 24-h sleep–wake cycle in WT, $MT_1^{-/-}$, $MT_2^{-/-}$, and $MT_1^{-/-}/MT_2^{-/-}$ mice ($n = 7$ for all genotypes). NREMS (A), REMS (B) and Wakefulness (C). Data are expressed as mean (min) ± SEM. Gray backgrounds denote the dark phase. $^{*}P < 0.05$ *vs.* WT; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ between pairs of genotypes, Student–Newman–Keuls test for *post hoc* comparison.

Figure 2

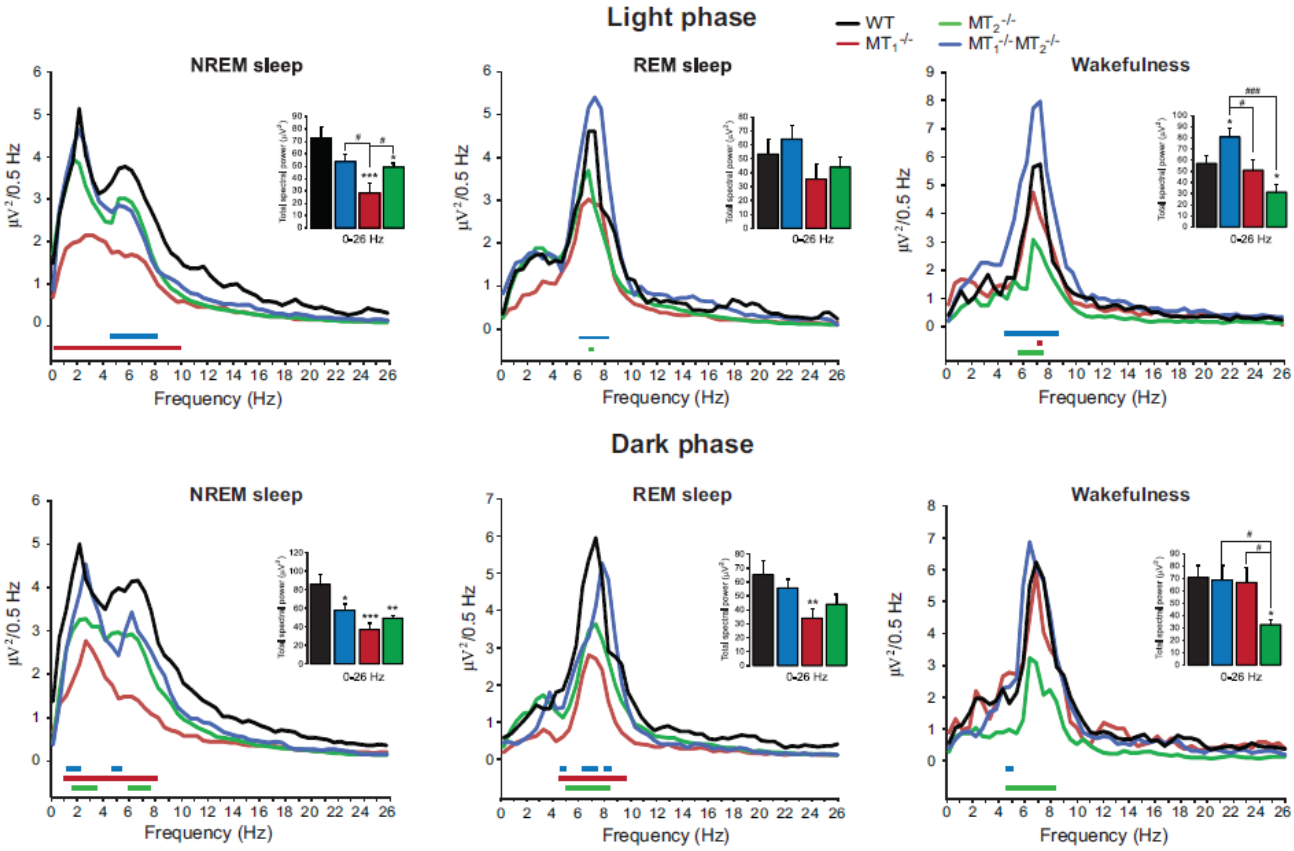

Fig. 2. Power spectra of NREMS, REMS and wakefulness in WT (dark line), $MT_1^{-/-}$ (red line), $MT_2^{-/-}$ (green line), and $MT_1^{-/-}/MT_2^{-/-}$ (blue line) mice (n = 7 for all genotypes) during the light (upper part) and dark (lower part) phase. SEM values have been omitted to have a clearer view of the EEG power. Bars at the bottom indicate frequency bins in which the power spectra of knockout mice significantly ($P < 0.05$, Student–Newman–Keuls test for *post hoc* comparison) differs from that of WT. Blue indicates $MT_1^{-/-}/MT_2^{-/-}$ *vs.* WT, red $MT_1^{-/-}$ *vs.* WT, and green $MT_2^{-/-}$ *vs.* WT. (Insets) Total spectral power (0–26 Hz). Data are expressed as mean (μV^2) ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* WT, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ between pairs of genotypes, Fisher's PLSD test for *post hoc* comparison.

Figure 3

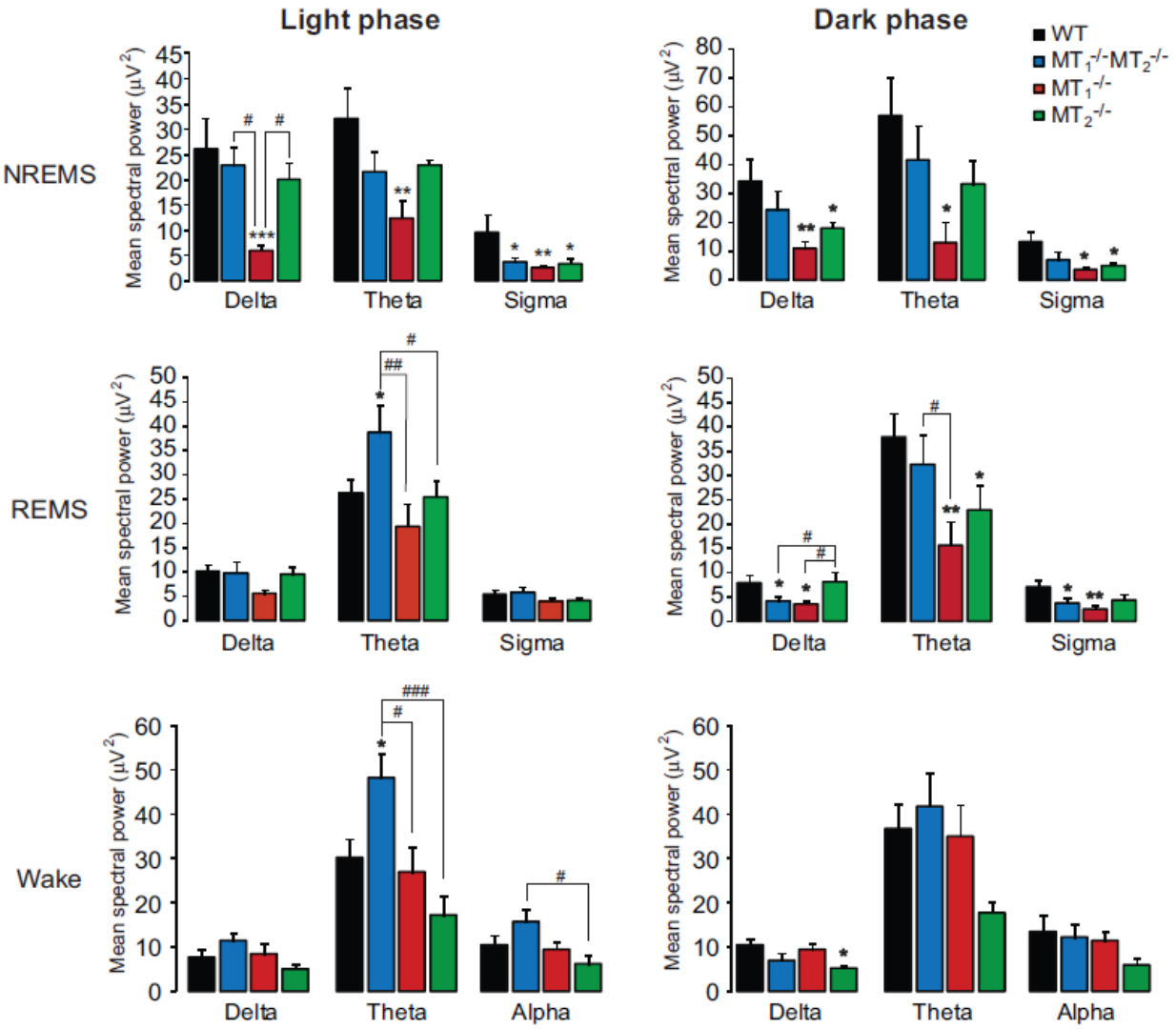

Fig. 3. Raw spectral power changes in delta, theta, sigma and alpha bands of NREMS (upper part), REMS (center part) and wakefulness (lower part) during the light and dark phases in WT, $MT_1^{-/-}/MT_2^{-/-}$, $MT_1^{-/-}$ and $MT_2^{-/-}$mice. Data are expressed as mean (μV^2) ± SEM; $n = 7$ for all genotypes. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* WT, $^{\#}P < 0.05$ between pairs of genotypes, Fisher's PLSD test for *post hoc* comparison.

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3.2 Interim discussion

In chapter III we investigated for the first time the sleep profile of MT_1/MT_2KO mice in comparison to MT_1KO, MT_2KO and WT mice. Our 24 h analysis in MT_1/MT_2KO mice revealed the total time of wakefulness to be increased. MT_1/MT_2KO mice also showed differences in the power spectrum analysis; the theta power was increased during REMS and wakefulness states.

During the light phase, the duration of REMS was decreased in MT_1KO mice, while NREMS was decreased in MT_2KO mice. These effects in MT_1KO or MT_2KO mice are associated with a decrement in the REMS theta power or NREMS delta power, respectively. Together, the findings in MT_1KO and MT_2KO mice suggest that the MT_1 receptor is involved in REMS and that the MT_2 receptor is involved in NREMS regulation. In agreement, in chapter II, MT_2KO mice showed a reduction in NREMS while MT_1KO mice showed a tendency to decrease REMS total time during the light phase. In line with these results, the single activation of MT_2 receptors with UCM765 increased NREMS in rats, an effect inhibited by the selective MT_2 receptor antagonist 4P-PDOT.

Interestingly, during the dark phase, the duration of NREMS was increased in MT_1KO mice. Similar results were obtained in chapter II, in which the genetic inhibition of MT_1 receptors in MT_1KO mice increased the NREMS time during the dark phase. These results suggest that MT_1 and MT_2 receptors might play opposite roles in the regulation of NREMS, either through inhibition or promotion, respectively. Therefore, the selective activation of a single receptor, in this case the MT_2 receptor, may represent a therapeutic advantage to increase NREMS. For instance, in chapter II, the selective activation of MT_2 receptors, but not the simultaneous activation of MT_1/MT_2 receptors, increased the duration of NREMS in rats. These findings could possibly explain the lack of effects on sleep maintenance of non-selective MT_1/MT_2 receptors and MLT itself.

Based on our previous results in chapter II, at first we hypothesized that NREMS, REMS and wakefulness durations might be impaired in MT_1/MT_2KO mice. Interestingly, in contrast to our hypothesis, the total time of wakefulness was increased but no significant changes in either NREMS or REMS durations were detected in MT_1/MT_2KO mice. A reasonable explanation is that changes seen in MT_1KO or MT_2KO mice resulted from not only the lack of one receptor, but

also may be a result of complementary effects due to the presence of the other receptor subtype. Consequently, the reduction of NREMS and REMS due to the lack of MT_2 or MT_1 receptors respectively, becomes minimal but sufficient to significantly increase the wakefulness total time in MT_1/MT_2KO mice, only in the whole 24 h analysis.

In agreement with the fact that the inactivation of both MT_1 and MT_2 receptors affects wakefulness rather than sleep stages, in chapter II, we demonstrated that the pharmacological activation of both MT_1/MT_2 receptors with UCM793 did not modify REMS or NREMS durations. However, the wakefulness total time was not decreased or modified by UCM793, likely because the effects of UCM793 in chapter II were tested only for 3 h instead of 24 h, the time frame that revealed the increment in wakefulness in MT_1/MT_2KO mice. Together, these findings may also explain the lack of effects of MLT and analogs on sleep maintenance in animals as well as in human studies mainly focused on sleep states rather than wakefulness state. To test this hypothesis, we need to explore whether MLT or MLT analogs modify wakefulness rather than sleep states across the 24 h light-dark cycle (see chapter IV).

In conclusion, further experiments need to be done to fully understand how MLT and MLT receptors regulate the vigilance states. Electrophysiological and behavioural experiments involving different brain regions that express MLT receptors as well as studies testing the actions of selective MT_2 agonists, selective MT_1 agonists, non-selective MT_1/MT_2 agonists and MLT in sleep are needed. Nevertheless, MT_1 and MT_2 receptors should be considered as targets for different sleep disorders with impaired REMS and NREMS, respectively.

Foreword to Chapter IV

Melatonin, selective and non-selective MT_1/MT_2 receptors agonists: differential effects on the 24-hr vigilance states

Previously, several reports demonstrated that the activation of MT_1/MT_2 receptors by MLT reduces the latency to sleep and increases REMS and NREMS (Holmes and Sugden 1982; Wang et al., 2003b). However, other reports showed effects only on the latency to fall asleep (Yukuhiro et al., 2004). Such a discrepancy is probably explained by different protocols used, in particular the time of administration, which plays a significant role for melatonergic compounds. In chapter II, we proved that the selective activation of MT_2 receptors by UCM765 induces and maintains NREMS while the activation of MT_1/MT_2 receptors by the non-selective MT_1/MT_2 agonist UCM793 produces only a mild effect on the latency to sleep. Nonetheless, the effects of UCM793 were assessed only for a short period of time (3 h). Besides, our experiments in MLT KO mice demonstrated that the MT_2 receptor is involved in NREMS regulation while the MT_1 receptor is involved in the regulation of REMS. Interestingly, only wakefulness, but not NREMS or REMS, was impaired in double MT_1/MT_2KO mice. These findings suggest that the dual activation of MT_1/MT_2 receptors might exert different effects on the sleep-wake cycle than the effects of the selective actions in MT_2 receptors. Consequently, further experiments have to compare the effects of MLT or non-selective MT_1/MT_2 agonists with those of selective MT_2 agonists. Additionally, it has been reported that the effects of MLT might occur not only through a receptor dependent mechanism, but also through a receptor independent mechanism such as ion channels, transcription factors and intracellular second messengers (Reiter el al., 2010). Therefore, it would be interesting to test whether MLT exert its sleep promoting effects in a different manner than UCM793, a compound that exclusively activates MT_1 and MT_2 receptors.

To address these questions, in this chapter we used the same protocol used in chapter II and examined the effects of MLT on the 24 h sleep-wake cycle. Then we compared these effects with those of UCM793 and those produced by the novel MT_2-selective partial agonist UCM924. Notably, this chapter demonstrates that only the selective activation of MT_2 receptors by UCM924, but not double activation of MT_1/MT_2 receptors by MLT or UCM793, enhances sleep maintenance. Interestingly, MLT and UCM793 differentially influence the three vigilance states, suggesting that the hypothesis of receptor-independent mechanism cannot be ruled out for MLT.

Chapter IV

Melatonin, selective and non-selective MT_1/MT_2 receptors agonists: differential effects on the 24-hr vigilance states

4.1 Research paper

Melatonin, selective and non-selective MT_1/MT_2 receptors agonists: differential effects on the 24-hr vigilance states.

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Abstract

Melatonin (MLT) is a neurohormone implicated in several physiological processes such as sleep. Contrasting results have been produced on whether or not it may act as an hypnotic agent and still to be elucidated is the neurobiological mechanism through which it controls the vigilance states. It has been proposed that both a receptor dependent (through melatonin MT_1 and MT_2 receptors) and a receptor-independent mechanisms are involved. In this study we investigated the effect of MLT (40 mg/kg), a non-selective MT_1/MT_2 receptor agonist (UCM793, 40 mg/kg), and a selective MT_2 partial agonist (UCM924, 40 mg/kg) on the 24-hr vigilance states. EEG and EMG sleep-wake patterns were registered across the 24-h light-dark cycle in adult Sprague–Dawley male rats. MLT decreased (-37%) the latency to the first episode of non rapid eye movement sleep (NREMS), enhanced the power of NREMS delta band (+33%), but did not alter the duration of any of the three vigilance states. Differently, UCM793 increased the number of episodes (+52%) and decreased the length of the episodes (-38%) of wakefulness leaving the 24-hr duration of wakefulness unchanged and did not alter the duration of NREMS and REMS. UCM924 instead reduced the latency (-56%) and increased (+31%) the duration of NREMS. Moreover, it raised the number of REMS episodes (+57%) but did not affect REMS duration. Taken together, these findings show that MLT and non-selective MT_1/MT_2 receptor agonists do not increase the quantity of sleep but differently influence the three vigilance states. In addition, they support the evidence that selective MT_2 receptor agonists have significant hypnotic properties compared to MLT and non-selective MT_1-MT_2 agonists.

1. Introduction

Insomnia is characterized by any of the following symptoms: difficulty falling asleep, waking during the night, problems getting back to sleep, waking up too early, and fatigue in the morning [1, 5]. Insomnia represents a public health problem that affects approximately 9% of the general population [11]. The neurobiology of sleep is still matter of ongoing research and current available hypnotics present several pitfalls such as tolerance, dependence, sedation [5], next-day cognitive impairment [13] and alteration of the physiological sleep architecture [9]. Melatonergic system has been studied for decades to understand how it modulates the sleep-wake cycle. While melatonin (MLT) itself has produced contrasting results on whether it may be used as hypnotic [7, 12, 21, 24], compounds targeting MLT receptors, namely MT_1 and MT_2, seem to produce substantial effects on sleep [3, 4, 10, 15]. In particular, MT_2 selective agonists/partial agonists such as IIK7 [4] and UCM765 [15] modulate non rapid eye movement sleep (NREMS). On the contrary, non-selective MT_1/MT_2 agonists such as ramelteon mainly shorten the latency to fall asleep [3]. It has been hypothesized that MLT may induce sleep at the neuronal level independently of its membrane receptors [8]. MLT effects can also be mediated by ion channels, transcription factors, and intracellular second and third messengers [18]. Here we aimed to assess how MLT affects the three vigilance states during the 24-h light/dark cycle. In addition, we want to compare the effects of MLT (MT_1 receptors: $pK_i = 9.85$; MT_2 receptors: $pK_i = 9.62$) with those of UCM793, a non-selective MT_1/MT_2 receptor agonist (MT_1 receptors: $pK_i = 9.09$; MT_2 receptors: $pK_i = 9.19$), and of UCM924, a selective MT_2 receptor partial agonist (MT_1 receptors: $pK_i = 6.76$; MT_2 receptors: $pK_i = 9.27$)[19, 20]. UCM924 is a class-cogener of UCM765 which conserves the same binding profile to the MT_2 receptor but compared to UCM765 it is more metabolically stable. In particular, UCM924 has the free phenyl ring of UCM765 protected by the introduction of a fluorine atom and the methoxy group bioisosterically replaced with bromine [20].

In this study we examined the hypnotic properties of UCM924 since *in vitro* experiments showed that it was less likely than UCM765 to be vulnerable to oxidative metabolism and consequently it displayed a significant longer half-life.

2. Material and methods

All experimental procedures were conducted in accordance with the standard and ethical guidelines mandated by the Canadian Institutes of Health Research, the Canadian Council on Animal Care, and McGill University Animal Care Center.

2.1 Animals

Adult Sprague–Dawley male rats (Charles River, Canada) weighing 250–260 g at the moment of arrival were housed in pairs in standard polypropylene cages under a 12 h:12 h light:dark cycle (light on at 7:00 a.m.). Room temperature was kept between 20 and 21 °C and access to food and water was ad libitum. Once they reached a weight of 300 g, animals underwent the surgery for the implantation of the electroencephalogram/electromyogram (EEG/EMG) electrodes. Surgery protocol, position of the electrodes, recovery time and habituation to the cables and the recording chamber were performed according to Ochoa-Sanchez et al. [15].

2.2 Monitoring of sleep-wake cycle and data analysis

EEG/EMG signals were amplified at a total gain of 10000 and filtered locally (EEG, low filter, 1 Hz; high, 1 kHz; EMG, low filter, 30 Hz; high, 3 kHz; Grass, P55), digitized using a CED power 1401 converter and Spike 2 software (CED), and stored in a PC with a resolution of 128 Hz. Consecutive 2 s epochs were subjected to a fast Fourier transform, and EEG power spectra density was computed in the frequency range of 0–64 Hz. The three classical vigilance states (NREMS; rapid eye movement sleep (REMS); wakefulness) were scored manually for consecutive 10-s period epochs using Spike 2 software, and were discriminated on the basis of the cortical EEG and neck EMG. The scoring was performed in a blinded manner by two expert raters (R.O.S. and S.C.). NREMS was distinguished by high-voltage delta waves (1–4 Hz) and a weak EMG activity. REMS was characterized by a low-amplitude EEG with a pronounced theta rhythm (4–9 Hz) and a complete loss of nuchal muscle tone. Wakefulness was identified by a low-amplitude and desynchronized EEG, with sustained EMG activity. To avoid transitional periods such as drowsiness, only epochs of typical stationary EEG and EMG were considered (97.4±1.3% of the total epochs).

For the analysis of the EEG spectra, the power in the following frequency bands was examined: delta: 1–4 Hz; theta: 4–9 Hz; alpha: 8–12 Hz; sigma: 11–15 Hz.

The latency to NREMS or REMS was defined as the time from the first injection (6:00 P.M.) to the first 10 s NREMS or REMS episode, respectively.

2.3 Drugs and Pharmacological treatments

UCM793 (*N*-{2-[(3-methoxyphenyl)methylamino]ethyl]acetamide}) was synthesized by the University of Urbino and UCM924 (*N*-{2-[(3-bromophenyl)(4-fluorophenyl)amino]ethyl}acetamide) by the University of Urbino and partially by BioQudrant Inc (Montreal, Qc). MLT was provided by Sigma-Aldrich Canada. Vehicle (DMSO 70%:30% saline, 0.2 ml), MLT (40 mg/kg) or UCM793 (40 mg/kg) were injected subcutaneously every 4 h for 24 h with the first injection at 6:00 P.M. UCM924 (40 mg/kg) was injected at 6:00 P.M. and 6:00 A.M., and in order to mimic the sequence of MLT and UCM793 injections, these rats received vehicle at 10:00 P.M., 2:00 A.M., 10:00 A.M. and 2:00 P.M.. In our previous research, UCM765 increased NREMS duration at the dose of 40 mg/kg when injected every 4 h [15]. MLT and UCM793 showed binding properties to MT_2 receptors similar to those of UCM765 [19], therefore the same dosage as well as the same injection protocol were chosen. In addition, we recently demonstrated that using *in vivo* behavioral paradigms, both MLT and UCM765 produced anxiolytic-like effects at the same dose [16]. On the other hand, since UCM924 displayed an half-life in the presence of rat liver S9 fraction much longer than that of UCM765 (40 min *vs* 8 min) [20], it was injected every 12 h at the dose of 40 mg/kg.

2.4. Statistical analyses

SigmaPlot software (version 12.0) was used to analyze the data. Results are expressed as the mean percentage of variation compared to vehicle ± S.E.M. Since assumptions of normality and variance homogeneity were satisfied for all data, one-way ANOVA followed by the Student–Newman–Keuls (SNK) *post hoc* comparisons was used to determine the differences between treatments. To analyze hour-by-hour and light/dark differences on the three vigilance states due to the treatments, two-way ANOVA for repeated measures (factors treatment and time of the day or phase of the day) followed by SNK *post hoc* comparisons was employed. Statistical values reaching $p \leq 0.05$ were considered significant.

3. Results

3.1. MLT and UCM924 reduce the latency to NREMS but not to REMS

The latency to NREMS was affected by treatment (One-way ANOVA, $F(3,16)=10.08$; $p<0.001$). Compared to vehicle, MLT (40 mg/kg) and UCM924 (40 mg/kg) significantly decreased the latency to NREMS by respectively 37±11 % ($p=0.009$) and 56±4 % ($p<0.001$). Interestingly, the effect of UCM793 (40 mg/kg) on NREMS latency was only marginal and not significant (-19±6 %, $p=0.097$). On the contrary, the main effect of treatment on REMS latency ($F(3,16)=2.04$; $p=0.148$) was not significant.

3.2. UCM924 but not MLT nor UCM793 affects 24-hr NREMS

Fig.1 (left side) presents the effect of MLT and the melatonergic ligands on 24-hr NREMS duration, number of episodes and mean episodes duration. In particular, one-way ANOVA revealed a significant main effect of treatment on NREMS duration ($F(3,16)=6.82$; $p=0.004$) and NREMS mean episodes duration ($F(3,16)=3.81$; $p=0.031$), but not on the number of episodes of NREMS ($F(3,16)=1.86$; $p=0.176$). UCM924 (40 mg/kg) significantly increased the 24-hr NREMS duration (+31%, $p=0.003$) and the 24-hr mean episodes duration of NREMS (+44%, $p=0.024$) compared to vehicle. In addition, 24-hr NREMS duration was longer in rats treated with UCM924 compared to those treated with MLT ($p=0.012$) and UCM793 ($p=0.015$). No effects due to MLT or UCM793 injections were observed.

3.3. MLT, UCM924 and UCM793 do not alter 24-hr REMS duration

As showed in Fig. 1 (center), the treatment with MLT, UCM793 or UCM924 does not affect REMS duration ($F(3,16)=0.94$; $p=0.441$) and REMS mean episodes duration ($F(3,16)=2.06$; $p=0.146$) during the 24-hr sleep-wake cycle. Interestingly, UCM924 significantly increased (+57%, $p=0.005$) the number of REMS episodes during 24-hr ($F(3,16)=5.48$; $p=0.009$). The number of REMS episodes was also higher in rats treated with UCM924 than those treated with UCM793 ($p=0.041$). Moreover, the difference between UCM924 and MLT treatment was close to significance ($p=0.062$).

3.4. UCM924 but not MLT nor UCM793 decreases the duration of wakefulness.

The 24-hr duration ($F(3,16)=6.51$; $p=0.004$), number of episodes ($F(3,16)=4.71$; $p=0.015$) and mean episodes duration ($F(3,16)=8.55$; $p=0.001$) of wakefulness were significantly affected by the treatments (Fig. 1, right side).

Rats treated with UCM924 but not with UCM793 or MLT spent less time awake than those receiving vehicle (-12%, $p=0.003$). Moreover, UCM924 also decreased the 24-hr duration of wakefulness compared to MLT ($p=0.018$) and UCM793 ($p=0.016$) treatments.

In addition to the decrease of the 24-hr duration of wakefulness, UCM924 produced an increase in the 24-hr number of episodes (+41%, $p=0.042$) and a decrease in the 24-hr mean episodes duration (-38.5%, $p=0.003$) of wakefulness compared to vehicle. Similarly to UCM924, UCM793 led to an increase in the 24-hr number of episodes (+52%, $p=0.016$) and a decrease in the 24-hr mean episodes duration (-38 %, $p=0.002$) of wakefulness compared to vehicle. Unlikely UCM924 and UCM793, MLT treatment did not affect these two parameters.

3.5. UCM924 but not MLT nor UCM793 affects the hour-by-hour NREMS and wakefulness profiles

Two-way ANOVA revealed that treatment with MLT and the other melatonergic compounds significantly affected the hour-by-hour profile of NREMS ($F(3,276)=7.83$; $p=0.004$) and wakefulness ($F(3,276)=7.68$; $p=0.004$) but not of REMS ($F(3,276)=1.14$; $p=0.372$). SNK post-hoc analysis showed that NREMS hour-by-hour profile in the group of rats treated with UCM924 was different than vehicle ($p=0.004$), MLT ($p=0.006$) and UCM793 ($p=0.007$) (Fig 2A). Similarly, the hour-by-hour profile of wakefulness was altered after UCM924 compared to vehicle ($p=0.004$), MLT ($p=0.008$) and UCM793 ($p=0.007$)(Fig. 2B). No interaction treatment x time of the day was observed for any of the three vigilance states (NREMS: $F(69,276)=0.76$, $p=0.909$; REMS: $F(69,276)=0.78$, $p=0.893$; wakefulness: $F(69,276)=0.70$, $p=0.961$).

3.6. UCM924 increases NREMS and decreases wakefulness during the light phase only.

Two-way repeated measures ANOVA analysis showed a significant phase of the day x treatment interaction for the duration of NREMS ($F(3,16)=3.67$, $p= 0.035$) and wakefulness ($F(3,16)=4.68$, $p=0.016$) but not for REMS. In addition, an effect due to the phase of the day and

treatment for NREMS (phase of the day: $F(1,16)=101.85$, $p<0.001$; treatment: $F(3,16)=3.29$, $p=0.048$) and wakefulness (phase of the day: $F(1,16)=102.87$, $p<0.001$; treatment: $F(3,16)=3.19$, $p=0.051$) was found. SNK post-hoc analysis revealed that NREMS duration was longer in rats treated with UCM924 compared to those receiving vehicle ($p=0.006$), MLT ($p=0.003$) and UCM793 ($p=0.004$) during the light phase (Fig. 2A, inset). The duration of wakefulness was instead decreased after treatment with UCM924 compared to vehicle ($p=0.004$), MLT ($p=0.003$) and UCM793 ($p=0.003$) during the light phase (Fig. 2B, inset).

3.7. Effects of MLT and melatonergic compounds on the 24-hr EEG power of delta, theta, sigma and alpha bands of the three vigilance states

Fig. 3 reports the effects of MLT, UCM793 and UCM924 on the 24-hr EEG power of delta, theta, sigma and alpha bands of NREMS (upper part), REMS (center), and wakefulness (lower part). The treatment with MLT, UCM793 or UCM924 significantly affected the 24-hr EEG power of NREMS delta ($F(3,16)=4.38$; $p=0.020$) and sigma ($F(3,16)=3.33$; $p=0.046$) bands. In particular, MLT and UCM924 increased NREMS EEG power of delta band compared to vehicle ($p=0.018$ and $p=0.043$, respectively). Unlikely MLT, NREMS EEG power of sigma band was higher in rats treated with UCM924 than in those with vehicle (+37 %, $p=0.032$).

No significant effects on the 24-hr EEG power of REMS were detected. However, it is worthy to mention that the treatment effect on REMS EEG power of theta ($F(3,16)=2.47$; $p=0.099$) and sigma ($F(3,16)=3.10$; $p=0.056$) bands was not far from significance.

EEG power of delta band of wakefulness was influenced by the treatments ($F(3,16)=7.29$; $p=0.003$) and in particular, UCM793 decreased the EEG power compared to vehicle (-27%, $p=0.003$) but also to MLT ($p=0.033$) and UCM924 ($p=0.004$). A tendency to a main effect due to the treatment with MLT or the melatonergic ligands was observed on theta EEG power of wakefulness ($F(3,16)=2.85$; $p=0.070$).

4. Discussion

The aim of this study was to investigate the effects of MLT on the 24-hr vigilance states and then to compare these effects with those produced by a non-selective MT_1/MT_2 receptor agonist (UCM793) and a selective MT_2 receptor partial agonist (UCM924). Our work shows that

MLT reduces the latency to fall asleep but does not affect the duration of the three vigilance states during the 24-hr sleep-wake cycle. Very interestingly, UCM793 altered the architecture of the awake state by increasing the number and decreasing the length of the episodes of wakefulness, but it did not affect NREMS and REMS latencies and durations as well as the 24-hr duration of wakefulness. Conversely, the selective MT_2 receptor partial agonist UCM924 [20] similarly to its class congener UCM765 [15] reduced the latency to NREMS, increased the 24-hr duration of NREMS, and decreased the 24-hr duration of wakefulness. Noteworthy, these differences were the result of changes occurring during the light/inactive phase.

Previous animal studies have demonstrated that MLT reduces time to sleep onset and increases NREMS and REMS [6, 14] both effects being blocked by the $GABA_A$ receptor antagonists flumazenil and picrotoxin [23]. On the contrary, other experiments showed little or no effect [12, 21]. These disagreements between studies likely derive by the different experimental protocols used such as the dose of MLT, the time of administration, the length of sleep recording. Very importantly, the discrepancies are mostly related to the possible effects of MLT on the duration of NREMS and REMS, because almost all studies, including the present research, agree showing that MLT reduce the latency to fall asleep.

We recently demonstrated that the selective MT_2 receptor partial agonist UCM765 favours NREMS through the stimulation of the neural activity of the reticular thalamic neurons, which bear MT_2 receptors [15]. Similarly, other authors showed that the MT_2 receptor agonist IIK7 selectively increased NREMS [4]. Since these important novel findings, we decided to test, using the same experimental protocol employed for UCM765, whether MLT affects the 24-hr vigilance states in a similar or different manner than a non-selective MT_1/MT_2 receptors agonist (UCM793) and a selective MT_2 receptor partial agonist (UCM924).

We need to remind that MLT acts through a receptor dependent but also a receptor independent mechanism [18] and it has been hypothesized that both mechanisms concurred to the sleep effect of MLT [8]. Therefore, the comparison between the effects on the 24-hr vigilance states determined by MLT and UCM793 allowed us to test this hypothesis. Our results corroborate this hypothesis since the two compounds differently affect the 24-hr vigilance states. Indeed, MLT treatment decreased the latency to NREMS and increased NREMS EEG power of delta band, while UCM793 altered the architecture of wakefulness. Future studies aiming at

testing the effects of MLT on the vigilance states in MT_1/MT_2 receptors knockout mice will provide additional evidence for this hypothesis.

Recently, by studying the 24-hr vigilance states in MLT receptors knockout mice, we showed that MT_1 and MT_2 receptors play selective, differential, and sometimes opposite roles in sleep [2]. In particular, we found that MT_2 receptors are mainly implicated in the regulation of NREMS sleep, while MT_1 receptors of REMS. However, we could not exclude that MT_1 receptors were also implicated in NREMS. While it is out of the scope of the present paper to enter into the debate of whether the vigilance states in mice should be scored using 10 s or 4 s epochs, we found that with a 10 s epoch analysis MT_1 receptor knockout mice had significantly increased 12-hr duration of NREMS during the active/dark phase [15]. Following the 4 s epoch analysis we observed that even though the 12-hr duration of NREMS during the active/dark phase was not altered in MT_1 receptor knockout mice, these animals displayed significantly higher NREMS duration compared to WT controls at the middle of the dark phase. Consequently, since MT_1 receptor knockout mice displayed increased NREMS duration whereas MT_2 receptor knockout mice decreased NREMS duration, it might be that the two receptors modulate NREMS in opposite manner. Due to the opposite effects of MT_1 and MT_2 receptors on NREMS duration, it is not surprising that we found that non selective MT_1/MT_2 receptor agonists such as MLT and UCM793 did not increase NREMS duration. On the contrary, acting selectively on one of the two receptors subtypes, in this study on MT_2 receptors by using UCM924, a significant effect on NREMS can be achieved. However, this hypothesis warrants further research since it may explain decades of debate on why MLT and non selective MLT agonists do not seem to be good hypnotics.

Our study on double MT_1/MT_2 receptors knockout mice [2] also highlighted that the genetic inactivation of both MT_1 and MT_2 receptors did not affect the 24-hr duration of NREMS and REMS but only that of wakefulness. In agreement with this finding that acting on both MT_1 and MT_2 receptors an effect on wakefulness rather than on the sleep stages is obtained, we found that the pharmacological activation of both MLT receptor subtypes with UCM793 produces significant effects on the awake state and not on NREMS and REMS. Ramelteon, a non-selective MT_1/MT_2 receptors agonist, instead was found to reduce NREMS latency and to produce a modest and short-lasting increase of NREMS duration in rats [3]. The different effects produced on NREMS by the two compounds very likely derive by the different time and protocol of

administration which, as above reported, may influence the hypnotic activity of melatonergic compounds; however, we found a trend toward a reduction of the NREMS latency even with UCM793.

The pharmacological activation of MT_2 receptors with UCM765 [15] and IIK7 [4] as well as the genetic inactivation of MT_2 receptors [2] did not produce significant effects on REMS parameters. On the contrary, we found that the MT_2 receptor partial agonist UCM924 increased the number of REMS episodes. UCM924 has an affinity more than 100 times lower for MT_1 than for MT_2 receptors, but in comparison to UCM765 it acts at the level of MT_1 receptor as an antagonist [19]. MT_1 receptor knockout mice displayed altered REMS [2] and consequently, we hypothesized that the observed effects of UCM924 on REMS very likely rely on its residual antagonistic activity towards the MT_1 receptors. Furthermore, it also may be that the increase in the number of REMS episodes derives by a compensatory mechanism, which, as the result of the significant augmentation of NREMS duration, aims at maintaining the physiological NREMS/REMS architecture pattern.

MLT did not affect the hour-by-hour curves of the three vigilance states and consequently, MLT had no effects on sleep or wakefulness even though we considered a specific time or phase of the light/dark cycle. And similarly to what we observed in MT_1/MT_2 receptors knockout mice [2], the changes occurring in the awake state after UCM793 treatment were significant only when considering the whole 24-hr.

MLT even though did not affect NREMS duration, augmented NREMS EEG delta power in keeping with the findings by Tzischinsky and Lavie [22] in healthy males. However, other authors did not find MLT affecting NREMS EEG delta power [17].

Altogether our data indicate that the mechanism underlying the modulation of the vigilance states by MLT does not only involve MT_1 and MT_2 receptors. New research is thus needed to assess the sleep-promoting mechanisms of MLT that are independent from its receptors. The present results also corroborate our recent findings in MT_1/MT_2 receptors knockout mice [2] showing that the modulation of both MT_1 and MT_2 receptors yields no effects on sleep stages because of the selective and opposite role of each MLT receptor subtype on NREMS. Targeting selectively MT_2 receptors may instead provide significant effects on sleep. Indeed, we confirm that selective MT_2 receptor agonists have high potential to be further developed as novel hypnotic agents since, unlike MLT, they promote and increase NREMS.

Acknowledgements

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Figures

Figure 1

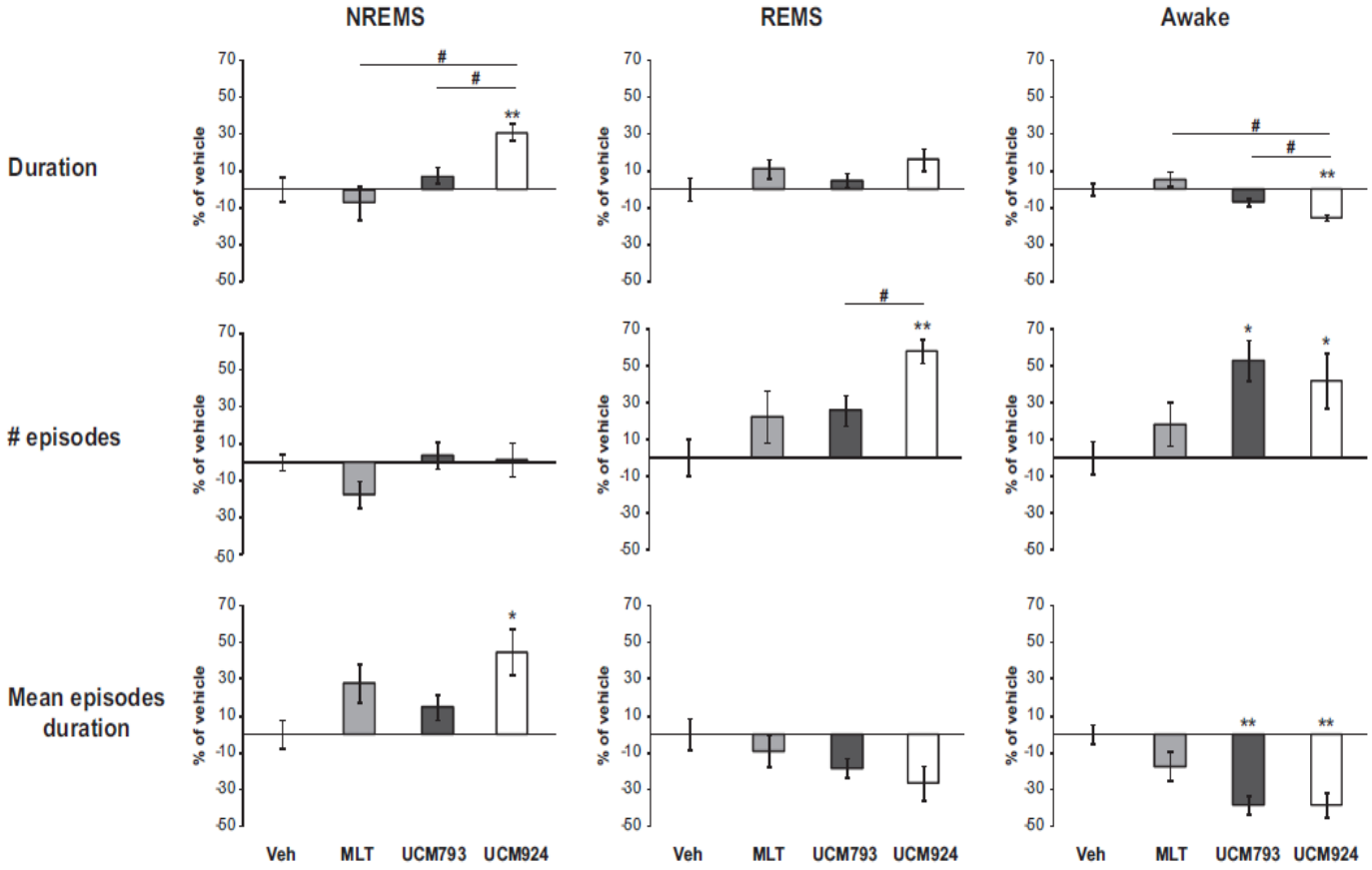

Fig. 1. Effects of MLT, UCM793 and UCM924 on the 24-hr vigilance states. (Left side) NREMS variables. (Center) REMS variables. (Right side) Wakefulness variables. Data are expressed as mean (percentage of variation *vs.* vehicle) ± S.E.M. of five rats per group. *p<0.05 and **p<0.01 *vs.* vehicle; #p<0.05 between treatments, SNK *post hoc* comparisons.

Figure 2

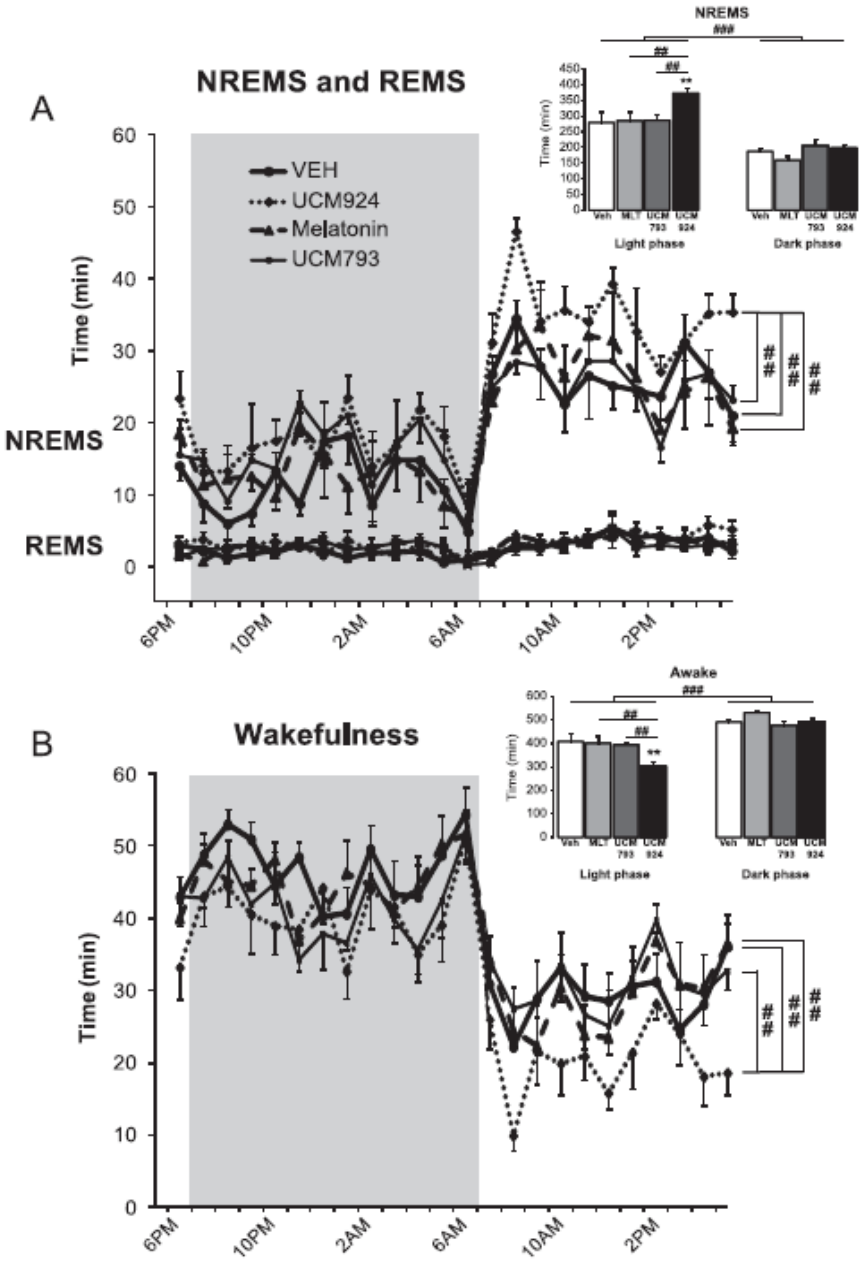

Fig. 2. Time course of NREMS, REMS, and wakefulness during the 24-h sleep–wake cycle after vehicle, MLT, UCM793 and UCM924 treatments. (A) NREMS and REMS. (A, inset) NREMS light/dark differences. (B) Wakefulness. (B, inset) Wakefulness light/dark differences. Data are expressed as mean (min) ± S.E.M of five rats per group. Gray backgrounds denote the dark phase. ***p*<0.01 *vs.* Vehicle; ##*p*<0.01, ### *p*<0.001 between treatments or light *vs*. dark phase, SNK *post hoc* comparisons.

Figure 3

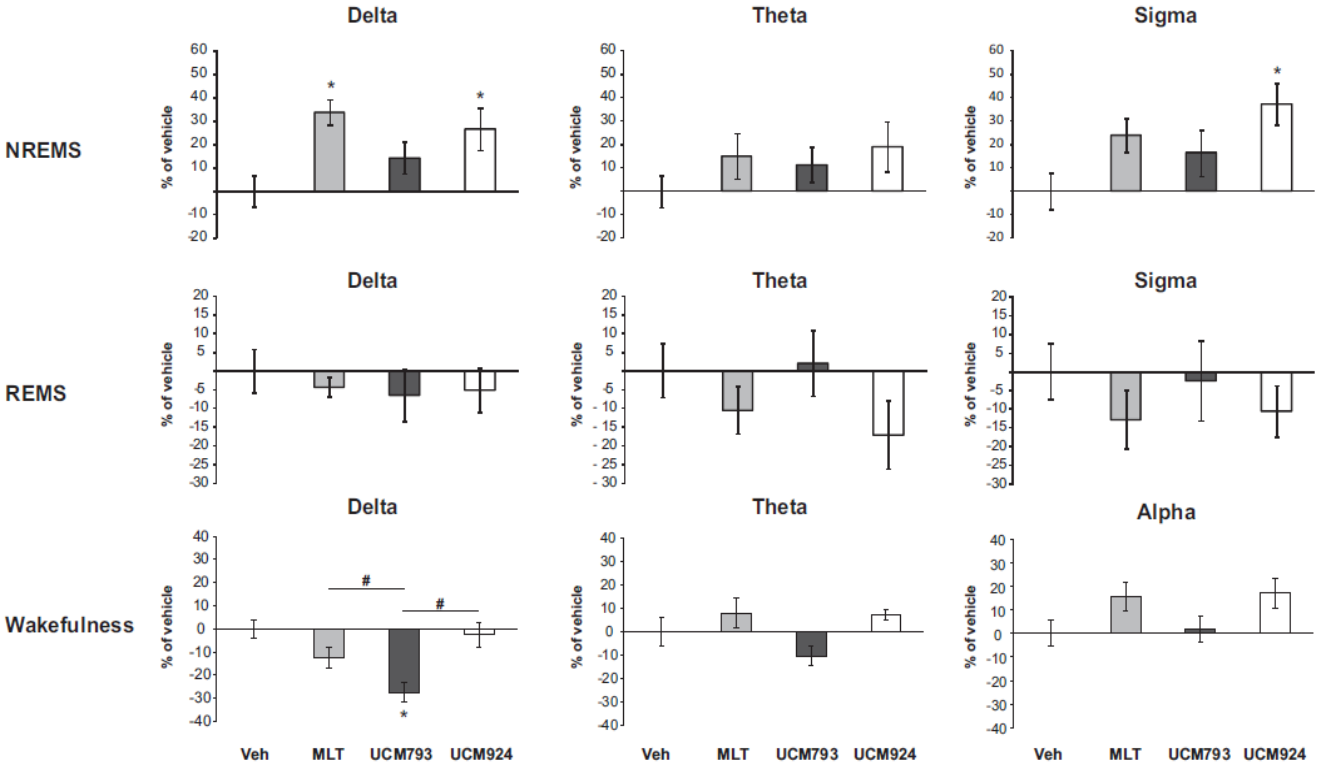

Fig. 3. Effects of MLT, UCM793 and UCM924 on the EEG power of delta, theta, sigma and alpha bands. (Left side) Delta band. (Center) Theta band. (Right side) Sigma or alpha bands. Data are expressed as mean (percentage of variation *vs.* vehicle) ± S.E.M. of five rats per group. *$p<0.05$ *vs.* vehicle; #$p<0.05$ between treatments, SNK *post hoc* comparisons.

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4.2 Interim discussion

Previous findings reported that MLT reduces the latency to sleep and increases the NREMS and REMS durations (Holmes and Sugden 1982; Wang et al., 2003b). On the contrary, other reports along with our findings demonstrated that MLT reduces the latency to sleep while having no changes in the duration of the three vigilant states.

In this chapter, we found that UCM793 shows a tendency to decrease the latency to sleep, as reported in chapter II. Interestingly, the 24 h sleep-wake analysis demonstrated that UCM793 modifies the wake state rather than NREMS or REMS. In particular, UCM793 increases the number and duration of wakefulness episodes. In keeping with this view, our results in MT_1/MT_2KO mice demonstrated that the dual MT_1/MT_2 genetic inactivation was associated with the regulation of wakefulness.

Although, the mechanism of action of both UCM793 and MLT involves the activation of MT_1/MT_2 receptors, we found that MLT and UCM793 exert different effects on sleep parameters, suggesting that the effects of MLT might involve not only the activation of MT_1/MT_2 receptors, as does UCM793, but also a membrane receptor-independent mechanism.

Then, similar to our results in chapter II with UCM765, here, the analog UCM924 reduces the latency to sleep and increases the duration and number of episodes of NREMS during the light phase. Differentially, UCM924 also increased the number of REMS episodes, an effect likely associated with different intrinsic activity towards MLT receptors. UCM924 is 100 times more selective for MT_2 than for MT_1 and acts as an antagonist for MT_1 and as a partial agonist for MT_2. In concordance, the genetic deletion of MT_1 receptors in MT_1KO mice impairs REMS duration. Therefore, we hypothesized that the effects of UCM924 on REMS episodes might be associated with its antagonistic actions on MT_1 receptors. Nevertheless, the increment in NREMS duration might also activate a compensatory mechanism that also increases the number of REMS episodes to maintain the NREMS/REMS pattern.

Interestingly, the analysis of MLT, UCM793 and UCM924 demonstrated that the selective activation of MT_2 receptors by UCM924 results in a more incisive effect not only on

sleep induction but also on sleep maintenance than does of dual activation of MT_1/MT_2 receptors by MLT or UCM793.

Overall, these results confirmed the role of the MT_2 receptor in the induction and maintenance of NREMS. Notably, differences at the level of intrinsic activity and affinity for MLT receptors may play a key role in the pharmacological actions of melatonergic compounds. Further research with selective ligands, including experiments with novel selective MT_1 agonists, may clarify the role of MT_1 and MT_2 receptors in the sleep-wake cycle. Nevertheless, selective agonists for the MT_2 receptor as well as for the MT_1 receptor should be considered in further research for the development of new hypnotics as well as to understand the role of MLT receptors in insomnia and other associated disorders.

Foreword to Chapter V

Anxiolytic effects of the melatonin MT_2 receptor partial agonist UCM765: comparison with melatonin and diazepam

In Chapters II-IV we focused on exploring the role of MLT receptors, in particular the MT_2 receptor, in sleep. We proved that both receptors, MT_1 and MT_2, are involved in sleep regulation and we proposed that these receptors may be considered as new targets in the pharmacological treatment of insomnia. Interestingly, animal and human studies have shown that MLT is also involved in the regulation of anxiety (Crupi et al., 2010). However, the single role of the MT_2 receptor in the regulation of anxiety remains to be defined. For these reasons, the goal of chapter IV was to explore the role of MLT and the MT_2 receptor in the regulation of anxiety.

It was reasonable to start with attempts at ascertaining the effects of the MT_2-selective partial agonist UCM765 in rats. Considering that several reports indicate that BZs tend to exert sedative-hypnotic effects at high doses while at low doses exert anxiolytic effects, herein we hypothesized that a lower dose than the hypnotic dose (40 mg/kg) of UCM765 may induce anxiolytic-like effects. First, we carried out the dose-response experiment testing doses from 10 to 40 mg/kg of UCM765 in two animal models of anxiety, elevated plus maze (EPMT) and novelty suppressed feeding (NSFT), widely used to validate the acute effects of putative anxiolytic compounds. Then, the effects of the effective dose of UCM765 (10 mg/kg) were compared to those of MLT (20 mg/kg) and to those of the clinically effective anxiolytic, DZ (1 mg/kg). In order to better understand the anxiolytic-like effects of UCM765 and MLT, the open field test (OFT) was also used to assess locomotion in order to confirm or discard a sedative effect. Moreover, in order to gain new insights into the neurobiological mechanism through which the melatonergic system regulates the anxiolytic-like effects of MLT, we characterized the single role of MT_1 and MT_2 receptors using a pharmacological approach employing two MLT receptor antagonists: the non-selective MT_1/MT_2 antagonist luzindole and the selective MT_2 antagonist 4P-PDOT. Notably, this chapter demonstrates the anxiolytic-like effects of UCM765 and MLT in the EPMT and NSFT with no sedative effects in the OFT. In addition, it shows that the MT_2 receptor is responsible for the anxiolytic-like effects of MLT and UCM765. Therefore, the MT_2 receptor may be considered a novel target for the treatment of anxiety disorders.

Chapter V

Anxiolytic effects of the melatonin MT_2 receptor partial agonist UCM765: comparison with melatonin and diazepam

5.1 Research paper

Anxiolytic effects of the melatonin MT_2 receptor partial agonist UCM765: comparison with melatonin and diazepam

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Abstract

Melatonin (MLT) is a neurohormone known to be involved in the regulation of anxiety. Most of the physiological actions of MLT in the brain are mediated by two high-affinity G-protein-coupled receptors, denoted MT_1 and MT_2. However, the particular role of these receptors in anxiety remains to be defined. Here we used a novel MT_2-selective partial agonist, UCM765 to evaluate the involvement of MT_2 receptors in anxiety. Adult male rats were acutely injected with UCM765 (5-10-20 mg/kg), MLT (20 mg/kg) or diazepam (DZ, 1 mg/kg). Anxiety-related behaviors were assessed in the elevated plus maze test (EPMT), novelty suppressed feeding test (NSFT) and open field test (OFT). UCM765 at the dose of 10 mg/kg showed anxiolytic-like properties by increasing the time spent in the open arm of the EPMT, and by reducing the latency to eat in a novel environment in the NSFT. In the EPMT, animals treated with UCM765 (10 mg/kg) or MLT (20 mg/kg) spent more time in the open arms compared to vehicle-treated animals, but to a lesser extent compared to DZ (1 mg/kg). In the NSFT, all treatments similarly decreased the latency to eat in a novel environment compared to vehicle. UCM765 and MLT did not affect the total time and the number of entries into the central area of the OFT, but unlike DZ, did not impair locomotion. The anxiolytic effects of UCM765 and MLT in the EPMT and the NSFT were blocked using a pre-treatment with the MT_1/MT_2 antagonist luzindole (10 mg/kg) or the MT_2 antagonist 4P-PDOT (10 mg/kg). These results demonstrated, for the first time, the anxiolytic properties of UCM765 and suggest that MT_2-receptors may be considered a novel target for the development of anxiolytic drugs.

1. Introduction

Anxiety is a physiological and psychological state characterized by unpleasant emotional state, fear and distressing physical symptoms in response to stressors. According to the DSM-IV (American Psychiatric Association, 1994), generalized anxiety disorder (GAD) is characterized by excessive anxiety and worry about a variety of topics occurring more days than not for at least six months. People with GAD find it difficult to control their worry, and often experience other related symptoms including restlessness, irritability, and muscle tension. Anxiety disorders represent a public health problem with a lifetime prevalence of 16.6 % (Somers et al., 2006). Benzodiazepines (BZs) are among the most clinically prescribed medications for the treatment of anxiety, but their use is correlated with unwanted side effects such as sedation, tolerance, dependence, next-day impairments, and abuse liability (Whiting, 2006). For this reason, there is a growing interest in exploring novel pharmacotherapeutic targets for the treatment of anxiety. Among these emerging targets, the melatonergic system has gained considerable attention. The neurohormone melatonin (MLT) is synthesized by the pineal gland during the dark period of the light-dark cycle, and in mammals modulates many physiological functions such as mood, circadian rhythms, sleep, and reproduction (Reiter, 1991; Simonneaux and Ribelayga, 2003). In addition to its neuroendocrine functions, MLT has also been linked to stress and anxiety (Caumo et al., 2009; Rios et al., 2010). The physiological actions of MLT are mainly mediated by two G protein-coupled receptors, MT_1 and MT_2, which are widely expressed in the mammalian brain (Dubocovich and Markowska, 2005; Reppert, 1997), but their selective role in anxiety has yet to be identified. MLT has elicited anxiolytic effects in both preclinical (Crupi et al., 2010; Golombek et al., 1993; Golus and King, 1981; Papp et al., 2006) and human studies (Caumo et al., 2009; Srinivasan et al., 2006) and recently, the non-selective MT_1/MT_2 receptor agonists agomelatine and Neu-P11 have been shown to possess anxiolytic properties in preclinical models of anxiety (Millan et al., 2005; Rainer et al., 2011; Tian et al., 2010). In addition, ramelteon (a non selective MT_1/MT_2 receptor agonist) and agomelatine, two drugs currently on the market as a hypnotic and an antidepressant, respectively, have also produced anxiolytic effects in humans (den Boer et al., 2006; Gross et al., 2009; Stein et al., 2008). Due to the lack of selectivity towards MT_1 and MT_2 receptors, these melatonergic agonists do not allow pharmacological investigation on the specific role of each MLT receptor subtype on anxiety. We recently demonstrated the hypnotic effect of the MT_2-selective partial agonist UCM765 in rats and mice,

and the pivotal role of MT_2 receptors in the promotion of non rapid eye movement sleep (Ochoa-Sanchez et al., 2011). UCM765 belongs to the class of *N*-(anilinoethyl)amides (Rivara et al., 2007), whose structure can be further modulated, improving metabolic stability while maintaining the same receptor profile (Rivara et al., 2009). UCM765 exhibits greater affinity for MT_2 receptors (pKi = 10.18) than for MT_1 receptors (pKi = 8.38), showing even greater affinity than MLT itself (pKi = 9.62) (Rivara et al., 2007). In this study, we tested whether UCM765 at a lower dose (10 mg/kg) than that having hypnotic effects (Ochoa-Sanchez et al., 2011) could possess anxiolytic-like properties. This dose was compared with a low dose of the BZ diazepam (DZ) (1 mg/kg) and with an equivalent dose of MLT (20 mg/kg). The effects of UCM765 and MLT were then blocked by selective antagonists toward MLT receptors, thus offering insight into the role of MT_1 and MT_2 receptors in anxiety.

2. Material and methods

All experimental procedures were conducted in accordance with standards and ethical guidelines mandated by the Canadian Institutes of Health Research, the Canadian Council on Animal Care, and McGill University Animal Care Center.

2.1. Animals

Adult Sprague-Dawley male rats (Charles River, Canada) weighing 250-260 g at the time of arrival were used in these experiments. Rats were housed in standard polypropylene cages, in pairs, under a 12h:12h dark: light cycle (lights on at 7:00 a.m.). Room temperature was kept between 20-21°C and access to food and water was *ad libitum*. Once they reached a weight of 300 g, all animals were submitted to a habituation period into the behavioral room for 3 h prior to the beginning of each experiment. All experiments were carried out between 4 to 7 p.m., when the physiological function and the expression of MT_2 receptor are maximal (Ochoa-Sanchez et al., 2011; Witt-Enderby et al., 2003).

2.2. Drugs and pharmaceutical treatments

MLT 20 mg/kg (Sigma-Aldrich, Oakville, ON, Canada), Diazepam (DZ) 1 mg/kg (Valium) and UCM765 (*N*-{2-[(3-methoxyphenyl)phenylamino]ethyl}acetamide) 5, 10 and 20 mg/kg were dissolved in a vehicle composed of 50 % dimethyl sulfoxide (DMSO, MP Biochemicals, Solon, OH, USA) and 50 % saline solution (NaCl 0.9 %). Luzindole 10 mg/kg (Tocris, Ellisville, MO, USA) and 4P-PDOT 10 mg/kg (Tocris, Ellisville, MO, USA) were dissolved in DMSO. These doses were chosen in accordance with the literature (Golombek et al.,

1993; Ochoa-Sanchez et al., 2011, Papp et al., 2006; Sudhakar et al., 2011; Domínguez-Lopez et al., 2011).

Drugs were injected subcutaneously (s.c.; 0.2 ml) 15 min prior to the behavioral tests. In the experiments aimed at exploring the selective role of MLT receptors on anxiety, rats were treated with either vehicle (0.2 ml; s.c.), the MT_1/MT_2 antagonist luzindole (0.2 ml; s.c.) or the MT_2 antagonist 4P-PDOT (0.2 ml; s.c.) 30 min before testing, followed by either vehicle, MLT or UCM765 (0.2 ml; s.c.) 15 min later,.

2.3. Elevated plus maze test (EPMT)

The EPMT apparatus consisted of two open arms (50 cm × 10 cm) crossed at right angles with two opposed arms of the same size. Two of the opposed arms were enclosed by wooden walls (40 cm high). The maze was raised 80 cm above the floor and had a 10 × 10 cm central area where the arms crossed. The rats were placed on the central platform facing one of the open arms. EPMT behavior was recorded for 5 min. The total time spent in the open arms and percentage of visits into the open arms was analyzed using an automated behavioral tracking system (Videotrack, View Point Life Sciences, Inc., Canada). Anti-anxiety behavior was assessed on the basis that anxiolytic drugs significantly increase the time spent and visits into the open arms (Pellow et al., 1985). Arm entries depended on whether the animal's center of gravity defined by the automated tracking system (commonly localized on the back) crossed the predefined borders of each of the open and closed arms (Bambico et al., 2010).

2.4. Novelty-suppressed feeding test (NSFT)

The NSFT has been used to validate the acute effects of putative anxiolytics by measuring anxiety-induced hyponeophagia, which is the inhibition of ingestion and approach to food when exposed to an anxiety-provoking novel environment. Modified from Bambico et al. (2010), rats were weighed and food-deprived for 24 hours, after which they were placed in the same corner of an open arena composed of black walls (80 × 80 × 30 cm) with a floor covered by layer (1 cm) of sawdust. The center of the arena was lightened with a white lamp suspended 2 m above the arena in order to reinforce the aversion for this area. The center of the arena contained three laboratory chow pellets placed on a circular white paper (12 cm diameter). The latency to initiate a feeding behavior (in seconds) was recorded, and used as an index of anxiety-like behavior. The test was stopped immediately after the first feeding episode and the cut-off time was 600 seconds. It was found that anxiogenics and anxiolytics increase and decrease,

respectively, the latency to eat. These effects are observed in the novel environment and not in the familiar home cage (Bodnoff et al., 1988; Gross et al., 2000). For this reason, immediately after the NSFT, each rat was returned to their home cage, which contained 3 chow pellets placed on the cage lid. The latency to eat in the home cage as well as food consumption was noted, as rats were allowed to continue eating for 5 min. Food consumption was measured by weighing the food pellet before and after the test in the home cage. Rats were weighed again at the end of the session. Only data from rats that lost weight (≥ 6.0 % body weight) were considered for statistical analysis.

2.5. Open field test (OFT)

Rats were each placed in the same corner of a black-painted wooden open field arena (80 × 80 × 30 cm) illuminated with a white light lamp suspended 2 m above the arena. The experiment was recorded for 10 min and anxiety-like behavior was measured by the total time spent and the frequency of visits into the central zone (40 × 40 cm). Anxiogenic drugs decrease these behavioral endpoints, whereas anxiolytics conversely increase them (Prut and Belzung, 2003). Locomotor activity (total distance travelled) was also measured. The parameters were analyzed using an automated behavioral tracking system (Videotrack, View Point Life Sciences, Inc., Canada) equipped with infrared-sensitive cameras.

2.6. Statistical analysis

Data analysis was carried out using SigmaPlot 11.0. Results were expressed as mean ± SEM. After testing for assumptions of normality of data distribution and homogeneity of variance, one-way or two-way ANOVA, followed by the Student-Newman-Keuls (SNK) post-hoc comparisons were used for analysis. Statistical values reaching $P \leq 0.05$ were considered significant.

3. Results

3.1. Dose response effects of UCM765 in the EPMT and NSFT

In the EPMT, one-way ANOVA on the time spent in the open arms (Fig. 1A) revealed a significant effect of the treatment ($F(3,31)=9.4$, $p<0.001$). SNK post-hoc test indicated that acute injection of UCM765 at 10 mg/kg significantly increased the time spent in the open arms compared to vehicle ($p<0.001$) and UCM765 at 5 mg/kg ($p=0.02$) and 20 mg/kg ($p<0.001$). No effect of different doses of UCM765 was found for the percentage of entries into the open arms ($F(3,31)=1.5$, $p=0.2$; Fig. 1B).

In the NSFT, one-way ANOVA on the latency to eat in a novel environment showed a significant effect of treatment ($F(3,25)=3.0$, $p=0.04$). Remarkably, UCM765 at the dose of 10 mg/kg significantly decreased the latency to eat compared to vehicle ($p=0.03$; Fig. 1C). The latency to eat in the home cage (Fig. 1D) was not affected by any dose of UCM765 ($F(3,25)=2.30$, $p=0.1$).

3.2. Melatonin and UCM765 display anxiolytic properties

The effects of MLT, UCM765 and DZ in EPMT and NSFT are shown in Fig. 2. In the EPMT, one-way ANOVA revealed a main effect between groups for the time spent in the open arms ($F(3,32)=18.50$, $p<0.001$) and the percentage of entries into the open arms ($F(3,32)=7.29$, $p=0.001$). Compared to vehicle, SNK post-hoc comparisons revealed that MLT ($p=0.03$) and UCM765 ($p=0.004$) significantly increased the time spent in the open arms similar to DZ ($p<0.001$); however, the effect of DZ was of higher magnitude than MLT ($p<0.001$) and UCM765 ($p=0.001$; Fig. 2A). The percentage of entries into the open arms was not affected by MLT and UCM765, but only by DZ ($p<0.001$ vs. vehicle, $p=0.007$ vs. MLT; $p=0.004$ vs. UCM765; Fig. 2B). One-way ANOVA on the percentage of entries into the closed arms showed significant main differences between treatments ($F(3,32)=7.53$, $p<0.001$; data not shown). Post-hoc analysis indicated that compared to vehicle, only DZ decreased the percentage of entries into the closed arms (65.8 ± 4.8 vs. 30.8 ± 5.3, $p<0.001$).

In the NSFT, one-way ANOVA revealed a significant effect of treatment on the latency to feed ($F(3,28)=5.37$, $p=0.005$). The SNK post-hoc test indicated that MLT, UCM765 and DZ all significantly decreased the latency to eat in a novel environment compared to vehicle (Fig. 2C). Conversely, these pharmacological treatments did not affect the latency to eat ($F(3,28)=1.54$, $p=0.2$; Fig. 2D) and the amount of food intake per animal body weight in the home cage ($F(3,28)=1.22$, $p=0.3$; data not shown).

The effects of MLT, UCM765 and DZ in the OFT are reported in Fig. 3. One-way ANOVA performed on the OFT showed a significant main effect of treatment for the total distance travelled ($F(3,36)=3.52$, $p<0.02$) and the number of entries ($F(3,36)=2.98$, $p<0.04$), but not for the time spent in the central area ($F(3,36)=2.02$, $p=0.12$; Fig. 3B). SNK post-hoc comparisons revealed that MLT and UCM765 did not affect locomotion compared to vehicle. On the contrary, DZ significantly decreased the total distance travelled compared to vehicle ($p=0.02$), MLT ($p=0.04$) and UCM765 ($p=0.02$; Fig. 3A). The number of entries into the central

area also remained unchanged after MLT or UCM765 treatment, whereas DZ produced a significant decrease in comparison to vehicle (p=0.01; Fig. 3C).

3.3. Anxiolytic effect of melatonin and UCM765 is blocked by Luzindole and 4P-PDOT

To study the involvement of MT_1 or MT_2 receptors in the anxiolytic profile shown by MLT and UCM765, two MLT receptor antagonists were used: the non-selective MT_1/MT_2 antagonist luzindole and the MT_2-selective antagonist 4P-PDOT. Neither of these compounds displayed an anxiety-related effect: the time spent in the open arm ($F(2,17)=0.2$, $p=0.8$), the percentage of entries into the open arm ($F(2,17)=0.6$, $p=0.5$), and the latency to eat in a new environment ($F(2,18)=0.5$, $p=0.5$) were not different than vehicle (Fig 4A-C). However, an effect on feeding behavior was found after blocking both MT_1 and MT_2 receptors with luzindole (Fig. 4D). The latency to eat in the home cage ($F(2,18)=4.74$, $p=0.02$) was significantly higher when rats were treated with luzindole instead of vehicle ($p=0.01$) or 4P-PDOT ($p=0.03$).

In EPMT, a two-way ANOVA performed on the time spent in the open arms showed no significant main effect for pre-treatment (luzindole or 4P-PDOT; $F(1,36)=0.007$, $p=0.9$) and treatment (vehicle, MLT, or UCM765; $F(2,36)=0.76$, $p=0.4$), with no interaction between these two factors ($F(2,36)=0.09$, $p=0.9$). As reported in Fig. 5A, MLT and UCM765 in the presence of luzindole or 4P-PDOT did not lead to the increase in the time spent in the open arm shown in Fig. 2A. Two-way ANOVA on the percentage of entries into the open arms yielded no significant differences between groups for pre-treatment ($F(1,36)=0.006$, $p=0.9$) and treatment ($F(2,36)=0.76$, $p=0.4$), with no interaction ($F(2,36)=0.09$, $p=0.9$, Fig. 5B).

In the NSFT, two-way ANOVA performed on the latency to eat food in a novel environment showed no significant main effect for pre-treatment ($F(1,34)=2.09$, $p=0.1$) and treatment ($F(2,34)=0.7$, $p=0.4$), with no interaction ($F(2,34)=0.02$, $p=0.9$). The effects on the latency to eat in a novel environment displayed by MLT and UCM765 (Fig. 2C) were blocked by both luzindole and 4P-PDOT (Fig. 5C). Two-way ANOVA on the latency to eat food in the home cage (Fig. 5D) showed no significant differences between groups in any of the factors, pre-treatment ($F(1,34)=3.92$, $p=0.6$), treatment ($F(2,34)=0.78$, $p=0.4$) and their interaction ($F(2,34)=1.27$, $p=0.2$). Two-way ANOVA on food consumption per animal body weight in the familiar environment revealed no significant differences between groups for pre-treatment ($F(1,34)=1.34$, $p=0.2$) and treatment ($F(2,34)=0.4$, $p=0.6$), with no interaction ($F(2,34)=0.78$, $p=0.4$; data not shown).

4. Discussion

The aim of this study was to investigate the anxiolytic-like effect of the novel selective MT_2 receptor partial agonist UCM765 and MLT. We examined the effects of UCM765 and MLT in three well-established animal paradigms of anxiety: EPMT, NSFT, and OFT. Moreover, in order to gain new insights in the neurobiological mechanism through which the melatonergic system may regulate anxiety, we used a pharmacological approach employing the non-selective MT_1/MT_2 receptor antagonist luzindole and the selective MT_2-selective antagonist 4P-PDOT to determine which MLT receptor subtype mediates the anxiolytic properties of MLT.

The dose response of UCM765 in the EPMT and NSFT indicates a maximal anxiolytic-like effect at 10 mg/kg, a dose that does not affect locomotor behavior in the OFT. However, a higher dose of UCM765 (20 mg/kg) does not show anxiolytic properties as previously reported for Neu-P11 (Tian et al, 2010) and agomelatine (Papp et al., 2006), two non selective melatonergic drugs. In agreement with our findings, previous reports showed that MLT induces an anxiolytic-like effect in the EPMT by increasing the time spent in the open arms (Golombek et al., 1993) and in NSFT by decreasing the latency to eat (Crupi et al., 2010). In spite of the anxiolytic properties of MLT in the EPMT and NSFT, we did not find any effect of MLT in the OFT. In contrast, Golus and King (1981) found that MLT increased the activity within the central area of the OFT. This disagreement may be due to the different experimental protocols used. Indeed, Golus and King (1981) employed a lower dose of MLT (1 mg/kg) that was injected 1 h before the test, which lasted 30 min. Nevertheless, the half-life of MLT in rats is 23 min (Gibbs and Vriend, 1981), and consequently, factors other than MLT could have influenced their findings. It seems that different animal models could be tapping different aspects of anxiety (Green, 1991), thus explaining why we found an anxiolytic effect of MLT and UCM765 only in the EPMT and NSFT, but not in the OFT. In agreement with other molecules, some have shown anxiolytic properties depending on the animal paradigm employed. As an example, the antidepressant fluoxetine has an anxiolytic profile in the NSFT but not in the EPMT (Borsini et al., 2002).

Other MLT receptor agonists such as Neu-P11, agomelatine and S23478, displayed anxiolytic-like effects in rodents. Neu-P11, an agonist to both MT_1/MT_2 receptors, promotes anxiolytic-like effects in EPMT in mice (Tian et al., 2010). Agomelatine, a MT_1/MT_2 receptors agonist but also a 5-HT_{2C} receptor antagonist (de Bodinat et al., 2010; Millan et al., 2005), showed anxiolytic-like effects in rats in the EPMT, conditioned foot-shock-induced ultrasonic vocalization test

(Papp et al., 2006) and punishing drinking test (Loiseau et al., 2006). S23478, another non-selective MT_1/MT_2 receptor agonist, decreased anxious reactions in the free-exploratory and light/dark choice test in mice (Kopp et al., 2000). Moreover, the authors clearly demonstrated that the anxiolytic properties of MLT could involve the activation of MT_1 and/or MT_2 receptors, but they could not provide information whether only one or both MLT receptor subtypes are involved in such effect. Altogether these findings argue that the activation of MLT receptors is a good strategy to reduce anxiety, and to our knowledge this work is the first attempting to elucidate which MLT receptor subtype is involved.

Our results show that UCM765, a MT_2-selective partial agonist, produces anxiolytic-like effects in the EPMT and NSFT at lower doses than MLT. This effect may be due to the higher affinity for the MT_2 receptor, since the anxiolytic-like behavior is mostly blocked by the MT_2-selective antagonist, 4P-PDOT.

This finding allows us also to speculate that only activation of MT_2 receptors may be sufficient to elicit an anxiolytic effect. To test such a hypothesis, we used the non-selective antagonist toward MT_1 and MT_2 receptors luzindole, and 4P-PDOT. While blockade of both MT_1 and MT_2 receptors or MT_2 receptors alone did not show any effect, the anxiolytic properties of MLT and UCM765 were blocked by both luzindole and 4P-PDOT. Interestingly, blockade of MT_1 and MT_2 receptors by luzindole increased the latency to eat in the home cage, whereas selective blockade of MT_2 receptor by 4P-PDOT did not influence this behavior. Therefore, a tonic effect of MLT on food intake mediated by MT_1 receptors may be implicated in this effect. Indeed, it has been shown that a feedback system between serotonin and MLT may regulate appetite and digestive processes by endocrine as well as paracrine effects in both the brain and the gastrointestinal tract (Bubenik and Pang, 1994). We have recently shown that activation of MT_2 receptors by UCM765 at high doses selectively induces hypnotic effects (Ochoa-Sanchez et al., 2011). However, while in sleep the concomitant activation of both MT_1 and MT_2 receptors by UCM793 (a non selective MT_1/MT_2 receptor agonist) and MLT produces only a slight effect, because of their opposing roles (Ochoa-Sanchez et al., 2011; Comai et al., submitted), in the anxiety paradigm, the co-activation of both receptors by MLT still elicits a significant pharmacological response, indicating that in the case of anxiety, MT_1 receptor has not preventing effect over the MT_2 receptor.

Evidence exists for an interaction of the melatonergic system with the γ-aminobutyric acid (GABA), serotonin and glutamate neurotransmissions (Eison et al., 1995; Mantovani et al., 2003; Raghavendra et al., 2000). Indeed, it is noteworthy that these different neurotransmitters are involved in the neurobiology of anxiety (Millan, 2003) and are under the regulation of MLT. In fact, MLT administration increased GABA levels in several brain regions such as the hypothalamus, cerebellum and cerebral cortex (Rosenstein and Cardinali, 1986), and the administration of flumazenil, a benzodiazepine receptor antagonist, blocked the anxiolytic-like effect of MLT in the EPMT (Golombek et al., 1993). Moreover, neurons of the reticular thalamus, that are known to be exclusively GABAergic in rodents, are endowed with MT_2 receptors. A selective activation of MT_2 receptors within this nucleus elicits hypnotic effects (Ochoa-Sanchez et al., 2011). In addition, Eison et al. (1995) showed an interaction between MLT and 5-HT_{2A} receptors in the central nervous system, which are known to play a key role in anxiety-like state modulation in mice (Weisstaub et al., 2006). Despite this evidence, further studies are necessary to explain first the molecular and cellular connections between the melatonergic system and the other neurotransmitter systems involved in anxiety, and second, the specific role played by MT_2 receptors in this complex neurobiological framework.

Another important finding in this study is that activation of MT_2 receptors seems to be a different anxiolytic strategy compared to the direct activation of the GABAergic system by anxiolytic drugs such as benzodiazepines and their derivatives. Indeed, we found that even though the anxiolytic effects of MLT and UCM765 in the EPMT and NSFT were milder than DZ, they did not impair locomotion in the OFT and the percentage of entries into the closed arms in the EPMT, a reliable index of locomotor activity (Cruz et al., 1994). Therefore, UCM765 and MLT, even though they possess mild anxiolytic properties, do not lead to sedation, one of the most common and unwanted side effects of benzodiazepines and derivates. In addition, UCM765 compared to DZ did not affect the percentage of entries into the open arms of the EPMT but only altered the time spent into the open arms, a parameter more sensitive to an anxiolytic drug effect (Pellow et al., 1985). These findings suggest that the melatonergic system, and in particular MT_2 receptors, most likely promote anxiolytic effects through the activation of different brain networks/pathways than benzodiazepines. Future research should examine whether these differences are at regional, cellular, or subcellular levels.

In the OFT, DZ significantly decreased locomotion without affecting the frequency and total duration of central zone visits. While our results in the EPMT and the NSFT after DZ treatment are in accordance with previous literature (Bodnoff et al., 1988; Gross et al., 2000; Rex et al., 1996; Sudhakar et al., 2011), they are contradictory to some findings reported on the effect of DZ in the OFT. We found that DZ decreased locomotion and the number of entries into the central area without affecting the time spent in the center. Siemiątkowski et al. (2000) showed that DZ (0.05, 0.02 and 1.5 mg/kg) decreased locomotion without affecting the number of entries into the central area. In another study, DZ, at doses of 0.05 and 0.1 mg/kg, increased the time spent and the number of entries into the central area without affecting locomotor activity, whereas a higher dose (0.5 mg/kg) showed no effect (Stefański et al., 1992). These contradictory findings in the effect of DZ in the OFT may rely on the different doses employed, light conditions and time of administration before the behavioral session.

5. Conclusion

We showed for the first time that a selective activation of MT_2 receptors by UCM765 is able to elicit a mild anxiolytic-like effect similar to MLT. Moreover, we demonstrated that the anxiolytic properties of MLT rely on its activation of MT_2 receptors only. Indeed, anxiolytic properties of UCM765 and MLT were both blocked by the MT_1/MT_2 receptor antagonist luzindole and by the MT_2-selective antagonist 4P-PDOT. Therefore, these results provide the evidence that the MT_2 receptor may represent a novel potential target for the treatment of anxiety with low sedation and lack of abuse potential (Lemoine et al., 2011).

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Figures

Figure 1

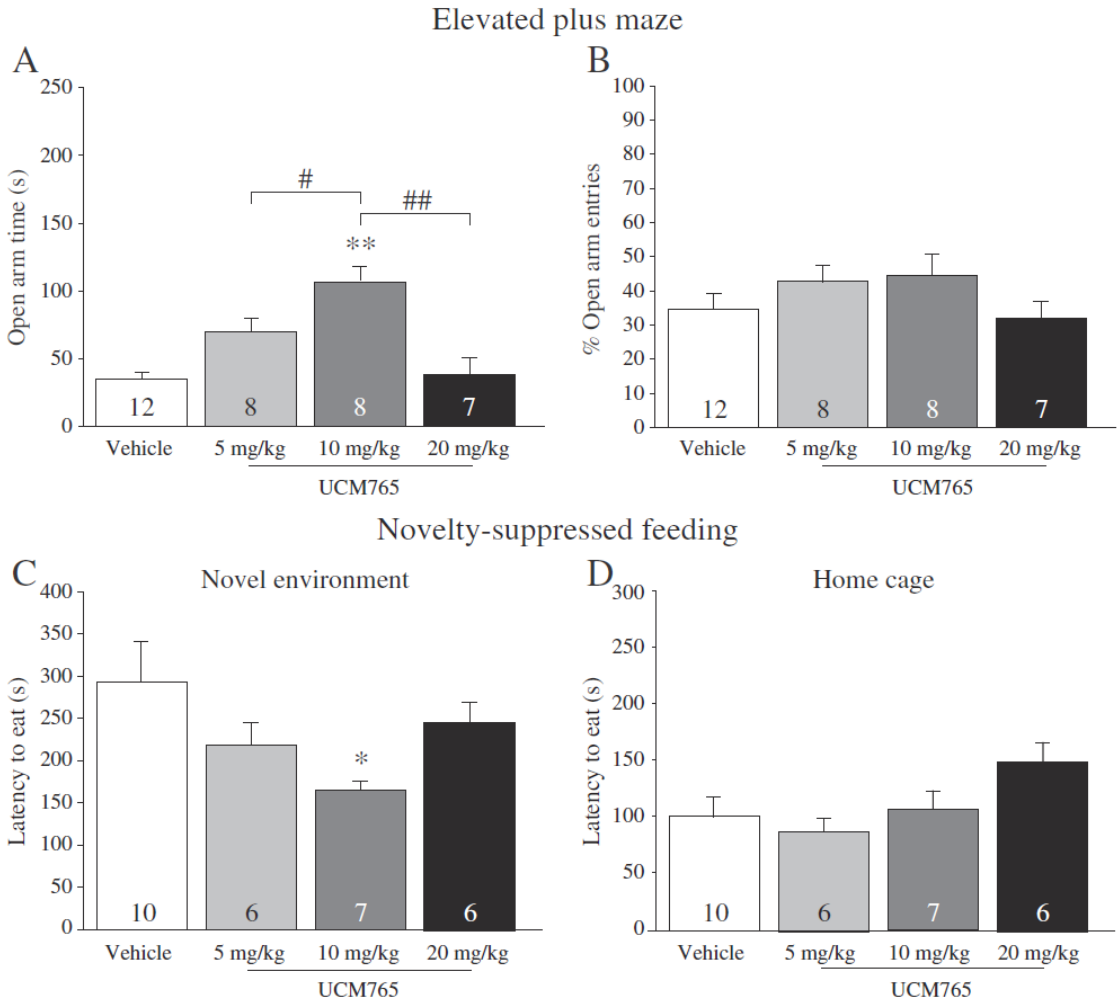

Fig. 1. Dose response of UCM765 (5, 10 and 20 mg/kg, s.c.) in the elevated plus maze test (EPMT) and novelty suppressed feeding test (NSFT). All doses were administered 15 min prior to the test. UCM765 at 10 mg/kg increased the time spent in the open arms in comparison with vehicle and UCM765 at 5 and 20 mg/kg (A). No significant difference was found in the percentage of entries into the open arms (B). UCM765 at 10 mg/kg decreased the latency to eat in a novel environment in comparison to vehicle (C). Any dose of UCM765 has modified the latency to eat in the home cage (D). Data are expressed as Mean ± SEM; numbers within bars indicate the number of rats per group. **$p<0.01$ vs vehicle; #$p<0.05$, ##$p<0.01$ vs UCM765 5 mg/kg or 20 mg/kg, one-way ANOVA, followed by SNK post-hoc test.

Figure 2

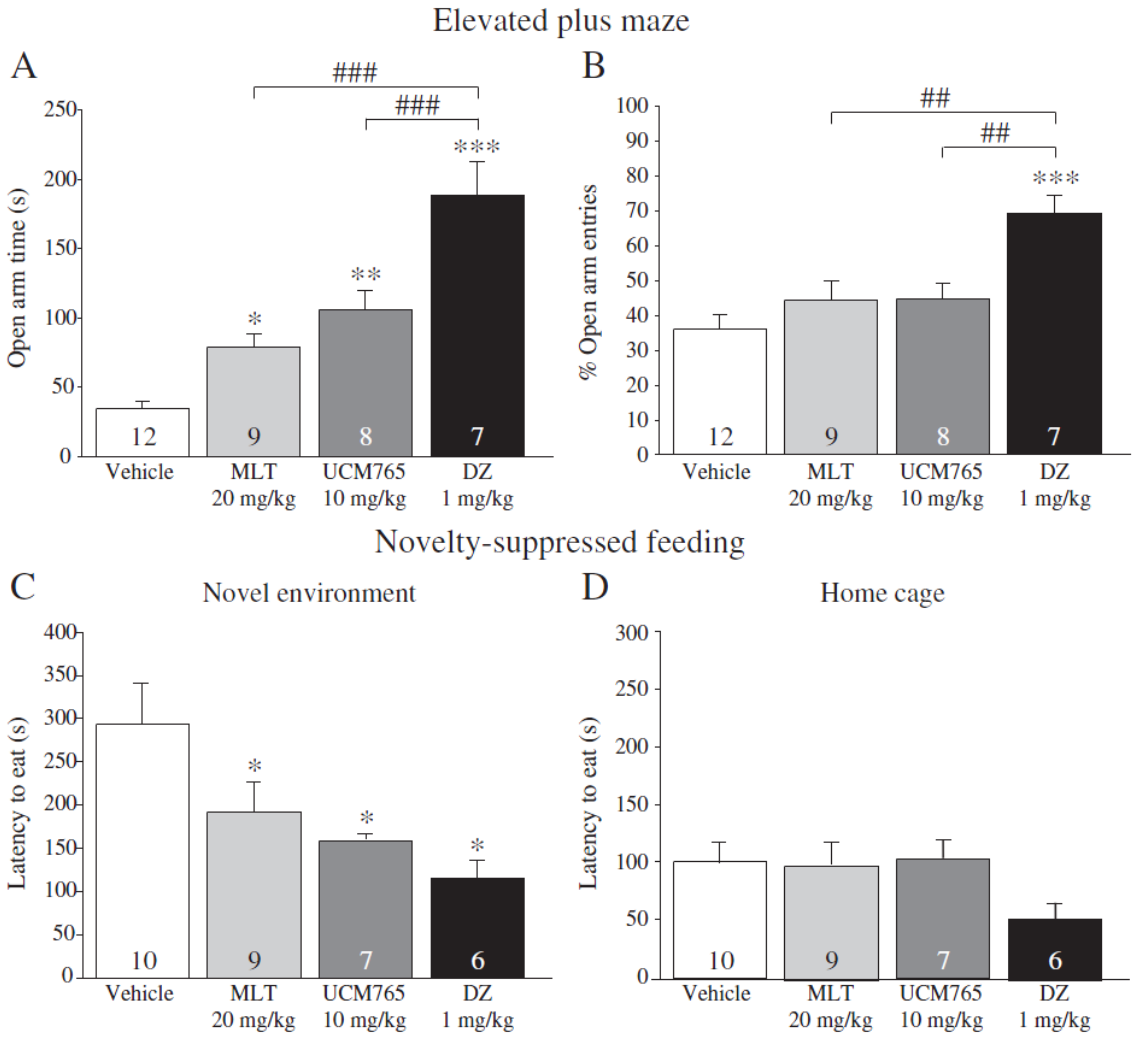

Fig. 2. Effects of melatonin (MLT, 20 mg/kg, s.c.), UCM765 (10 mg/kg, s.c.) and DZ (1 mg/kg, s.c.) on the elevated plus maze test (EPMT) and novelty suppressed feeding test (NSFT). The drugs were injected 15 min prior to the tests. MLT and UCM756 as well as DZ increased the time spent in the open arms compared to vehicle (A). DZ, but not MLT and UCM765, increased the percentage of entries into the open arms in comparison to vehicle (B). MLT, UCM765 and DZ, decreased the latency to initiate a feeding episode in comparison with vehicle in a novel environment (C) while they did not affect the latency to eat in the home cage (D). Data are expressed as Mean ± SEM; numbers within bars indicate the number of rats per group. *p<0.05, **p<0.01, ***p<0.001 vs vehicle; ##p<0.01, ###p<0.001 vs MLT or UCM765, one-way ANOVA, followed by SNK post-hoc test.

Figure 3

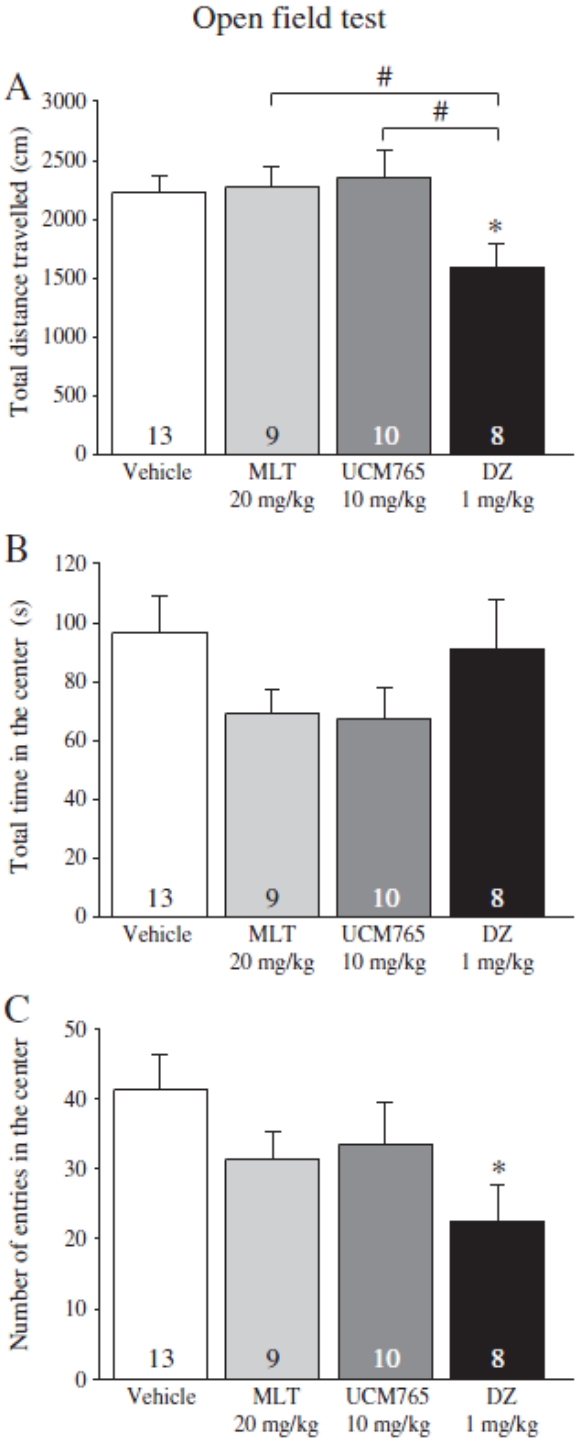
Open field test
A
Total distance travelled (cm)
3000
2500
2000
1500
1000
500
0
#
#
*
13
9
10
8
Vehicle
MLT 20 mg/kg
UCM765 10 mg/kg
DZ 1 mg/kg
B
Total time in the center (s)
120
100
80
60
40
20
0
13
9
10
8
Vehicle
MLT 20 mg/kg
UCM765 10 mg/kg
DZ 1 mg/kg
C
Number of entries in the center
50
40
30
20
10
0
*
13
9
10
8
Vehicle
MLT 20 mg/kg
UCM765 10 mg/kg
DZ 1 mg/kg

Fig. 3. Effects of MLT (20 mg/kg, s.c.), UCM765 (10 mg/kg, s.c.) and DZ (1 mg/kg, s.c.) in the open field test (OFT). The drugs were injected 15 min prior to the test. DZ, but not MLT and UCM765, decreased the total distance travelled in the OFT (A) and the number of entries into the central area (C). Any of the treatments produced changes in the time spent into the central area of the OFT (B). Data are expressed as Mean ± SEM; numbers within bars indicate the number of rats per group. *$p<0.05$ vs vehicle; #$p<0.05$ vs MLT or UCM765; one-way ANOVA, followed by SNK post-hoc test.

Figure 4

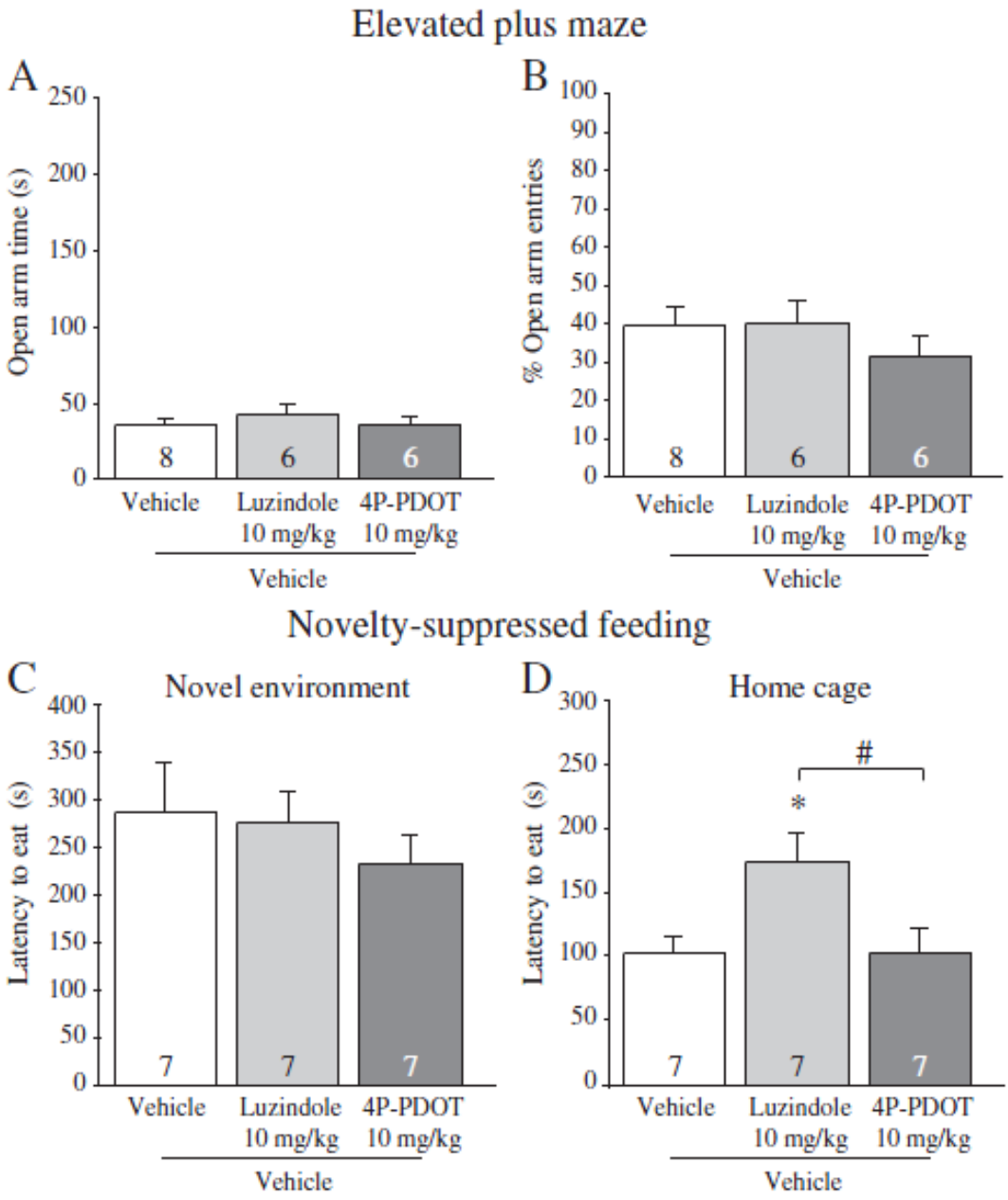

Fig. 4. Blockage of MT_2 and MT_1/MT_2 receptors does not affect anxiolytic-like behavior. The time spent in the open arms (A), the percentage of open arms entries (B), and the latency to eat in a novel environment (C) were not modified by luzindole or 4P-PDOT. The latency to eat in the home cage (D) was increased after treatment with luzindole. Data are expressed as mean ± SEM; numbers within bars indicate the number of rats.

Figure 5

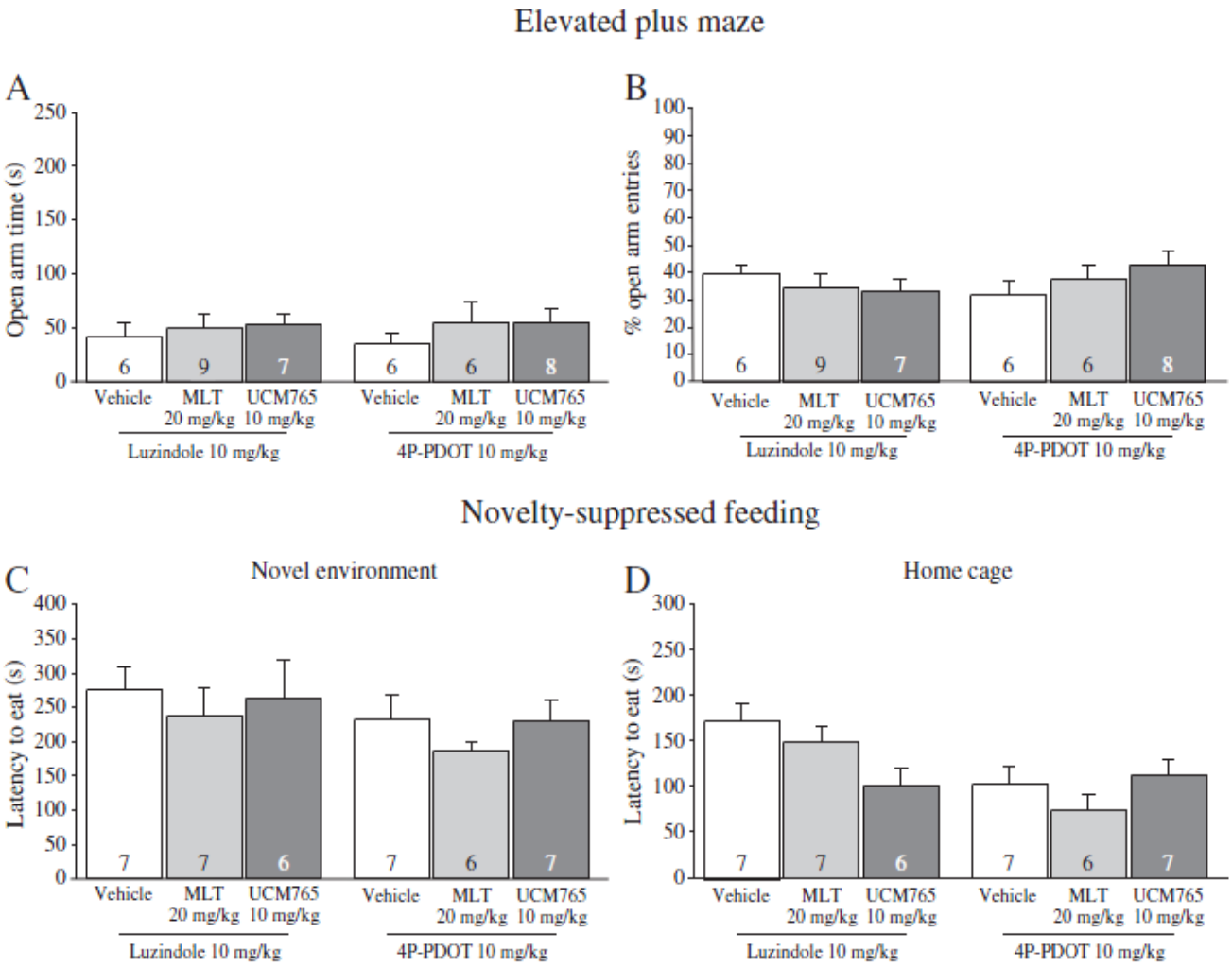

Fig. 5. Anxiolytic-like activity of MLT and UCM765 is due to activation of MT_2 receptors. The time spent in the open arms (A) and the percentage of open arms entries (B) were not modified by MLT or UCM765 in the presence of luzindole or 4P-PDOT. MLT and UCM765 in the presence of luzindole or 4P-PDOT did not affect the latency to eat in a novel environment (C) as well as in the home cage (D). Data are expressed as Mean ± SEM; numbers within bars indicate the number of rats.

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5.2 Interim discussion

The aim of this chapter was to examine the role of the MT_2 receptor in the regulation of anxiety. We tested the effects of UCM765 and MLT in the EPMT, NSFT and OFT. Similarly to DZ, UCM765 and MLT reduced the time spent in the open arm of the EPMT and reduced the latency to eat in a novel environment in the NSFT. In agreement, previous studies with MLT have reported an anxiolytic-like effect in the EPMT and NSFT. The anxiolytic-like effects of MLT and UCM765 were milder than those of DZ. Nevertheless, the anxiolytic-like effects of DZ were associated with sedative effects, one of the most unwanted side effects of BZs. In particular, DZ but not MLT or UCM765 decreased the total distance travelled and number of entries into the central area of the OFT. Therefore, MLT and UCM765 might represent a therapeutic option with no sedative effects.

Later, the pre-treatment with either the selective MT_2 antagonist 4P-PDOT or the non-selective MT_1/MT_2 antagonist luzindole inhibited the anxiolytic-like effects of UCM765 and MLT, suggesting that the anxiolytic-like effects of MLT and UCM765 are mediated by the MT_2 receptor. Notably, the single administration of luzindole or 4P-PDOT showed no significant effects in the EPMT and NSFT. Similarly, previous reports with lunzindole at higher doses (30 and 60 mg/kg) than in our study did not modify anxiety levels (Nava and Carta 2001). Regarding 4P-PDOT, it will be interesting to explore whether 4P-PDOT at different doses modifies the behaviour in animal models of anxiety.

In relation to the single role of the MT_2 receptor in anxiety, in chapter II, we demonstrated that the pharmacological actions of UCM765 on sleep are mediated by the MT_2 receptor, effects confirmed by pharmacological and genetic inhibition of MT_2 receptors. Moreover, our unpublished data in MT_2KO mice demonstrates that the genetic deletion of the MT_2 receptor modifies behavior in the NSFT, EPMT and OFT (see Comai and Gobbi 2014). Compared to the WT, MT_2KO mice showed no effects in the number of entries and time spent in the open arms, but MT_2KO mice increased the time spent in the center of the maze, an effect likely associated with decision memory impairment in this strain. In the NSFT, MT_2KO mice increased the latency to eat in the novel environment with no changes in the latency to eat and food consumption in the home cage. In the OFT, MT_2KO mice increased the number of entries and time spent in the central area, with no changes in locomotion when compared to WT mice.

Although, the genetic inactivation of MT_2 receptors in mice shows complex effects in anxiety paradigms depending on the test, in general these results support the participation of the MT_2 receptor in the regulation of anxiety.

Interestingly, the results obtained in the role of MLT receptors in sleep and anxiety suggest that the selective or non-selective activation of MT_2 or MT_1/MT_2 receptors, respectively, is relevant for hypnotic effects but not for anxiety-like effects in animal models. In particular, our results in sleep regulation show that the selective activation of MT_2 receptors, but not the double activation of MT_1/MT_2 receptors, enhances quality of sleep, suggesting that the selective activation of MT_2 receptors results in a more incisive effect on sleep. In fact, in chapter II we hypothesized that the activation of MT_1 receptors might inhibit the effects associated with the activation of MT_2 receptors. Differentially, in anxiety both the single activation of MT_2 and simultaneous activation of MT_1/MT_2 receptors induce anxiolytic-like effects, suggesting that in anxiety, the activation of the MT_1 receptor does not interfere with the anxiolytic-like actions associated with the MT_2 receptor. Nevertheless, experiments using selective MT_1 ligands are needed to better understand the influences of interaction between MT_1 and MT_2 receptors in sleep and anxiety.

Overall, the MT_2 receptor may be considered as a new target for the treatment of anxiety disorders, while UCM765 should be considered a good candidate to treat the problems with anxiety and other associated medical conditions such as insomnia.

Chapter VI

General conclusion

In chapter II, we investigated the effects of the MT_2-selective partial agonist UCM765 on the 24-hr sleep-wake cycle of rats. The first key finding was that the selective activation of MT_2 receptors by UCM765 reduced the latency to sleep and increased the amount of NREMS during the light phase. In particular, the dose-response effects of UCM765 indicated significant changes with high doses, 40 and 60 mg/kg, and no effects with a lower dose (20 mg/kg). The effects of UCM765 were more evident at the end of the light phase with no changes during the dark phase. The sleep promoting effects of UCM765 are similar to those of the $GABA_A$ agonist DZ, an effective hypnotic, except for the reduction in number of NREMS episodes observed with DZ. With respect to REMS, both UCM765 and DZ increased the latency of sleep with no changes in the duration and number of episodes. In concordance, the hypnotic effects of UCM765 during the light phase might be associated with the positive expression of MLT receptors during the light phase, when levels of MLT are low and there is no desensitization of MLT receptors. On the other hand, during the dark phase MLT levels rise and such an elevation desensitizes MLT receptors (Witt-Enderby et al., 2003), which might explain our null effects observed during the dark phase.

Then, in order to determine whether the MT_2 receptor mediates the sleep promoting effects of UCM765, we used a pharmacological approach employing the selective MT_2 antagonist 4P-PDOT to confirm the role of the MT_2 receptor in sleep regulation. Thus, we found that the pre-treatment with 4P-PDOT inhibited the sleep promoting effects of UCM765, thereby suggesting that UCM765 induces and maintains sleep via activation of the MT_2 receptor during the light phase in freely-moving rats.

Considering that UCM765 not only displays affinity for the MT_2 receptors, but also a low affinity for MT_1 receptors, in chapter II we carried out 3 h sleep recordings in MT_1KO and MT_2KO mice and we tested UCM765 in order to confirm that the sleep promoting effects of UCM765 are mediated by the MT_2 and not by the MT_1 receptor. We found that UCM765 increases the amount of NREMS in MT_1KO but not in MT_2KO mice, confirming the role of MT_2 receptors in NREMS regulation and its independent role in the actions of UCM765.

Later, we questioned whether the selective activation of MT_2 receptors, but not the dual activation of MT_1/MT_2 receptors, might be responsible for such hypnotic effects, induction and maintenance of NREMS. To address this question, in chapter IV, we examined the effects of the

MT_2-selective partial agonist UCM924 (UCM765' analog) on sleep in comparison to those of MLT and UCM793, a non-selective MT_1/MT_2 agonist. Similar to our previous results with UCM765, the selective activation of MT_2 receptors by UCM924 reduced the latency to sleep and increased the amount of NREMS. However, UCM924 also increased the number of REMS episodes with no effects on REMS latency, suggesting that a different affinity toward the MLT receptors may account for such a discrepancy. In particular, one of the differences between UCM924 and UCM765 is at the level of the MT_1 receptor; UCM924 acts as an antagonist rather than an agonist like UCM765 (Rivara et al., 2007). In concordance, the genetic inactivation of MT_1 receptors in mice produced an impairment in REMS. Regarding MLT, we found that the double activation of MT_1/MT_2 receptors by MLT reduces the latency to sleep but it does not increase the duration of NREMS. In agreement other reports of MLT have shown little or no effects in sleep maintenance (Tobler et al., 1994; Mailliet et al., 2001). Similarly, UCM793 showed only a tendency to decrease the latency to sleep with no changes in the amount of NREMS and REMS. Correspondingly, other reports with non-selective MT_1/MT_2 agonists, such as ramelteon, only showed effects on the latency to sleep (Fisher et al., 2008). Interestingly, UCM793 modified the number and length of episodes of wakefulness without effects in wakefulness duration (Ochoa-Sanchez et al., 2014). Overall, these findings confirm the hypothesis that the modulation of one single receptor is more effective than the modulation of both of them. In other words, since two receptors for the same neurotransmitter may have divergent functions, selectivity for only one of the receptors can provide a therapeutic advantage reflected not only in sleep induction but also in sleep maintenance (Ochoa-Sanchez et al., 2011; 2014).

As mentioned above, UCM793 and MLT exert different effects on vigilance states, in particular on wakefulness, suggesting that the mechanism of action of MLT might involve not only the activation of MT_1/MT_2 receptors, as does UCM793, but also a membrane receptor-independent mechanism. Nevertheless, further experiments need to investigate the role of these receptor-independent mechanisms on sleep.

In chapter II, we also performed 24 h EEG recordings in MT_2KO mice to better characterize the role of MT_2 receptors in the 24 h light/dark cycle. The sleep profile of MT_2KO mice were compared to those of WT and MT_1KO mice. Notably, the genetic deletion of MT_2

receptors in MT_2KO mice impairs the duration of NREMS during the light phase in comparison to WT mice, and no changes in REMS duration and in the sleep-wake cycle were observed. MT_1KO mice increased the duration of NREMS with a concomitant reduction in wakefulness total time during the dark phase and a tendency to decrease the amount of REMS during the light phase. The overall analysis between light and dark phases in MT_1KO mice suggests an impairment in the 24 h sleep-wake cycle. Likely, the disruption of the 24 h sleep-wake cycle in MT_1KO mice might explain the enhancement of NREMS during the dark phase. Moreover, MT_1KO mice spent an equal amount of time in REMS during both light and dark phases, suggesting that the MT_1 receptor is involved in the sleep-wake cycle regulation. In keeping with this view, the presence of MT_1 receptors in MT_2KO mice left the 24 h sleep-wake cycle intact. Hence, similar to our findings in rats, the results in KO mice confirmed the role of MT_2 receptor in the regulation of NREMS during the light phase, while the MT_1 receptor is mostly involved in the circadian rhythm of sleep stages.

Then, in order to prove that the reduction of NREMS in MT_2KO mice is actually due to the lack of MT_2 receptors, rather than to an impairment in other sleep related neurotransmitter systems such as GABA, MT_2KO mice were treated with the $GABA_A$ agonist DZ. In this case, DZ reduced the latency and increased the duration of NREMS in MT_2KO mice indicating that the impairment of NREMS in this strain is indeed due to the lack of MT_2 receptors.

Presumably, the regulation of NREMS not only involves the activation of MT_2 receptors but also an interaction with MT_1 receptors. To address this question, in chapter III, we aimed to better characterize the sleep microarchitecture of MT_1KO and MT_2KO mice, including the first experiments in MT_1/MT_2KO mice. At first, we hypothesized that the double genetic inactivation of MT_1/MT_2 receptors in MT_1/MT_2KO mice might impair the duration of the three vigilance states. However, in contrast with our hypothesis, our 24 h sleep analysis demonstrated that MT_1/MT_2KO mice only showed significant differences in wakefulness duration, with no significant changes in NREMS and REMS durations. In particular, MT_1/MT_2KO mice spent more time awake, an effect detected only in the whole 24 h analysis but not in the 12 h light/dark or 1 h interval analyses. When we looked at the sleep stages in MT_1/MT_2KO, we saw a minimal and not a significant reduction in NREMS and REMS durations. The changes in MT_1/MT_2KO mice were also associated with changes in the EEG power spectrum in all vigilance states. In

concordance, in rats, the dual activation of MT_1/MT_2 receptors by UCM793 modifies the number and length of wakefulness episodes without changes in NREMS and REMS parameters. Interestingly, these results might explain animal as well as human studies that were not able to demonstrate robust effects of MLT and non-selective MT_1/MT_2 analogs on sleep maintenance.

Then, in agreement with our first results in KO mice, in chapter III we confirmed that the single genetic inactivation of MT_2 receptors in MT_2KO mice reduces the duration of NREMS with a concomitant increase in wakefulness time during the light phase. We also demonstrated that the single inactivation of the MT_1 receptor in MT_1KO mice impaired the sleep-wake cycle. However, the new analysis in MT_1KO mice showed a decrement in REMS time during the light phase, while the duration of NREMS during the dark phase was not enhanced, as reported in chapter II. Nevertheless, in chapter III, the 1 h interval analysis showed that NREMS was increased in the middle of the night in MT_1KO mice. Therefore, MT_1 and MT_2 receptors might play opposite roles in the regulation of NREMS, either through inhibition or promotion, respectively. This is another factor that might explain the lack of effects on sleep maintenance of non-selective MT_1/MT_2 receptors and MLT itself.

The discrepancies in KO mice, in particular MT_1KO, between chapter II and III are likely produced by different EEG analysis used to score the three vigilance states, 10 s and 4 s epochs, respectively. Therefore, the second analysis (4 s) was able to characterize the sleep microarchitecture in KO mice, scoring for instance several micro-episodes undetectable with the 10 s epoch analysis. In fact, the comparison between the two EEG analyses revealed several episodes of micro-wakefulness within the NREMS in MT_1KO mice suggesting a sleep fragmentation in this strain. This sleep fragmentation in MT_1KO mice was accompanied by a reduction in NREMS delta, theta and sigma EEG power spectra that likely impairs NREMS quality.

Overall, our results in KO mice suggest that MT_1 and MT_2 receptors differentially, and sometimes in complementary ways, modulate the vigilance states: MT_2 receptors mainly influence NREMS, whereas MT_1 receptors mainly influence REMS. Interestingly, it appears that genetic inactivation of both MT_1/MT_2 receptors in MT_1/MT_2KO mice likely contributes to increased wakefulness time rather than NREMS and REMS parameters. However, these changes in wakefulness may be produced by minimal changes in NREMS and REMS due to the lack of

MT_2 or MT_1 receptors, respectively, contributing to a significant increment in wakefulness duration in the 24 h analysis in MT_1/MT_2KO mice. Nonetheless, our results in KO mice support a key role of MT_2 receptors in the regulation of NREMS.

Notably, we are one of the first groups to demonstrate that the MT_2 receptor is mainly implicated in the regulation of NREMS. Previously Fisher and Sugden (2009) also demonstrated that the activation of MT_2 receptors by the selective MT_2 full agonist IIK7 induces and promotes NREMS during the light phase in rats. However, *in vitro* and *in vivo* studies have implicated the MT_2 receptor in the regulation of circadian rhythms and phase-shift, rather than sleep regulation. In particular, Dubocovich et al. (1998) demonstrated that the MT_2 receptor is responsible for the phase advances of circadian rhythms of MLT, and Hunt et al. (2001) showed that the MT_2 receptor mediates the phase advances of the circadian clock of MLT on the SCN activity. In contrast, Liu et al. (1997) showed that MLT decreases the SCN firing rate in WT and MT_2KO but not in MT_1KO mice. Then, Jin et al. (2003) reported that the circadian rhythm in MT_2KO mice is unaltered. Besides, *in vivo* studies have shown that MLT induces phase shifts in the circadian clock in WT and MT_2KO (Hudson et al., 2005) but not in MT_1KO mice (Dubocovich et al., 2005). Overall, these findings suggest that the MT_1 but not MT_2, is the receptor subtype related to the circadian regulation associated with MLT.

In chapter II, we also explored the localization of MT_2 receptors in the rat brain by carrying out immunohistochemical experiments using MT_2-selective antibodies. We found that MT_2 receptors are located in different brain regions including the Rt nucleus, a brain region widely involved in sleep regulation, in particular in the generation of sleep spindles in the EEG during NREMS (Steriade and Timofeev, 2003; Steriade 2005). Given the presence of MT_2 receptors in this nucleus, we hypothesized that UCM765 may exert its sleep behavioral effects by acting on Rt GABAergic neurons. Therefore, we carried out single unit extracellular recordings of Rt neurons in anesthetized rats and we examined the effects of UCM765 on the firing rate and burst activity of these neurons. Interestingly, the systemic administration of UCM765 produced an enhancement in firing rate and burst activity of these neurons. Moreover, the effects of UCM765 were blocked by the pre-infusion of 4P-PDOT into the Rt nucleus, suggesting that MT_2 receptors expressed in the Rt neurons are involved in the mechanism of action of UCM765 and in all likelihood, in the sleep promoting effects of this ligand.

The question remains as to whether or not the activation of MT_2 receptors expressed in Rt neurons by UCM765 are actually responsible for its sleep promoting effects. To address this question and discard the involvement of other regions that also express MT_2 receptors, we bilaterally microinfused UCM765 into either the Rt nucleus or into the substantia nigra pars reticulata in freely moving rats. The analysis of sleep EEG showed that the local administration of UCM765 into the Rt nucleus reduces the latency and increases the total time of NREMS, while the administration into the substantia nigra pars reticulata showed no effects on sleep behavior. Interestingly, our 24 h sleep analysis also demonstrated that the systemic administration of UCM765 increases the number of sleep spindles, one of the EEG characteristics associated not only with NREMS (stage 2) but also with the electrical activity of the Rt nucleus (Steriade 2005). Consequently, the sleep promoting effects of UCM765 may involve a connection between the firing rate of Rt neurons and generation of sleep spindles in the EEG during NREMS. Together, these results suggest that MT_2 receptors located in Rt GABAergic neurons should be considered as key components in sleep regulation and most likely represent the neurophysiological substrate by which UCM765 promotes NREMS enhancement.

In addition to the sleep architecture analysis, we analyzed the EEG power spectrum, a complementary tool to study sleep quality and evaluate the effects of putative hypnotics on sleep. We examined the effects of UCM765 and DZ on sleep EEG power spectrum in rats. UCM765 produced a slight increase in NREMS delta power spectrum during the light phase, whereas DZ significantly reduced it. Previous studies with DZ correlated a reduction of sleep EEG delta power with a reduction in sleep quality (van Lier et al., 2004). Therefore, this increment in the NREMS delta power and the sleep promoting effects of UCM765 might be associated with an enhancement of sleep quality. Consequently, other physiological functions associated with sleep quality such as memory consolidation, learning or restoring/refilling energy (Diekelmann and Born 2010; Wilhelm et al., 2013) might be improved by UCM765. Since the firing and bursting activity of Rt neurons have also been involved in the generation of delta waves in the EEG during sleep (Steriade et al., 1993), the activation of MT_2 receptors and the consequent activation of Rt neurons by UCM765 might explain this increment in NREMS EEG delta power spectrum. However, the effects of UCM765 on NREMS EEG delta power were also observed during the dark phase, a period of time when UCM765 is not active and the expression of MT_2 receptors is

down-regulated. Nevertheless, such a down-regulation of MLT receptors likely occurs after prolonged exposure (≥ 5 hours) to high levels of MLT (Witt-Enderby et al., 2003).

Additionally, the basal EEG power spectrum was also examined in MT_2KO mice and compared to those of MT_1KO, MT_1/MT_2KO and WT mice. In particular, the EEG total power of NREMS was decreased in MT_1KO and MT_2KO mice during the light and dark phases, while in MT_1/MT_2KO mice the reduction was detected only in the dark phase. MT_2KO mice also showed a decreased wakefulness power during the light and dark phases and it was increased in MT_1/MT_2KO mice during the light phase. The REMS total power was reduced during the dark phase in MT_1KO mice.

The analysis by frequency bands revealed that the EEG delta power spectrum of NREMS was decreased during the dark phase in MT_1KO and MT_2KO mice but not in MT_1/MT_2KO mice. Moreover, in MT_1KO mice the EEG delta power was also decreased in the light phase. In agreement, in humans, the administration of MLT and the subsequent activation of MT_1/MT_2 receptors do not affect the EEG delta power spectrum of NREMS (Dijk et al., 1995), whereas this frequency band was enhanced after the selective activation of MT_2 receptors by UCM765 (Ochoa-Sanchez et al., 2011). These findings suggest that the selective genetic inhibition or pharmacological activation of MT_1 or MT_2 but not simultaneous MT_1/MT_2, modifies the delta power spectrum during NREMS. Nonetheless, the effects of selective MT_1 agonists in the EEG power spectra activities have to be tested.

Furthermore, the sigma frequency band of NREMS was decreased not only in MT_1KO and MT_2KO mice, but also in MT_1/MT_2KO mice during the light phase. Accordingly, the administration of MLT in humans enhances the sigma power spectrum of NREMS, a frequency band related to sleep spindles (Dijk et al., 1995). In rats, we showed that the selective activation of MT_2 receptors by UCM765 increases sigma power spectrum and the number of sleep spindles during NREMS (Ochoa-Sanchez et al., 2011). Together, these findings suggest that both MT_1/MT_2 receptors synergistically contribute to the spindle promotion effect of MLT and likely, the genetic inhibition of MLT receptors in KO mice may affect the occurrence of sleep spindles during NREMS.

Further studies are needed to clarify the role of the MT_2 receptor and the actions of UCM765 on EEG power spectra. Nevertheless, the activation or inhibition of MT_2 receptors, together with direct or indirect changes in EEG power spectra may be related to the sleep promoting effects of UCM765 and, to the impairments in NREMS and wakefulness in MT_2KO mice.

As mentioned in the introduction, the Rt nucleus plays an important role in the regulation of sleep and in particular, it is considered a spindle pacemaker. In addition, the network in the Rt nucleus is involved in the low frequency (0.5-4 Hz) thalamic and cortical rhythm in NREMS (Steriade et al., 1993). During NREMS, TC relay neurons show a burst-train pattern that activates and interacts with cortical thalamic neurons to generate the slow delta waves. In addition, TC burst activity is involved in the transition from wakefulness to sleep. Furthermore, TC neurons also activate Rt GABAergic neurons which generate sleep spindles in the EEG (Franks 2008). Rt neurons show a burst-train pattern of activity characterized by rhythmic, low and high frequency intervals (accelerando-ritardando) during NREMS. During the awake state these neurons fire in the single-spike mode (Marks and Roffwarg 1993). As mentioned above, the bursting activity in Rt neurons has been associated with the induction of spindle frequency (7-14 Hz) thalamic discharges (Steriade et al., 1993). In this case, bursting Rt neurons send back an inhibitory postsynaptic response to the same TC neurons whose inhibition is mediated by the $GABA_A$ receptor. After recovering from hyperpolarization, TC neurons start bursting and reactivate Rt neurons, thus closing the loop TC-Rt responsible for the generation of sleep spindles in the EEG (Franks 2008). Interestingly, spindles are considered the landmark of sleep onset and aid the facilitation of NREMS (Steriade et al., 1993). In agreement with these findings, the bilateral lesion of the Rt nucleus in freely-moving rats increases the latency to sleep onset, diminishes the total time of NREMS, and gradually eliminates delta rhythm (Marini et al., 2000). These findings along with our results in sleep behaviour and the electrical activity in the Rt nucleus may explain the potential mechanism by which the Rt nucleus plays an important role to induce and enhance sleep (see Figure 3). Nevertheless, besides TC and cortico thalamic neurons, afferent brainstem modulatory systems and other regions might be involved in the generation of spindles, and sleep enhancement associated with the activation of MT_2 receptors.

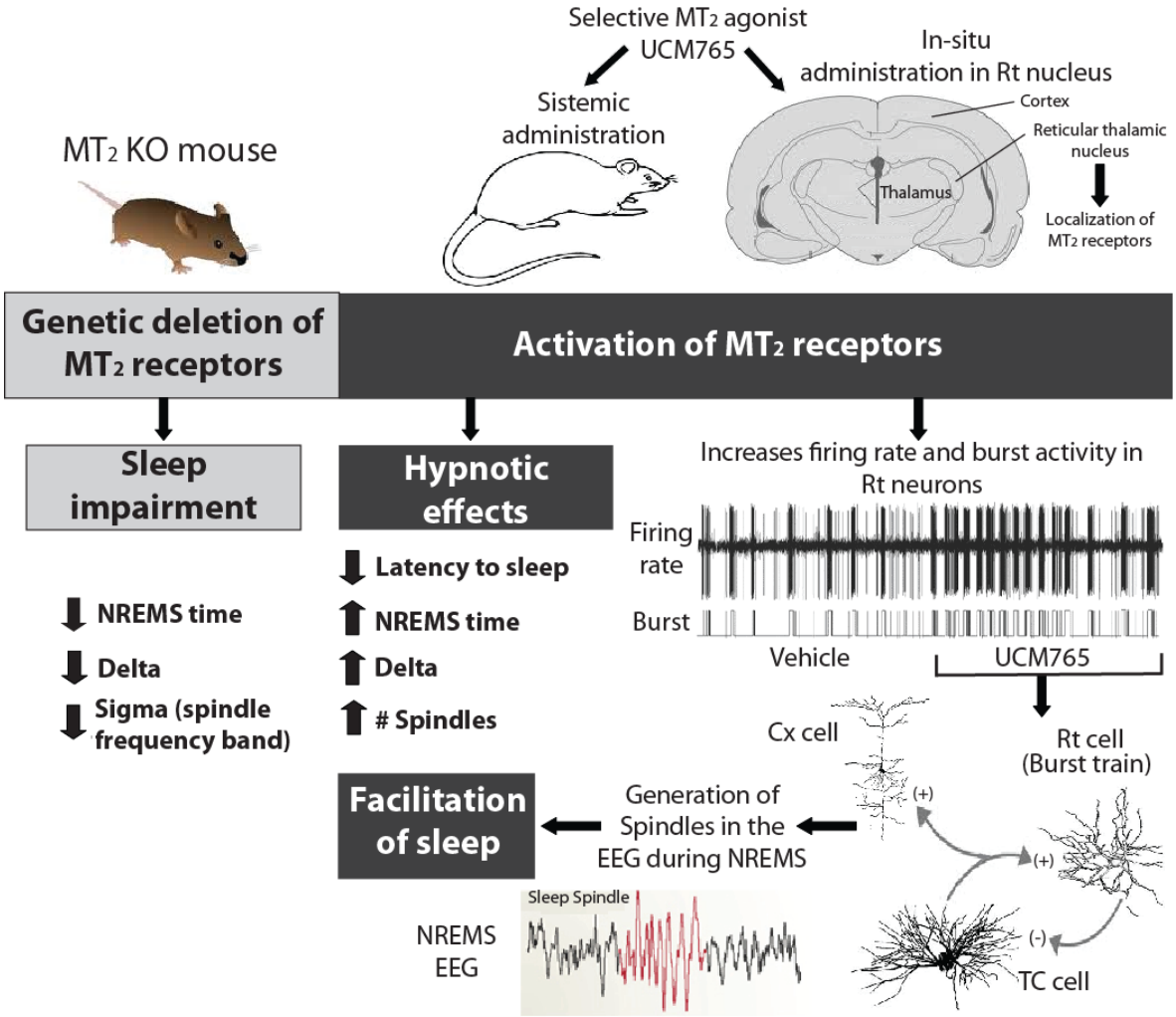

Figure 3. Summary diagram of the effects in sleep behaviour and electrophysiology after the genetic deletion of MT_2 receptors in mice and after the selective activation of MT_2 receptors in rats. The duration of NREMS and power spectra of sigma and delta frequency bands are impaired in MT_2KO mice. Interestingly, delta frequency band is involved in deep sleep (delta waves 1-4 Hz), while sigma represents the frequency for sleep spindles (7-14 Hz), one of the EEG features associated with the transition from wakefulness to sleep. In contrast, the activation of MT_2 receptors increases NREMS time and the number of sleep spindles (in red). These effects are associated with the activation of MT_2 receptors localized in the Rt nucleus. Such MT_2 activation increases the firing rate and burst activity of Rt neurons, a pattern involved in the generation of sleep spindles. In particular, Rt bursting neurons inhibit thalamocortical neurons (TC) which eventually recover from hyperpolarisation and respond back with a barrage of spikes, establishing an oscillation. The post-inhibitory rebound burst also activates cortical cells (Cx), generating the sleep spindles. Likely, the activation of Rt neurons, sleep spindles generation and the interaction with TC neurons might explain the enhancement of NREMS in freely moving rats (Steriade et al., 1993; Bryant et al 2004).

Interestingly, the enhancement of sleep spindles in the EEG during sleep along with the activation of Rt neurons might suggest that the activation of MT_2 receptors by UCM765 or UCM924 might particularly enhance the stage 2 (light sleep) which is enriched with spindle activities (Bryant et al., 2004). For instance, in humans, the administration of the non selective MT_1/MT_2 agonist agomelatine increases the stages 3 and 4 of sleep (Quera-Salva et al., 2010). Nonetheless, further analyses for each of the NREMS stages (1-4) are needed to reveal which sleep stage or stages are augmented by the selective activation of MT_2 receptors.

Aditionally, we demonstrate that the effects of UCM765 on NREMS depend on the phase of day, with sleep enhancement only in the light/resting phase in rodents. However, the question remains as to whether or not the activation of MT_2 receptors by UCM765 or UCM924 will have similar and positive results during the resting (night) time in humans. It is difficult to infer the results in rats to the human specie, in particular because in contrast to rats and mice, the sleep/resting time for humans is at night while the active phase occurs during the day. One of the differences with humans is that in rats the activite or rest period is not well consolidated because of the ultradian rhythm in sleep. In particular, the consolidated active phase in humans redults from the interaction between the homeostatic sleep pressure and the circadian signal. This interaction promotes sleep during the night and waking during the day. In rodens, the circadian modulation is smaller with little effects on the duration of wakefulness or sleep (Yasenkov and Deboer 2012).

The administration of MLT during the light phase, 2-3 h before bedtime, promotes sleep during the dark or resting phase in humans (Zhdanova et al.,1997). Similarly, the advance phase shift of exogenous MLT is observed when it is administered in the afternoon, probably because exogenous and endogenous MLT overlap at this time (Burgess et al., 2010). Also, in humans, the treatment with non-selective MT_1/MT_2 agonists, such as ramelteon or agomelatine, before bedtime reduces the latency to sleep and promotes sleep during the night/resting phase (Roth et al., 2005b; Quera-Salva et al., 2010). These results demostrate that the administration of MLT or non-selective MT_1/MT_2 agonists at the end of the day/active phase in humans induces and promotes sleep during the night/resting time. Therefore, one would expect that the administration of UCM765 in humans, 2-3 h before bedtime, would induce and maintain sleep during the night/resting but not during the day/active phase. Considering that the effects of UCM765 were

not observed during the active phase in rats, one can hipothetized that in humans, the effects of UCM765 will be observed only at night. In particular, as our results showed, the MT_2 receptors are mostrly expressed during the rest time and not in the active phase. Nevertheles, further studies are required to investigate the sleep promoting effects of UCM765 or UCM924 during the resting and active phase in humans in order to gain a better understanding of the effects of these selective MT_2 agonists and understand the underlying regulatory mechanisms in different species.

Another limitation that we faced in this study was the reading of the EEG recordings because although the program Spike 2 (CED) automatically separates the different vigilance states (wakefulness, NREMS and REMS) according to the EEG signal and power spectra, the outcome presents an important number of errors or episodes non-identified that have to be corrected manually. Since the precision of manual state identification is always questionable because it might bias the results, each record was analysed by 2 experienced readers using the same criteria and comparing the results at the end. In addition, the recordings that did not show an identified stage in the EEG more than 15 % of the time were discarded from analysis. Different research groups are developing and improving software to better analyse and discriminate the vigilance states as well as the detection of spindles and other features of interest in order to decrease analysis time and human error which is always questionable.

As discussed in the introduction, sleep is a complex phenomenon regulated by many molecules and specific receptor subtypes. Based on this fact, many molecular targets that play a role in sleep have been proposed to treat insomnia and sleep disorders (Wafford and Ebert 2008). One of the most important drugs to treat insomnia are BZs that act on the GABAergic system. For instance, $GABA_A$ agonists have showed efficacious effects on induction and maintenance of sleep but their use is correlated with important side effects (Lancel, 1999). Notably, recent research in the mechanism underlying sleep regulation is influencing the development of new pharmacological treatments for insomnia and other sleep disorders. In fact, many alternative options have been used to treat sleep disorders and others are still in the process of being approved for use in humans (Wafford and Ebert 2008).

For instance, drugs acting on the histaminergic system, which play a major role in wakefulness maintenance, have been used to treat sleep disorders such as narcolepsy (Lin et al.,

2011). The histamine H_3 autoreceptor has been shown to be involved in the reduction of the synthesis and release of histamine along with a reduction in the firing rate of histamine neurons (Lin et al., 2011). For instance, inverse agonists for the H_3 autoreceptor including the wake-enhancing molecule pitolisant are being developed for the treatment of cataplexy, narcolepsy and other sleep disorders (Dauvilliers et al., 2013; Leu-Semenescu et al., 2014). In addition, modafinil and armodafinil (Teva Pharmaceuticals), which presumably act on dopamine transporters, have been used to treat excessive sleepiness caused by sleep apnea, narcolepsy or shift work sleep disorder (Turner et al., 2013; Howard et al., 2014). Another alternative used in patients with excessive daytime sleepiness and cataplexy is the $GABA_B$ and possibly γ-hydroxybutyrate receptor agonist sodium oxybate (Xyrem; Jazz Pharmaceuticals) (Huang and Guilleminault 2009; Alshaikh et al., 2011).

Regarding the orexin system, which is associated with sleep stabilization and functions mainly in the promotion of wakefulness via the activation of brain areas involved in wakefulness and arousal, different orexin OX_1/OX_2 antagonists including suvorexant and MK-6096 (Merck & Co.) are under development to treat insomnia and other sleep disorders (Winrow et al., 2012; Bennett et al., 2014). In particular, suvorexant was better at helping patients fall asleep and stay asleep in comparison to a placebo; this candidate is currently waiting for marketing approval from the FDA to be used in cases of insomnia (Bennett et al., 2014).

Additionally, clinical and pre-clinical trials are focused on the development of new MLT ligands to treat insomnia and sleep disorders. For instance, the non-selective MT_1/MT_2 agonists, tasimelteon (Vanda Pharmaceuticals) and the beta-methyl-6-chloromelatonin improve sleep latency with a modest efficacy on sleep maintenance in patients with insomnia (Zemlan et al., 2005; Rajaratnam et al., 2009). Similar effects are observed with ramelteon and agomelatine, two drugs currently on the marked as a hypnotic and an antidepressant, respectively (Mini et al., 2007; Quera-Salva et al., 2007). Although, these novel compounds display relatively modest efficacy to induce and maintain sleep, in terms of unwanted effects, recently approved drugs that act on novel molecular targets may be better tolerated than BZs, in particular for long-term use (Wafford and Ebert 2008). For example, MLT ligands such as ramelteon and agomelatine show a good safety profile and they might be used for long-term treatment, one of the restrictions of BZs (Quera-Salva et al., 2007; Rajaratnam et al., 2009).

In this thesis, we have included data demonstrating that the selective activation of MT_2 receptor by UCM765 or UCM924 may represent a better therapeutic advantage than non-selective MT_1/MT_2 agonists to treat insomnia, which is characterized not only by having problems falling sleep but also by problems staying asleep. Moreover, UCM765 and UCM924 might enhance sleep likely with fewer side effects than conventional pharmacological options on the market such as GABAergic hypnotics (see *p*.174-175).

As discussed in the introduction, the melatonergic system is also associated with the regulation of anxiety. In fact, the deficiency in MLT has been correlated with anxiety and sleep disorders, whereas the administration of MLT (Zhdanova 2005; Buscemi et al., 2006; Caumo et al., 2009) and other non-selective MT_1/MT_2 analogs (den Boer et al., 2006; Stein et al., 2008; Gross et al., 2009) induce anxiolytic and hypnotic effects in animals as well as in humans. However, the single role of MT_2 receptors in anxiety had not been previously studied. Consequently, in chapter V, we turned our attention to the role of the MT_2 receptor on the regulation of anxiety. We assessed the putative anxiolytic-like effects of the novel MT_2-selective partial agonist UCM765 in animal models of anxiety. Firstly, the dose-response of UCM765 in the EPMT and NSFT in rats showed that 10 mg/kg but not higher doses increased the time spent in the open arms of EPMT and reduced the latency to eat in the NSFT. Secondly, the effects of UCM765 were compared to those of MLT (20 mg/kg) and the anxiolytic drug DZ (1 mg/kg) in the EPMT, NSFT and OFT. In the EPMT, UCM765 and MLT reduced the time spent in the open arms compared to vehicle treated rats, but to a lesser extent compared to DZ. Although the dose of UCM765 was lower than MLT, its anxiolytic-like effects were greater than MLT, probably due to its higher affinity for the MT_2 receptor. In the NSFT, all treatments similarly decreased the latency to eat in a novel environment with no changes in a familiar environment. In the OFT, any treatment showed anxiolytic-like effects, so the time spent in the central area was not modified, but interestingly, we proved that only DZ decreased the number of entries into the center and the total distance travelled revealing the sedative effects of this $GABA_A$ agonist. Together, these results suggest that UCM765 and MLT, promote anxiolytic-like effects with no sedative effects, one of the most common side effects of BZs and derivatives (Whiting, 2006).

Later on, in order to gain new insights into the neurobiological mechanism through which the melatonergic system regulates anxiety, we used a pharmacological approach employing the

non-selective MT_1/MT_2 receptor antagonist luzindole and the selective MT_2 antagonist 4P-PDOT to determine which MLT receptor subtype mediates the anxiolytic properties of MLT. We found that both antagonists inhibit the anxiolytic-like effects of UCM765 and MLT, suggesting that the MT_2 receptor is indeed involved in the regulation of anxiety and it mediates the anxiolytic-like effects of MLT and UCM765. In addition, we found that 10 mg/kg of 4P-PDOT or luzindole alone did not change anxiety levels. These findings are in line with a previous report showing no effects of luzindole (30-60 mg/kg) in anxiety levels (Nava and Carta 2001). Moreover, the anxiolytic-like actions of MLT are in agreement with previous data demonstrating the anxiolytic-like effects of MLT in humans (Srinivasan et al., 2006; Caumo et al., 2009) and animal models (Golombek et al., 1993, Papp et al., 2006; Crupi et al., 2010). Remarkably, this is the first study showing the anxiolytic-like effects of UCM765 and the first demonstrating that such anxiolytic effects, including those of MLT, are regulated by the MT_2, but not by the MT_1 receptor subtype. Consequently, the MT_2 receptor may represent a novel potential target for the treatment of anxiety and, unlike BZs and antidepressants, UCM765 could induce anxiolytic effects with low sedation and lack of abuse potential.

In agreement with the role of the MT_2 receptor in anxiety, our preliminary results in MT_2KO mice showed that the lack of MT_2 receptors impairs the levels of anxiety in the EPMT, NSFT and OFT (unpublished data; see Comai and Gobbi, 2014). In the EPMT, MT_2KO mice spent more time in the center of the maze but the time spent and entries into the open arm were not modified in comparison to WT mice. However, the time spent in the central platform of the EPMT has been linked to the decision-making process rather than to anxiety itself, normally detected by differences in the time spent in open arms. Consequently, a cognitive impairment at the level of "goal-oriented" behavior in MT_2KO mice has been suggested (Comai and Gobbi 2014). Previously, WT but not MT_2KO mice showed shorter transfer latencies to enter a closed arm of the EPMT on the second day of evaluation suggesting synaptic plasticity and learning impairment (Larson et al., 2006). In addition, cellular mechanisms involved in learning and memory, such as long-term potentiation, were smaller in CA1 hippocampal slices of MT_2KO mice in comparison to slices of WT mice, supporting the decision-making impairment in MT_2KO mice (Larson et al., 2006). In the NSFT, the latency to eat in a novel environment was longer in MT_2KO mice than in WT mice with no changes in a familiar environment. In the OFT, MT_2KO mice spent more time in the center than WT mice with no effects on the total distance

travelled (unpublished data; see Comai and Gobbi, 2014). In spite of the complex results obtained in EPMT, NSFT and OFT, overall, these findings in MT_2KO mice along with the pharmacological effects of UCM765 in anxiety paradigms in rats prove the pivotal role of MT_2 receptors in the regulation of anxiety.

In relation to the role of single MT_2 or dual MT_1/MT_2 activation in sleep and anxiety regulation, our EEG recordings in rats showed that total sleep time was enhanced by selective MT_2 partial agonists but not by the non-selective MT_1/MT_2 agonist UCM793 or by MLT itself, indicating differential effects between single MT_2 or dual MT_1/MT_2 activation. Based on other reports showing opposite effects between MT_1 and MT_2 receptors, we hypothesized that in sleep the activation of MT_1 receptors might inhibits the effects associated with the activation of MT_2 receptors and vice versa. In contrast, in anxiety, the co-activation of MT_1/MT_2 receptors by MLT produces anxiolytic-like effects similar to UCM765, suggesting that the activation of MT_1 receptors did not prevent those effects associated with the activation of MT_2 receptors as reported for hypnotic effects (Ochoa-Sanchez et al., 2011).

In chapter II, we also demonstrated that MT_2 receptors are highly expressed in different brain regions including the ventral tegmental area, hippocampus, Rt nucleus and septum (Ochoa-Sanchez et al., 2011), all regions associated with the neurobiological mechanism that regulates anxiety and motivated behaviors via connectivity with the amygdala, the brain's emotional center (Menard and Treit 1996; Kalisch et al., 2006; Zikopoulos and Barbas 2012; Johansen 2013). For instance, the amygdala innervates the Rt nucleus through the amygdalar pathway, which provides the mechanism for shifting of attention to emotional stimuli, thus modulating anxiety (Zikopoulos and Barbas 2012). Interestingly, our electrophysiological experiments in the Rt nucleus, a region also involvement in the brain's attentional network, showed that UCM765 increases the firing rate and bursting activity, an effect associated with its hypnotic effects (Ochoa-Sanchez et al., 2011). Therefore, the modulation of cell excitability in the Rt nucleus may provide a mechanism by which MLT ligands are capable of inducing anxiolytic-like actions, likely associated with the modulation of the amygdala via the amygdalar pathway.

As noted earlier, the GABAergic system, in particular the activation of $GABA_A$ receptors, has been involved in the regulation of sleep and anxiety. For instance, the stimulation of $GABA_A$ receptors by the GABA receptor agonist muscimol, induces anxiolytic-like effects in

the EPMT (Zarrindast et al., 2001). Interestingly, the anxiolytic actions of MLT are associated with an enhancement of GABAergic activity (Golombek et al., 2003) and different studies have proposed that high doses of MLT (10-20 mg/kg) can directly interact with BZ-$GABA_A$ receptors (Niles 2004). In concordance, pharmacological blockage of $GABA_A$ receptors inhibits the hypnotic effects of MLT (Wang et al., 2003a). Interestingly, one of the brain regions that respond to the activation of BZ-$GABA_A$ receptors is the Rt nucleus, which is constituted of GABAergic cells. In this context, hypnotics and anxiolytics non-BZs, such as zolpidem and eszopiclone, modulate the activity of $GABA_A$ receptors in Rt neurons through binding to BZ sites (Jia et al., 2009). Here, we showed that the activation of MT_2 receptors expressed in Rt neurons by UCM765 are involved in sleep regulation (Ochoa-Sanchez et al., 2011). Therefore, MLT/$GABA_A$ interaction, the localization of MT_2 receptors in Rt neurons and its activation by UCM765 suggest an allosteric interaction between $GABA_A$ and MT_2 receptors (Wan et al., 1999). Moreover, the fact that MT_2 ligands have a distinct profile from BZs, and that BZs are still active in MT_2KO mice (Ochoa-Sanchez et al., 2011), indicates that MT_2 ligands might modulate GABAergic activity in a differential manner compared to BZ-$GABA_A$ receptors, using, for example, a different intracellular pathway (Wang et al., 2003a). These differences between the activation of MT_2 and $GABA_A$ receptors might be related to the fact that DZ, but not UCM765, reduced the locomotion in the OFT. Nevertheless, more research is required to fully elucidate the participation of MT_2 receptors and its interaction with the GABAergic system in anxiety. Other regions expressing MT_2 receptors and interacting with other neurotransmitter systems may be involved in the regulation of anxiety and likely the same pathways will be involved in the regulation of sleep.

Pathological and non-pathological anxieties are characterized by increased arousal or alertness in response to stress, producing sleep impairments. Conventional treatments that reduce such over-reaction to stress improve both anxiety and sleep disorders (Staner 2003). In general, low doses induce anxiolytic/sedative effects while high doses promote hypnotic effects (Short and Chui 1991; Sun et al., 2008). In concordance, UCM765 produces both anxiolytic-like and hypnotic effects; in particular we demonstrated that UCM765 at 10 mg/kg induces anxiolytic-like effects, while higher doses (40 and 60 mg/kg) promote hypnotic effects.

Overall, our results in sleep and anxiety suggest that the melatonergic system constitutes a common neurobiological substrate not only for the etiology of both anxiety and sleep disorders, but in the treatment of these medical conditions as well. Therefore, here we hypothesized that UCM765 may represent a better option than MLT and, possibly better than BZs to treat anxiety and insomnia. For instance, from a pharmacological point-of-view, UCM765 has acceptable pharmacokinetic and pharmacodynamic properties for hypnotic and anxiolytic drug development. In pharmacokinetics, UCM765 shows a good absorption and distribution profile with an optimal hydrophilic–lipophilic balance (Ochoa-Sanchez et al., 2011). However, the short half-life is one of the limitations for melatonergic compounds and although, UCM765 induces hypnotic and anxiolytic-like effects in rats, it shows a very short half-life (8 min) in the presence of rat liver S9 fraction (oxidative enzymes), which might limit its efficiency. Therefore, UCM924 a compound that express significantly longer half-life (40.6 min) than UCM765 might represent a better option to induce/maintain anxiolytic and/or hypnotic effects (Rivara el al., 2009). On the other hand, in comparison to DZ, whose long half-life has been associated with side-effects (Griffiths and Johnson 2005), the half-lives of UCM765 and UCM924 are significantly shorter.

In pharmacodynamics, UCM765 is a selective MT_2 partial agonist that does not display significant affinity for other receptors, ion channels, and transporters, including those known for their involvement in sleep and mood regulation, such as $GABA_A$, 5-HT_{2A}, and histamine receptors (Ochoa-Sanchez et al., 2011). In accordance with the relative intrinsic activity values that the partial agonist confers (Rivara et al., 2007), UCM765 may produce only a submaximal MT_2 receptor activation with lower or no receptor desensitization. Therefore, this submaximal MT_2 receptor activation might be associated with the fact that UCM765, but not MLT, enhances and maintains sleep. Unlike DZ, UCM765 induces anxiolytic-like effects with no sedative effects and promotes hypnotic effects without altering sleep architecture, two of the medical challenges in the drug development field for anxiolytics and hypnotics, respectively. Interestingly, it has been reported that non-selective MT_1/MT_2 ligands such as ramelteon and agomelatine showed lower side effects than BZs. For instance, the likelihood of abuse liability and other side effects such as memory impairment, acute sedation, withdrawal syndrome or lethality in overdose were present with several BZs including DZ but not with ramelteon (Griffiths and Johnson 2005). Considering that UCM765 also activates the melatonergic system, the use of UCM765 may not

be associated with abuse liability and toxic effects. Nevertheless, safety tests for UCM765 including acute, repeat-dose and long-term toxicity tests in animal models are necessary.

In order to fully understand the single role of MLT receptors in sleep and anxiety disorders, the further directions for this project includes the development and application of novel MLT ligands with selective affinities for MT_1 or MT_2 receptors. Recently, a selective MT_1 agonist named UCM871 was synthesized, therefore, we are already evaluating its effects in animal models of anxiety and later on we will test it in sleep experiments. As previously discussed, further experiments assessing the effects of UCM765 in other brain regions that express MT_2 receptors, in particular those involved in sleep and anxiety disorders such as the SCN and the hippocampus, will help us better understand the mechanism of action of selective MT_2 agonists as well as the pivotal role of MT_2 receptors in anxiety and sleep regulation. Besides, further studies are necessary to explain the molecular and cellular connections between the melatonergic system and other neurotransmitter systems involved in anxiety and sleep regulation. Considering the available information in pharmacodynamics and pharmacokinetics regarding UCM765 and UCM924, along with the fact that we proved their anxiolytic and hypnotic effects, it will be very interesting to screen the safety of these compounds in acute, repeat-dose and long-term toxicity tests in animal rodent and non-rodent species in order to decide whether these compounds are good candidates to be tested in humans. Likely, the clinical trials will confirm the role of MT_2 receptors in the regulation of anxiety and sleep disorders and might propose MT_2-selective partial agonists as effective treatment not only for anxiety and sleep disorders but also for other medical conditions with an urgent need for good pharmacological treatments.

In conclusion, the present thesis demonstrates that the MT_2 receptor is involved in the regulation of sleep and anxiety. In particular, we proved that the selective activation of MT_2 receptors by MT_2-selective partial agonists, UCM765 and UCM924, is sufficient to induce and maintain NREMS with no changes in sleep architecture, whereas the genetic inhibition of MT_2 receptors increases the latency to fall sleep and decreases the amount of NREMS in MT_2KO mice. In concordance, the pharmacological blockage of MT_2 receptors nullifies the sleep promoting effects of UCM765. Interestingly, the MT_2 receptor might interact with the MT_1 receptor to maintain the homeostatic mechanism that regulates sleep and wakefulness rather than

independent effects of each receptor subtype. Moreover, UCM765 and MLT induce anxiolytic-like effects with no sedative effects, whereas the pharmacological blockage of MT_2 receptors inhibits such effects, confirming that the MT_2 but not the MT_1 receptor mediates the anxiolytic-like effects of UCM765 and MLT itself. Notably, we demonstrated that the activation of MT_2 receptors expressed in Rt GABAergic neurons by UCM765, increases the firing rate and bursting activity of these cells; such effects were associated with sleep enhancement and likely associated with the anxiolytic-like effects induced by UCM765 and MLT. The submaximal receptor activation and probably the low/null desensitization of MT_2 receptors associated with UCM765 represent a better approach for treating anxiety and insomnia than MLT, which shows anxiolytic-like effects, reduces the latency to sleep but has no significant effects on sleep maintenance. Further research has to fully elucidate the single role of the MT_2 receptor in the regulation of sleep and anxiety as well as the mechanism of action and pharmacological effects of MT_2-selective partial agonists. Nevertheless, the MT_2 receptor should be considered as a new target for the treatment of anxiety and sleep disorders while MT_2-selective partial agonists should be considered good candidates to induce anxiolytic and hypnotic effects in humans, with low sedation likely and lack of abuse potential as observed with conventional treatments.

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