Novel melatonin MT_1 receptor agonists as antidepressants: In vivo electrophysiological and behavioural characterization

Ada R. Posner

Degree of Master of Science

Neurobiological Psychiatry Unit
Department of Psychiatry
Faculty of Medicine
McGill University
Montreal, Quebec, Canada
April, 2015

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science in Psychiatry

Table of Contents

Abstract

Background: Depression is a mental disorder second only to coronary heart disease as a cause of disability in industrialized countries. Most of the current antidepressants work by blocking the reuptake of serotonin (5-HT) and norepinephrine (NE), two monoamines impaired in mood disorders. Unfortunately, they are fully effective in only one third of patients and produce a wide range of adverse effects. Consequently, novel antidepressant targets are under examination. One promising candidate is the melatonergic system. Melatonin, a neurohormone involved in many physiological processes including mood, exerts its effects mainly by acting on two high-affinity G-protein coupled receptors, MT_1 and MT_2. The selective roles of MT_1 and MT_2 receptors in mood regulation are poorly understood, though recent studies have indicated MT_1 to be mainly responsible in the mood-related effects of melatonin. Here, we investigated the putative effects of MT_1 receptors on mood regulation by employing a novel selective MT_1 receptor ligand.

Methods: We investigated the MT_1 receptor selective partial agonist N-(2-{Methyl-[3-(4-phenylbutoxy)phenyl]amino}ethyl)acetamide (UCM871) using in vivo electrophysiological and behavioural paradigms. To determine the mechanism of MT_1 agonism in depression we employed in vivo electrophysiology to study the modulatory effect of acute UCM871 administration on 5-HT and NE neuronal firing and burst activity in the dorsal raphe nucleus (DR) and locus coeruleus (LC), respectively. We studied the effects of UCM871 in the forced swim test (FST) and the open field test (OFT), well-validated behavioural paradigms for depressive and anxiety-related behaviour.

Results and discussion: Remarkably, acute intravenous administration of UCM871 showed a bell-curve effect on firing of 5-HT DR neurons, increasing at lower doses and decreasing at higher doses, increased the number of bursts at its peak dose (7 mg/kg, i.v.), and attenuated the response of $5\text{-}HT_{1A}$ receptors. In NE LC neurons, increasing doses of UCM871 decreased both firing and burst activity in a dose-dependent manner, with, an increase in the response of alpha-2 adrenergic autoreceptors. The dose of UCM871 producing the peak of 5-HT firing (7 mg/kg) also increased the amount of climbing activity in the FST with no effect on immobility. On the other hand, doses of UCM871

that did not induce changes in 5-HT firing produced depressive-like effects in FST (decreased immobility). In the OFT, UCM871 at 7 mg/kg showed an anxiolytic-like effect, increasing the time in the centre and also locomotion.

In conclusion, our data shows that the novel melatonin MT_1 ligand UCM871 shares neurobiological features with other classes of antidepressants: increased 5-HT activity, decreased NE activity, increased climbing in FST and increased time spent in the central area in the OF, however it has a narrow therapeutic window, since with lower or higher doses the effect is reversed or nullified. This effect may be linked to its partial agonism activity. For the first time, these results suggest that the MT_1 receptor is involved in the pathophysiology of depression by modulating serotonergic and noradrenergic activity, and may be considered a target for the development of novel antidepressant drugs. Further research is needed to develop novel melatonin MT_1 ligands with larger therapeutic window allowing a safer profile for human therapeutics.

Résumé

Contexte : La dépression majeure, après les maladies cardiovasculaires, est à la deuxième position pour invalidité. Les antidépresseurs disponibles agissent sur le blocage de la recapture de la sérotonine (5-HT) et la noradrénaline (NE), deux neurotransmetteurs altérées dans la dépression et possèdent une efficacité limitée car seulement un tiers des patients répondent en plus génèrent plusieurs effets secondaires. Par conséquent de nouvelles cibles thérapeutiques sont nécessaires. La mélatonine, une neurohormone impliquée dans nombreux processus physiologiques, exerce ses effets pas le biais de deux récepteurs appartenant à la famille des récepteurs couplés à une protéine G (RCPG) et appelés MT_1 et MT_2. Le rôle respectif de ces récepteurs dans la régulation de l'humeur reste mal connu, mais des études récentes ont montré que le sous-récepteur MT_1 pourrait y être impliqué. Dans ce travail, nous avons étudié les effets antidépressifs des récepteurs MT_1 en utilisant un nouveau ligand MT_1 sélectif.

Méthode : Nous avons analysé les effets d'un nouveau ligand sélectif le *N*-(2-{méthyl-[3-(4-phénylbutoxy)phényl]amino}éthyl)acétamide (UCM871), agoniste partiel des récepteurs MT_1 en utilisant des modèles d'électrophysiologique et de comportement *in vivo*. Afin de déterminer le rôle de l'activité MT_1 agoniste dans la dépression, nous avons réalisé une étude d'électrophysiologie pour analyser l'effet modulateur du UCM871 sur l'activité électrique des neurones de la 5-HT dans le noyau du raphé dorsal (RD) et de la NE dans le locus coeruleus (LC) ainsi que dans les tests de la nage forcée et de l'espace ouvert, des modèles pré-validés pour tester la dépression et l'anxiété, respectivement.

Résultats et discussion: L'UCM871 (i.v), sur l'activité électrique neuronale de la 5-HT dans le RD, produit un effet en courbe en cloche, qui augmente avec les doses faibles et diminue avec les hautes doses. L'UCM871 montre également une augmentation de l'activé électrique à bouffées de neurones à la dose maximale de 7 mg/kg et atténue la réponse des récepteurs $5\text{-}HT_{1A}$. Dans le cas de neurones de la NE du LC, l'augmentation de la dose de l'UCM871 conduit à la diminution de l'activité électrique neuronale et des bouffées de façon dose-dépendante, en induisant une augmentation de la réponse des autorécepteurs alpha-2 adrénergiques. La dose de l'UCM871 lié au pic maximal de l'activité de la 5-HT (7 mg/kg), conduit également à l'augmentation de l'activité de grimpée dans le test de la nage forcée sans toutefois influencer l'immobilité. Autrement,

les doses de UCM871 qui n'induisent pas de changement dans le taux de l'activité neuronale 5-HT, produisent un effet dépressif dans le test de la nage forcée (réduction de l'immobilité). L'UCM871 (7 mg/kg) a montré un effet anxiolytique dans l'espace ouvert, augmentation de la locomotion et du temps passé dans le centre.

En conclusion, nos résultats montrent que le nouveau ligand MT_1 (UCM871) partage les mêmes traits que d'autres classes d'antidépresseurs : augmentation de l'activité sérotonergique, diminution de l'activité noradrénergique, augmentation respective de l'activité de grimpée dans le test de la nage forcée et du temps passé au centre dans le test de l'espace ouvert. Cependant, ce ligand possède une fenêtre thérapeutique très étroite car une dose supérieure ou inférieure à 7 mg/kg conduit à une inversion de ces effets ou leur simple annulation. Ce phénomène peut être lié à son profil d'agoniste partiel MT_1. Pour la première fois ces résultats suggèrent l'implication des récepteurs MT_1 dans la physiopathologie de la dépression en modulant les activités neuronales sérotonergique et noradrénergique. Ce récepteur peut donc constituer une cible de choix pour la recherche et le développement de nouveaux antidépresseurs. Cependant, d'autres études sont nécessaires pour synthétiser de nouveaux ligands MT_1 sélectifs avec une fenêtre thérapeutique plus large augmentant ainsi la sécurité clinique chez les humains.

Acknowledgements

First, I thank my research advisor Dr. Gabriella Gobbi for her excellent guidance, patience, support and general supervision throughout all aspects in planning and conducting the experiments and for her advice in composing this thesis. I also thank Dr. Stefano Comai for his assistance in editing and composing this thesis.

I thank my lab members for their support and friendship: Dr. Stefano Comai, Dr. Rafael Ochoa-Sanchez, Dr. Ali Nasr Esfahani, Dr. Mohammed Ettaoussi, Ms. Tania Sasson, Dr. Martin Van, Dr. Nico Nuñez, and Dr. Michael Ghabrash.

I am especially grateful to Drs Stefano Comai and Rafael Ochoa-Sanchez for training me in the experimental techniques and protocols and for their constant guidance, patience and invaluable support, and to Dr. Ali Nasr Esfahani, Dr. Rafael Ochoa-Sanchez, and Ms. Tania Sasson for their assistance in conducting electrophysiology experiments, and to Dr. Rafael Ochoa-Sanchez, Dr. Ali Nasr Esfahani, Dr. Mohamed Ettaoussi, Dr. Martin Van and Dr. Nicolas Nuñez for help in conducting behavioural experiments and analysing behavioural data. I especially thank Dr. Mohamed Ettaoussi for his French translation. Your expertise and assistance were greatly appreciated.

Finally I would like to thank my family, Posners Paul, Ilona, Serena, Adam, Eleanor, and Joseph, and Rotts Vladimir and Iya, as well as my dear friends Truphena Matunda, Saining Li, and Peter Collins for their love and support always and especially throughout this time.

Preface & Contribution of Authors

My supervisor Dr. Gabriella Gobbi was involved in all aspects of the experiments and thesis composition, including designing experiments, acquiring the compound, performing analyses, communicating with the Animal Research and Ethics departments, interpreting results, providing figures for use in the discussion section, and writing and editing this thesis.

I was responsible for designing and conducting experiments, organizing and coordinating lab members for experiments requiring multiple experimenters, analysing data, performing statistical analyses, constructing figures and writing this thesis.

Dr. Stefano Comai trained and assisted me in behavioural techniques, as well as assisted me with data analysis and thesis editing. Additionally he contributed figures from his publications for use in my introduction section.

Dr. Rafael Ochoa-Sanchez trained and assisted me in performing behavioural and electrophysiological experiments and techniques, and provided important advice and assistance in data analysis and interpretation. He also assisted in analyzing the videos in the behavioural tests.

Dr. Ali Nasr Esfahani provided substantial assistance in performing electrophysiological experiments as well as assisted in the behavioural experiments along with Drs. Martin Van and Nico Nuñez, who also analyzed the videos in the behavioural tests.

Ms. Tania Sasson assisted in performing electrophysiological experiments.

Dr. Mohamed Ettaoussi assisted in behavioural experiments and in French translation of the abstract of this thesis.

Drs. Gilberto Spadoni, Silvia Rivara, and Giorgio Tarzia designed and synthetized the melatonin ligand UCM871.

My research was supported by the Canadian Graduate Scholars' Master's Award from the Canadian Institutes for Health Research, as well as the Graduate Excellence Award from McGill University.

Abbreviations

[125I]Mel	2-iodo-melatonin
˚C	Degrees celcius
4P-PDOT	4-phenyl-2-propionamidotetraline
5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin (5-hydroxytryptamine)
5-HT1A	Serotonin 1A subtype receptor
5-HT2C	Serotonin 2C subtype receptor
8-OH-DPAT	(±)-8-Hydroxy-2-dipropylaminotetralin hydrobromide
AA-NAT	Arylalkylamine N-acetyl transferase
AAA-D	Aromatic amino acids decarboxylase
AD	Alzheimer's disease
AFK	N1-acetyl-5-methoxykynuramine
AFMK	N1-acetyl-N2-formyl-5-methoxykynuramine
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
ATG	Haplotype adenosine-thymine-guanosine
AUC	Area under curve (the integral in a plot of concentration of drug in blood plasma against time)
BZ	Benzodiazepines
CA1	Cornu Ammonis subfield 1
CA2	Cornu Ammonis subfield 2
CA3	Cornu Ammonis subfield 3
CA4	Cornu Ammonis subfield 4
cAMP	3'-5'-Cyclic adenosine monophosphate
CCD	Charged coupled device
cm	Centimetre
Cmax	Maximum (peak) serum concentration that a drug achieves in a specified compartment or test area of the body after the drug has been administrated and

	prior to the administration of a second dose
CNS	Central nervous system
CREB	Cyclic adenosine monophosphate response element-binding protein
CRH	Corticotropin-releasing hormone
CRY1	Cryptochrome protein 1
DA	Dopamine (3,4-dihydroxyphenethylamine)
DES	Desipramine
DM	Dorsomedial hypothalamic nuclus
DMSO	Dimethyl sulfoxide
DR	Dorsal raphe nucleus
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, Edition IV
EEG	Electroencephalogram
EPMT	Elevated plus maze test
FST	Forced Swim Test (Porsolt Test)
g	Grams
GAD	Generalized anxiety disorder
GDNF	Glial cell derived neurotrophic factor
Gi	Inhibitory regulative G-protein
GPCR	G-protein coupled receptor
GPR50	G protein coupled receptor 50
h	Hour (time)
HEK 293	Human embryonic kidney 293 cell line
HIOMT	Hydroxyindole O-methyltransferase
HPA	Hypothalamic-pituitary-adrenal axis
HPG	Hypothalamic-pituitary-gonadal axis
HPLC	High performance (/pressure) liquid chromatography
htt	Mutant huntingtin protein
Hz	Hertz
i.p.	Intraperitoneal administration
i.v.	Intravenous administration
IA	Intrinsic activity

kg	Kilograms
LC	Locus coeruleus
LogP	Partition coefficient; ratio of concentrations of a compound in a mixture of two immiscible phases at equilibrium; a measure of the difference in solubility of the compound in these two phases.
M	Molar
MDD	Major Depressive Disorder
Mel1c	Also called MT3, third melatonin receptor subtype found in non-mammalians
METH	Methamphetamine
mg	Milligrams
MHz	Mega hertz
min	Minutes
mL	Milliliters
MLT	Melatonin (N-acetyl-5-methoxytryptamine)
mRNA	Messenger RNA (ribonucleic acid)
ms	Milliseconds
MT_1	Melatonin receptor 1
MT_1-/-	Melatonin receptor 1 transgenic knockout mice
MT_2	Melatonin receptor 2
MT3	"Melatonin receptor 3", a MLT-sensitive form of quinone reductase 2
MUPP1	Multiple PDZ domain containing protein 1
MΩ	Mega ohms
NaCl	Sodium chloride
NE	Norepinephrine or noradrenaline (4,5-β-trihydroxy phenethylamine)
Neu-P11	Melatonin MT1/MT2 receptor agonist
ng	Nanograms
NIH3T3	Standard fibroblast cell line
NK1	Neurokinin 1
NMDA-R	N-methyl-D-aspartate receptor
NREMS	Non-rapid eye movement sleep
NRI	Norepinephrine reuptake inhibitor

NSC	Neural stem cells
NSFT	Novelty suppressed feeding test
OFT	Open Field Test
PCPA	Parachlorophenylalanine
PD	Parkinson's disease
PER1	Period circadian protein homolog 1 protein
pg	Picograms
PKA	Protein Kinase A
PKC	Protein Kinase C
pKi	Log dissociation constant
PVN	Paraventricular nucleus of the hypothalamus
RBD	REM-associated sleep behaviour disorder
REMS	Rapid eye movement sleep
ROR/RZR	RAR(retinoic acid receptor)-related orphan receptor
RT-qPCR	Real-time quantitative reverse transcription polymerase chain reaction
s	Seconds
s.c.	Subcutaneous administration
SAD	Seasonal affective disorder
SCG	Superior cervical ganglion
SCN	Suprachiasmatic nucleus
SEM	Standard error of the mean
SNK	Student Newman Keuls post hoc comparisons test
SNRI	Serotonin and norepinephrine reuptake inhibitor
SOD1	Transgenic mouse model of ALS
SPT	Sucrose preference test
SSRI	Selective serotonin reuptake inhibitor
SWS	Slow wave sleep
T-5M	Tryptophan 5-monooxygenase
TCA	Tricyclic antidepressants
TD	Tardive dyskinesia
TST	Tail suspension test

UCM765	*N*-{2-[(3-methoxyphenyl)phenylamino]ethyl}acetamide
UCM871	(N-(2-{Methyl-[3-(4-phenylbutoxy)phenyl]amino}ethyl)acetamide), selective MT_1 receptor partial agonist
VEGF	Vascular endothelial growth factor
VEH	Vehicle
VM	Ventromedial hypothalamic nucleus
WHO	World Health Organization
WT	Wild type
α2	Alpha-2 adrenergic receptor
µA	Micro amperes
µg	Microgram
µm	Micrometres

Introduction

Melatonin

The neurohormone melatonin (MLT, *N*-acetyl-5-methoxytryptamine) serves a functional role in both simple and complex organisms including bacteria, unicellular algae, fungi, plants, and animals such as humans. McCord and Allen in 1917 first demonstrated that an extract of the bovine pineal glands caused skin lightening in tadpoles. In 1958, the research group of the dermatologist Aaron Lerner isolated the active compound from the pineal gland extract, and found that it promoted the aggregation of dark melanin granules within melanophore cells of the dermis, and consequently named it melatonin (Aaron et al., 1958). They then elucidated the chemical structure of MLT the following year (Aaron et al., 1959).

In the biosynthetic pathway of MLT (see **Figure I**), the circulating precursor *L*-tryptophan is taken up from cerebral vessels by pinealocytes of the pineal gland, a small endocrine gland in the centre of the brain situated between the two halves of the thalamus and lying outside of the blood-brain barrier. In pinealocytes, aromatic hydroxylation occurs via the enzyme tryptophan 5-monooxygenase (T-5M) to yield 5-hydroxytryptophan, which is decarboxylated by aromatic amino acids decarboxylase (AAA-D) to yield serotonin (5-HT) which is acetylated by arylalkylamine *N*-acetyl transferase (AA-NAT) to *N*-serotonin in the rate-limiting step, and O-methylated by hydroxyindole *O*-methyltransferase (HIOMT) to yield melatonin (Zlotos et al., 2014). These enzymes are encoded and controlled by mRNAs in the pineal gland that are expressed with a day/night rhythm (Bernard et al., 1999). Furthermore, melatonin synthesis in the pineal gland is dependent on the availability of tryptophan, and is markedly reduced after acute depletion of tryptophan, and other nutritional factors such as folate and vitamin B6 (a coenzyme in tryptophan decarboxylation) influence melatonin production (Claustrat et al., 2005).

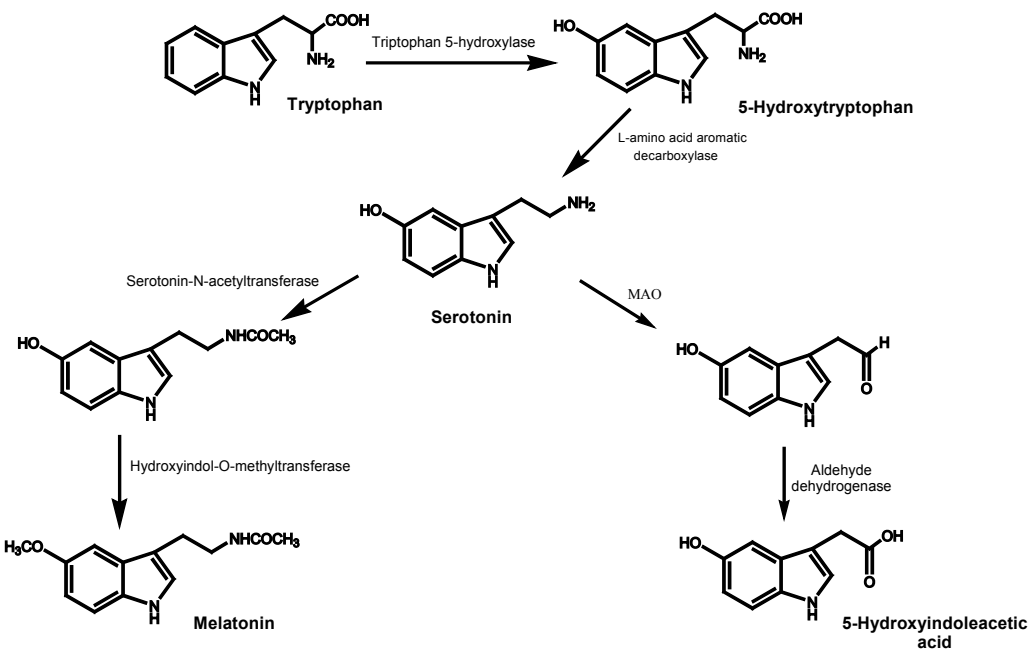


Figure I. Melatonin biosynthetic pathway (permission from Dr. Stefano Comai).

The pineal gland has limited amount of storage of MLT and so, from there, MLT is released into circulation, crossing the blood brain barrier and entering the central nervous system (CNS) as well as peripheral tissues (and, consequently, the fluctuating plasma concentration of MLT accurately reflects pineal gland activity (Reiter, 1991, Longatti et al., 2007). It is secreted in a circadian rhythm, characterized by acrophase (highest plasma concentration of MLT) at night (around 24:00h-3:00h) and reaching nadir (lowest point) during the day in both diurnal and nocturnal species (Karasek, 2007). Though the majority derives from the pineal gland, MLT is also synthesized at secondary sites including the retina, gut, skin, platelets, and bone marrow, where it is produced in amounts that are functionally insignificant (Claustrat et al., 2005).

In mammals, MLT serves both a "clock" function, signalling time of day information, and a "calendar" function, signalling time of year information to its target tissues throughout the body (Benarroch, 2008). MLT has been shown to have involvement in nearly all of the body's physiological processes including circadian rhythms, body temperature, sleep, mood, anxiety, pain perception, immunity, metabolism and cardiac functioning. It has been shown to be anti-inflammatory, antioxidative, neuroprotective and anti-tumour (Hardeland, 2012). While its main functions are related

to endocrine properties whereby the neurohormone is synthesized and secreted, then acts at distant sites, MLT may also work in an autocrine or paracrine manner in certain areas including the retina and gut (Tan et al., 2003).

In the liver, MLT is metabolized by aromatic C6-hydroxylation (to 6-hydroxymelatonin) and then undergoes sulphate conjugation; one minor conversion that occurs is the demethylation of MLT, reverting it back to *N*-acetylserotonin. In other tissues, enzymes can deacetylate MLT to 5-methoxytryptamine. Other nonenzymatic and enzymative oxidations can occur; in the brain, an oxidative ring is cleaved yielding N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) which can undergo further deformylation to N^1-acetyl-5-methoxykynuramine (AFK). In an intermediate step in the production of AFMK, two hydroxyl radicals can be scavenged by MLT to yield cyclic 3-hydroxymelatonin, demonstrating its anti-oxidant capability (Zlotos et al., 2014).

In many countries including Canada, MLT is sold as a dietary supplement, to promote sleep and treat jet lag. However its efficacy as a multi-purpose drug is limited by its pharmacokinetic profile. Though it possesses high lipid and water solubility, which facilitates passage across cell membranes (Pardridge and Mietus, 1980), circulating MLT has high first-pass metabolism and rapid elimination from the body, with a first distribution half-life of 2 min and a second metabolic half-life of 20 minutes (Geoffriau et al., 1999).

In humans, at the onset of MLT secretion (around 21:00-22:00h) circulating levels of MLT begin to rise to a peak level of 80-120 pg/mL (between 24:00 and 3:00h); the offset of MLT secretion is at 07:00-09:00h, when MLT serum levels begin falling to a low of 10-20 pg/mL in the light phase (Karasek, 2007). These fluctuations in circulating levels of MLT throughout the day are correlated with the entrainment of circadian rhythmicity of sleep-wake cycles, as well as numerous other diurnally varying physiological functions, and, accordingly, MLT has been shown to mediate these functions by signalling to the suprachiasmatic nucleus (SCN), the body's master clock. The SCN receives input from the retino-hypothalamic tract to synchronize with the light/dark phase, and its constituent neurons follow circadian rhythms of activity, centrally controlling all circadian activities via its output to the hypothalamic nuclei and the pineal gland. The nighttime synthesis and secretion of MLT in the pineal gland

follows these circadian rhythms governed by the SCN. A multisynaptic pathway (involving the paraventricular nucleus of the hypothalamus (PVN), sympathetic preganglionic neurons of the intermediolateral cell column of the spinal cord, and the noradrenergic sympathetic neurons of the superior cervical ganglion (SCG)) connecting the SCN to the pineal gland mediates SCN regulation of MLT secretion (Perreau-Lenz et al., 2003). Thus MLT plays a dual role, both acting on and being regulated by the SCN.

Recent research indicates that robust circadian rhythms of MLT, with sufficiently high MLT at night, are required in healthy individuals, and suggests that pathological deviations in circadian rhythms of MLT secretion may contribute to the sleep disorders found in aging, Alzheimer's disease and other disorders (Benarroch, 2008). In addition to the problems inherent to the underlying causes, considering the widespread targets of MLT, abnormalities in MLT signalling may further lead to an exacerbation of illness symptoms, especially in sleep, mood and in neurodegenerative conditions. Indeed, therapeutically administered MLT may treat irregular phase shifting and/or address abnormally long sleep onset latency. However, due to reasons including the poor bioavailablility and short half-life of orally administered MLT, MLT has not been found to be able to treat other MLT-related conditions such as mood disorders (Hardeland and Poeggeler, 2012).

Sites and mechanisms of melatonin action: receptors

The physiological effects of MLT in mammals, including humans, are mainly mediated by the activation of two high-affinity G-protein coupled receptors (GPCRs) named MT_1 and MT_2. These two MLT receptor subtypes were cloned in the 90s. MT_1 and MT_2 differ in a number of ways, including downstream receptor signalling pathways, affinities for MLT, and interactions with other proteins.

Both MT_1 and MT_2 trigger downstream effects upon activation by ligands, inhibiting adenylyl cyclase production via Pertussis toxin sensitive inhibitory G_i proteins, and leading to decreases in cAMP, PKA activity and CREB phosphorylation. Expression of cloned recombinant receptors in various cell lines have demonstrated that MT_1 inhibits adenylyl cyclase and forskolin stimulated cAMP formation by coupling to different proteins, including interactions with several G-proteins (i, o, q, z, 12, 13, 14, 16), and by

activating phospholipase C beta. In addition to inhibiting adenylyl cyclase, MT_2 activation also leads to the inhibition of the soluble guanylyl cyclase pathway (Masana and Dubocovich, 2001).

The two receptors can also be differentiated by their affinities to MLT. The pK_is of human MT_1 and MT_2 have been demonstrated to be 10.09 and 9.42 respectively (Hardeland and Poeggeler, 2012). The human MT_2 receptor has a lower affinity for the high-affinity radioligand and non-selective agonist [^{125}I]Mel than does the human MT_1 receptor (von Gall et al., 2002). Furthermore, MT_2, but not MT_1, desensitizes after exposure to the full agonist MLT (this desensitization is probably by an internalization mechanism), but it remains unclear whether this desensitization occurs in the short (minutes) or long (hours) term after in vivo exposure to MLT (Witt-Enderby et al., 2003).

MT_1 is modulated by the protein MUPP1, which does not interact with MT_2, and GPR50, the melatonin receptor homolog that has no affinity for human MLT, but has been identified as an ortholog of the MLT receptor Mel_{1c} found in non-mammalians. MUPP1 is required for high affinity binding of MT_1 to G_i, while GPR50 prevents high-affinity binding of MT_1 agonists and prevents G protein coupling. MT_1 and MT_2 have been found to heterodimerize; MT_1 has also been found to homodimerize under experimental conditions, and so too has MT_2, albeit to a lesser extent (Hardeland, 2012). Very recently, it was revealed that MT_2 also forms functional heteromers with the 5-HT_{2C} receptor, which amplify the 5-HT mediated G_q/PLC, and found that MLT triggered the unidirectional trans-activation of the 5-HT_{2C} protomer of the heteromer (Kamal et al., 2015).

Many further differences between MT_1 and MT_2 are species or tissue specific. In some mammals, experiments utilizing a MT_2-specific antagonist 4-phenyl-2-propionamidotetraline (4P-PDOT) have revealed that MT_2 also signals a stimulation of PKC in a mechanism involving phospholipase C, and unrelated to decreases in cAMP also observed in MT_1 signalling. In rodent experiments, this effect was required for the phase shift of circadian rhythm in the SCN (Lewy et al., 1992, Vansteensel et al., 2008), however this effect may not be occurring in human SCN, which express MT_2 very poorly. It is possible that, in humans, MT_1 may be capable of signalling through the same pathways as MT_2, or else this mechanism may involve MT_1- specific pathways.

Functional overlap between MT_1 and MT_2 and likely yields complementary effects in certain processes, but the separate receptors have been found to activate many distinct pathways controlling physiologically unique responses which mediate the distinct actions of MLT. For example, they have been shown to exert antagonist effects in the vasomotor system, wherein MT_1 activation leads to vasoconstriction whereas MT_2 activation leads to vasodilation (Doolen et al., 1998).

A third low-affinity MLT binding site, called MT_3, was once thought to be more involved in the melatonergic system, but is now characterized as a MLT-sensitive form of the quinone reductase 2 enzyme in humans. Furthermore, apart from the effects mediated by binding to its two specific high-affinity transmembrane receptors, MLT exerts effects via other mechanisms including by binding to intracellular proteins such as calmodulin or tubulin-associated proteins, by direct or indirect antioxidant effects, and by binding to receptors of retinoic acid receptor superfamily including RZR/ROR (Ekmekcioglu, 2006). While much less is known about the roles of these other components in the melatonergic system, these mechanisms may act in numerous general functions of MLT, and contribute to MLT's role as a pleiotropic regulator molecule.

Localization and distribution of melatonin receptors in the brain

The current research into the localization of specific MT_1 and/or MT_2 receptors in the brain have revealed both overlapping as well as distinctly separate expression domains. Techniques used to study localization include the use of quantitative autoradiography with the high affinity radioligand non-selective agonist [^{125}I]Mel (Dubocovich and Takahashi, 1987), real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR), polyclonal antibodies (Wu et al., 2013), in situ hybridization with selective/specific digoxigenin-labelled sense and antisense oligonucleotide probes (Hunt et al., 2001), and transgenic mice containing MT_1–promoter driven fluorescent protein, visualized with fluorescence microscopy (Adamah-Biassi et al., 2014). MT_1 and MT_2 are widely distributed throughout the CNS and peripheral tissues, and are expressed in separate as well as common regions. The highest receptor density in many mammals of MT_1 and MT_2 is found in the SCN, and also in the pars tuberalis of the pituitary, especially in seasonal breeders (Wu et al., 2006, Pandi-Perumal

et al., 2006). In humans, MT_1 is highly expressed in the SCN and particularly localized in vasopressin neurons, an important finding since one of the major outputs of SCN circadian functions is the release of vasopressin. For many years there had been no knowledge of MT_2 expression in the SCN, however recently MT_2 expression has been identified there, as well as in the supraoptic nucleus and paraventricular nucleus, notably in neurons but not in glial cells (Wu et al., 2013).

Other regions of both MT_1 and MT_2 expression in mammals include the prefrontal cortex, occipital cortex (pyramidal and non-pyramidal neurons), cerebellar cortex, vestibular nuclei, pineal gland hippocampal CA3 layer, amygdala, basal ganglia (including putamen, caudate nucleus and substantia nigra), ventral tegmental area, nucleus accumbers, retinal horizontal, amacrine and ganglion cells, and choroid plexus. In the hippocampus, MT_1 is found in the CA1 layer while MT_2 occupies the CA4 layer (Uchida et al., 1996, Mishima et al., 1999).

MT_2 in the cerebral cortex is expressed in neurons as well as in Bergmann glia and other astrocytes. Studies employing polyclonal antibodies (Angeloni et al., 2000) have detected MT_2 receptors in the reticular thalamus, pars reticulata of the substantia nigra, supraoptic nucleus, red nucleus, and CA2 and CA3 hippocampal areas (Ochoa-Sanchez et al., 2011).

MT_1 is expressed in other hypothalamic regions including paraventricular nucleus, periventricular nucleus, supraoptic nucleus, sexually dimorphic nucleus, diagonal band of Broca, nucleus basalis of Meynert, infundibular nucleus, VM and DM nuceli, tuberomammilary nucleus, mammillary bodies and paraventricular thalamic nucleus. In a transgenic mouse model expressing the red fluorescence protein under the control of the endogenous MT_1 promoter, MT_1 was located in the subcommisural organ, part of the ependymal lining lateral and third ventricles, aqueduct, hippocampus, cerebellum, pars tuberalis, habenula and habenula commissure (Adamah-Biassi et al., 2014). MT_1 was found to be colocalized with vasopressin, oxytocin and corticotropin-releasing hormone (CRH) in the hypothalamus, and weakly expressed in anterior and posterior pituitary, in addition to the aforementioned strong expression in the pituitary pars tuberalis (Wu et al., 2006); the colocalization with CRH may suggest a modulatory role of MT_1 in the hypothalamic-pituitary-adrenal axis (HPA), and this has implications

for MT_1 involvement in stress-related illnesses including mood and anxiety disorders. Niles et al. (2004) found that neural stem cells (NSCs) express MT_1, which is colocalized with neurotrophic factors and neuronal and glial markers. MT_1 was found to induce GDNF expression in these cells, supporting a functional role for MT_1 in NSCs, and, since GDNF has the capacity to promote survival of dopaminergic neurons, this finding has implications for neuroprotection in dopaminergic disorders such as Parkinson's. In addition, research suggests a regulatory role of MLT in dopaminergic behaviours such as drug-induced diurnal locomotor sensitization and drug seeking, and identified MT_1 protein and mRNA expression in specific regions of the central dopaminergic system (including nucleus accumbens) as well as in specific cells (tyrosine hydroxylase immunopositive cells), finding that MT_1 expression varied diurnally (Uz et al., 2005).

Important for this thesis, our group recently elucidated the precise anatomical and cellular localization of MLT receptors in the adult rat brain (Lacoste et al., 2015) and found MT_1 and MT_2 on neuronal cell bodies and dendrites throughout the brain, occurring in different and complementary regions. High levels of MT_1 receptors were observed in somatodendrites in the cerebral retrosplenial cortex, basal forebrain islands of Calleja, medial habenula, SCN (moderate- high), superior colliculi, substantia nigra pars compacta (moderate-high), and pars tuberalis of the pituitary gland; moderate levels of MT_1 were observed in medial forebrain bundle, ventral pallidum, anterodorsal thalamic nucleus, inferior colliculi (moderate-high), and dorsal raphe nucleus (DR); weak levels of MT_1 were observed in prefrontal and occipital cortices, CA1, CA2, CA3 (weak-moderate) and dentate gyrus of hippocampus, nucleus accumbens (absent to signal), globus pallidus, lateral habenula, ventromedial thalamic nucleus, medial eminence, ventrolateral PAG, and substantia nigra pars reticulata. For now, it is unclear whether MT_1 is expressed in the locus coeruleus (LC), but it is known that the DR projects to the LC. Additionally, high levels of MT_2 receptors were observed in the CA3 area of hippocampus, reticular thalamic nucleus, supraoptic nucleus, inferior colliculus (moderate-high), substantia nigra pars reticulata; moderate levels were observed in the medial forebrain bundle and ventral pallidum, dentate gyrus, CA2 (weak-moderate), globus pallidus (weak-moderate), ventromedial nucleus, medial eminence, and ventrolateral PAG; weak levels were observed in the retrosplenial cortex, prefrontal

cortex (absent to weak), Islands of Calleja, CA1, paraventricular hypothalamic nucleus, superior colliculi, DR nucleus, and substantia nigra pars compacta (Lacoste et al., 2015).

Indeed, a substantial amount of research has been devoted to the localization of MT_1 and MT_2 receptors, including many that have employed mRNA labelling techniques. It should be noted however that, depending on the system, the correlation between mRNA and protein expression levels may be as low as 40% (Vogel and Marcotte, 2012). Factors involved in this include differences in the regulation of mRNA and protein stability and degradation rates; for example, the half-life of proteins can be up to several days, whereas mRNA falls in the range of 2-7 hours in mammals (Vogel and Marcotte, 2012). Therefore, knowledge into the localization of mRNA expression for the MLT receptors is useful for knowing general regions of receptor expression, but cannot lead to assumptions regarding the receptor expression levels based on corresponding mRNA levels. Furthermore, due, in part, to the lack of selective antibodies and ligands to the receptors, a full understanding of their localization in the CNS remains somewhat elusive. It is clear, however, that the differential localization of MT_1 and MT_2 receptors in the brain contributes to their varied and collaborative physiological functions.

Depression

The Diagnostic and Statistical Manual of Mental Disorders IV-TR describes major depressive disorder (MDD) as a disease involving a complex mix of cognitive, affective, vegetative, somatic and neuroendocrine symptoms (DSM-IV, 1994). Predominantly characterized by low mood, anhedonia, anxiety, fatigue, diminished ability to concentrate, negative thoughts/feelings of worthlessness or guilt, weight changes, and suicidal ideation, depression is also frequently accompanied by changes in circadian rhythms of sleep, core temperature, behaviour (including psychomotor disturbances) and hormone secretion (e.g. cortisol). The global incidence of depression is increasing; affecting 350 million people each year including between 3-6% of Canadians (Thombs and Ziegelstein, 2013), depressive disorders are among the most prevalent mental illnesses, and are second only to coronary heart disease among the leading causes of disability and premature death in industrialized countries (WHO, 2012). Etiologically and clinically, depression is a heterogeneous condition with a huge variability of

symptoms between individuals, and is often inadequately recognized, diagnosed and/or treated. In addition to the associated health care costs, depression and other mood disorders have devastating physical and psychological implications on the quality of life for affected individuals and their families.

The neurobiology of depression and antidepressant mechanisms of action

For centuries, scientists and philosophers have attempted to understand both the causes and, indeed, the very nature of the illness. In the 1950s, the anti-tuberculosis drug Iproniazid was observed to improve patients' moods, leading to its sale as the first antidepressant drug (West and Dally, 1959). The precise etiology and pathophysiology of depression remain poorly understood due to the complex array of symptoms that overlap with other psychiatric conditions and suggest that the symptoms of depression result from the dysfunction of multiple but connected neurobiological systems. Researchers have linked depressive disorders to impairments resulting from neurodegeneration (especially in the hippocampus and prefrontal cortex), neuroendocrine dysfunction including disturbances in the hypothalamic-pituitary-adrenal (HPA) axis, and, most relevant to first-line antidepressant therapeutics, neurochemical imbalance of central monoaminergic neurotransmission (Bambico and Gobbi, 2008, Blier and de Montigny, 1994a).

The most prevalent model of depression hypothesizes that depression arises from an imbalance of monoamines in the brain, specifically the neurotransmitters serotonin (5-hydroxytryptamine, 5-HT) and norepinephrine (NE); alterations to physiological levels of 5-HT and NE disrupts synaptic and neural activity and consequently disturbs, mood (among other systems) (Hirschfeld, 2000). In the midbrain, the dorsal raphe (DR) nucleus and the locus coeruleus (LC) supply 5-HT and NE, respectively, and are therefore key sites in antidepressant action. The DR and LC project to and supply 5-HT and NE to regions of the limbic system and cortex that regulate emotion and mood (Bambico and Gobbi, 2008, Blier and de Montigny, 1994a).

Supporting the hypothesis that underactive monoamine functioning underlies depressive disorders, the 5-HT synthesis inhibitor parachlorophenylalanine (PCPA) has been shown to reverse the antidepressant effect, reduce 5-HT neuronal activity, NE synthesis, and alpha-1 adrenoreceptor activity in animal models; human studies have

shown that depleting the 5-HT precursor L-tryptophan in healthy volunteers with a history of depression results in mild dysphoria (Blier and de Montigny, 1994b, Shopsin et al., 1975).

Different classes of antidepressants target different components of 5-HT and NE neurotransmission. Targets include 5-HT and/or NE transporters, alpha-2-adrenoreceptors, 5-HT_{1A} receptors, NE release mechanisms, and oxidative deaminating enzymes (Blier and de Montigny, 1994b).

The selective serotonin reuptake inhibitor (SSRI) class of antidepressants prevent the reuptake of 5-HT into presynaptic terminals, thereby increasing the amount of 5-HT available in synapses. With acute SSRI treatment, the increased levels of 5-HT act on inhibitory 5-HT_{1A} autoreceptors on DR 5-HT somatodendrites, resulting in decreased electrical activity/neuronal firing of 5-HT neurons. Chronic treatment with SSRIs results in a desensitization of the 5-HT_{1A} autoreceptors and leads to an increase in 5-HT activity and a return to basal 5-HT firing levels; importantly, this effect of chronic treatment has been demonstrated to affect brain regions involved in mood regulation by increasing 5-HT outflow to these critical postsynaptic forebrain areas (Blier and de Montigny, 1994b). Separately, inhibitory alpha-2 receptors are located on NE terminals that synapse with DR 5-HT neurons, and antidepressants such as mirtazapine block these inhibitory receptors, increasing 5-HT firing in the DR, and leading to an increase in NE outflow that results in increased excitation of alpha-1 heteroreceptors on 5-HT neurons. (Haddjeri et al., 1998, Gobbi and Blier, 2005). Other systems involved include neurokinin 1 (NK_1) receptors, excitatory amino acids and sigma ligands and receptors (Gobbi and Blier, 2005, Bambico and Gobbi, 2008). Though they involve different mechanisms, all these antidepressants result in enhanced 5-HT neurotransmission.

The 5-HT and NE systems are intimately linked, with high numbers of projections running between them. The tricyclic (TCA) and norepinephrine reuptake inhibitor (NRI) classes of antidepressants influence 5-HT neurotransmission by directly modulating the central NE system. It is also possible that NE-active antidepressants act through their own distinct 5-HT independent mechanisms. Indeed, it has been shown that 5-HT active antidepressants such as SSRIs influence the NE system as well as the 5-HT system, and it is therefore possible that some of their antidepressant capabilities are mediated via

secondary effects involving NE-specific mechanisms (Szabo and Blier, 2001). In both acute and chronic treatments, all classes of antidepressant drugs influence NE neurotransmission--the majority decrease LC NE firing, but mirtazapine and techniques such as vagal nerve stimulation increase NE firing (Haddjeri et al., 1995, Manta et al., 2009)--though some mechanisms are not well understood. Notably, as NE systems are responsible for the regulation of vigilance, arousal and anxiety, antidepressant drugs that decrease NE activity may be important in addressing depression-related anxiety by yielding additional anxiolytic effects; an example of this is the approval of the serotonin and norepinephrine reuptake inhibitor (SNRI) antidepressant venlafaxine, which decreases NE firing and desensitizes the 5-HT_{1A} autoreceptor like SSRIs, for use in treating anxiety disorders such as general anxiety disorder, panic disorder and social phobia (Bambico and Gobbi, 2008). Contrarily, treatments that increase NE activity are important in addressing depression-related psychomotor retardation, motor speed, and executive functioning (Bambico and Gobbi, 2008).

Unfortunately, the most common first-line antidepressant therapeutics have slow onsets of action (2-3 weeks) and are only effective in a third of patients, while one third respond only partially and the rest do not respond at all (Thase, 2003). Additionally, they are associated with a host of side effects ranging from mild to severe; for example, SSRIs and SNRIs are associated with gastrointestinal, sexual and metabolic side effects that result from an induced increase in 5-HT levels. In one study, 15.8% of patients taking the SSRI citalopram developed an intolerable adverse effect, 38.6% developed moderate-to-severe difficulties caused by an adverse effect, 8.6% discontinued antidepressant treatment due to adverse events, and 4% developed a serious adverse event (Trivedi et al., 2006). More recently, the so-called “fast-acting” antidepressants such as the NMDA receptor antagonist ketamine have also been implemented in emergency settings for severe intractable cases (Li et al., 2010), but also have serious side effects including dependence and they have not been studied extensively for use in chronic treatment. Considering the recent advancements in research technology and techniques, it is possible and thus necessary to further elucidate the pathophysiology of the disease, and, furthermore, to discover novel putative targets for antidepressant drug development.

Circadian dysfunction and depression

Circadian dysfunction is intimately associated with many mood disorders such as major depressive disorder (MDD), atypical depression, seasonal affective disorder, and bipolar disorder (McClung, 2013). The precise chronobiological disruptions observed in depressed patients are heterogeneous, but are linked to many symptoms including delayed sleep onset, non-restful sleep, early morning wakening, daytime fatigue, and abnormal morning peaks in energy, mood, cognition and alertness (Hickie and Rogers, 2011). Additionally, circadian disruption resulting in internal desynchronization of various rhythms causes significant phase differences between these rhythms and critical environmental cues; some MDD patients display hypersomnia, fatigue, and excessive napping, as well as shortened periods of rapid eye-movement (REM) sleep, all results of circadian disruptions (non-REM sleep is regulated by other non-circadian homeostatic pathways) (Nutt et al., 2008). When prescribing antidepressants, clinicians endeavour to resynchronize circadian rhythms, restore the influence of external cues, especially light, increase the amount of REM sleep and normalize sleep architechture overall; the restoration of these areas can be tracked as a measure of a drug's effectiveness. However, the majority of antidepressants including SSRIs and SNRIs characteristically disrupt both REM and slow-wave sleep, and do not normalize chronobiology; until recently, the suppression of REM sleep was believed to be an essential feature of antidepressant drugs (Hickie and Rogers, 2011). Because of these difficulties, antidepressants are often prescribed along with another sedative drug. However, these sedatives only facilitate sleep onset and largely do not address long-term circadian function. Troublingly, long-term sedative use can lead to drug tolerance and/or addiction (Greenblatt and Shader, 1978). Furthermore, the inability of an antidepressant to normalize chronobiology is a strong prognostic that the patient maintains ongoing symptoms, or may suffer early relapse (Hickie and Rogers, 2011). To address the multitude of problems caused by common antidepressants and their inability to normalize circadian rhythms, treatment development should target alternative pathways that are implicated in depression. Consequently, research has begun investigating the important links between the melatonergic system and depressive disorders, and early studies have yielded promising results.

The melatonergic system and depression

In animals, supplemental MLT administration has been shown to reverse some long-term effects of chronic stress, and possesses anti-depressant-like activity; however these reports show inconsistencies with regards to dosing, time of administration, and reported efficacy (Binfaré et al., 2010, Bourin et al., 2004). Depression in humans is associated with alterations in the circadian rhythm of MLT output, notably a blunted nocturnal peak (Srinivasan et al., 2009). MLT production in humans may decrease in depression, and certain antidepressants may enhance circulating levels of MLT (Magnusson and Boivin, 2003). For example, the SSRI fluvoxamine increases the amplitude and duration of the plasma melatonin peak (Skene et al., 1994). Light exposure has been shown to suppress MLT synthesis (Lewy et al., 1980), and, during the fall and winter seasons, the decrease in photoperiod duration with its accompanying increase in MLT synthesis has been linked to the pathophysiology of seasonal affective disorder (SAD) (Srinivasan et al., 2006); in patients with SAD, studies have found MLT secretion to be extended/increased (Wehr et al., 2001). Unfortunately, in humans, while MLT does improve timing and length of sleep in certain groups of depressed patients, MLT alone has a weak or null antidepressant therapeutic effect, likely due to its low bioavailability and lack of selectivity towards either of its receptors (Hickie and Rogers, 2011). However, some positive effects have been demonstrated when administered synergistically with other antidepressant drugs such as buspirone (Fava et al., 2012).

In 2009, agomelatine, a structural analog of MLT, a MT_1 and MT_2 receptor agonist and 5-HT_{2C} receptor antagonist, was approved by the European Medical Agency for clinical use in depression, proving to be at least comparable in efficacy to conventional antidepressants (Kasper et al., 2010). While Servier Laboratories Ltd, the company that developed agomelatine, claimed that the antidepressant-like activity seen in the forced swim test (FST) in rodents resulted from interactions with MT_1, MT_2 and 5-HT_{2C} receptors, the antidepressant activity was not reproduced with MLT or a 5-HT_{2C} receptor antagonist in other behavioural paradigms of depression such as the learned helplessness model (Bertaina-Anglade et al., 2006). Indeed, antidepressant activity of agomelatine seems due to its effects on both MLT receptors and 5-HT_{2C} (Chenu et al.,

2014b). The approval of agomelatine support the hypothesis that the melatonergic system can be a novel target for antidepressant therapeutic development, but the lack of understanding with regards to its specific mechanisms of action reinforces the need for a more comprehensive understanding of the role of the MLT receptors in depression.

Melatonin and the depression-anxiety relationship

Generalized anxiety disorder (GAD) is a physiological and psychological state characterized by an unpleasant emotional state of excessive anxiety and worry that is difficult to control and related to several events or activities (DSM-IV), and is highly disabling, characterized by emotional, cognitive and psychological impairments. To meet diagnostic criteria, the anxiety be ongoing for more than six months and be accompanied by physical symptoms such as restlessness and fidgeting, easy fatigability, difficulty concentrating, headaches, irritability, muscle tension, nausea, numbness, sweating and sleep disturbances. Anxiety disorders have been linked with the melatonergic system. In animal studies, MLT has been shown to have anxiolytic effects (Golus and King, 1981, Golombek et al., 1993, Papp et al., 2006, Crupi et al., 2010, Srinivasan et al., 2006). In rodents, MLT increased the time spent in open arms in the elevated plus maze test (EPMT), increased the time spent in the central area in the open field test, and decreased latency to eat in the novelty suppressed feeding test (NSFT), all indicators of an anxiolytic effect of MLT. MLT analogues such as agomelatine and Neu-P11 promote anxiolytic-like effects in rodent behavioural models as well (Tian et al., 2010, Papp et al., 2006, Loiseau et al., 2006). In human studies, MLT has anxiolytic effects similar to benzodiazepines (BZs) but without side effects such as sleep disturbances (Samarkandi et al., 2005), and also aided in the discontinuation of BZs while improving sleep quality in elderly patients with sleep problems (Garfinkel et al., 1999). In addition to the reported anxiolytic effect of the antidepressant agomelatine, Ramelteon, a hypnotic and nonselective MT_1/MT_2 receptor agonist, has also yielded anxiolytic effects in humans (Stein et al., 2008). Importantly, our lab has recently demonstrated the anxiolytic effects of the selective MT_2 partial agonist UCM765 (Ochoa-Sanchez et al., 2012). Altogether, these results reinforce the relationship between MLT and anxiolytic effects.

With regards to the MLT system, just as serum MLT levels during the night were found to be lower in patients with major depression (Beck-Friis et al., 1984), elevated age is similarly associated with a decrease in MLT levels (Karasek, 2007), and GAD is common in older-adult populations (Miloyan et al., 2014). Interestingly, Harinath and colleagues found that relaxing exercises such as meditation and yoga increased plasma MLT, and, similarly, the well-being score associated with lower levels of anxiety was correlated with the maximum night time MLT level, and these exercises also reduced stress-related disorders such as anxiety, depression and insomnia (Harinath et al., 2004). Comorbidity of depressive and anxiety disorders is common, in part due to the overlap of many core symptoms, and the extent of this bidirectional overlap is such that it is difficult to study either of these disorders without also studying the other (Stein, 2009). Troublingly however, anxiety symptoms in major depression are correlated with more severe depressive symptoms, longer duration of illness, a greater impairment of function at work and at home, and increased suicide risk. A number of conventional antidepressants are used to treat anxiety disorders as well as anxiety symptoms of major depression, but it is also known that depressed patients with greater levels of anxiety symptoms respond worse to antidepressants and show an increased risk of relapse (Stein et al., 2001). Importantly, in a study that compared the efficacy of agomelatine vs the pooled efficacies of fluoxetine, sertraline, and venlafaxine (SSRIs/SNRIs that have also been used for treating anxiety disorders) on treating the anxiety symptoms of major depressive disorder, agomelatine was found to have a significantly greater effect on anxiety than both placebo and the other antidepressants, and in patients that were more anxious depressed, agomelatine had a greater effect on anxiety and depression than both placebo and the comparator antidepressants (Stein et al., 2013). As mentioned previously, we recently demonstrated the involvement of the MT_2 receptor in anxiety-like behaviours (Ochoa-Sanchez et al., 2012). These reasons emphasize the need for the development and study of novel selective MT_1 and MT_2 receptor agonists and antagonists in order to elucidate the role of each receptor in depressive disorders. Preliminary data (that will be discussed later) suggests that MT_1 (but not MT_2) receptors are involved in the pathogenesis of depression. Therefore the focus will now be on the known functions of the MT_1 receptor in the CNS and related systems.

Functions of MT_1 receptor

MT_1 in circadian rhythms

Much recent research has focused on the role of MT_1 in circadian rhythms in different species. Results from in vivo and in vitro experiments concerning the differential role of MLT receptors in circadian regulation and sleep, especially in the phase shift of SCN activity, produced controversial results. As mentioned previously, the mammalian SCN expresses both MT_1 and MT_2 receptors. Activation of MT_1 receptors were shown to inhibit SCN firing, while MT_2 receptor activation phase-shifted circadian rhythms of SCN activity (Hunt et al., 2001, Liu et al., 1997, Jin et al., 2003, Pfeffer et al., 2012). Results from our lab showed that genetic deletion of both MLT receptors resulted in increased wakefulness, and that MT2 receptor deletion likely caused a reduction in NREMS while MT_1 deletion likely caused a reduction in REMS (Comai et al., 2013). Other experiments involving mice kept under constant darkness and injected with MLT two hours before the onset of the active phase reported a phase shift in activity onset in wild type mice but not in MT_1 knockout mice ($MT_1^{-/-}$), and that in wild type but not knockouts, MLT accelerated the mice's entrainment to a new light-dark cycle (Dubocovich and Markowska, 2005). Therefore the involvement of MT_1 receptors in MLT-induced phase shift of SCN activity cannot be ruled out.

mRNA expression patterns of MLT receptor subtypes in the pineal organs of lunar-synchronized spawning fish have been shown to be influenced by moonlight; RT-qPCR revealed that MT_1 mRNA increased during the new moon phase and decreased in the full moon phase, and these changes corresponded with levels of plasma MLT, thus relating MLT and MT_1 to reproductive patterns (Park et al., 2014). In the fish species mudskipper that spawns once per season, both MLT receptors were found in diencephalon, pituitary glands, and ovaries, but only MT_1 was found in fully grown follicles, isolated follicle layers and denuded oocytes, and was overall found to be involved in the synchronization of semilunar spawning rhythms by acting through the hypothalamic-pituitary-gonadal (HPG) axis and/or directly on ovarian tissues (Hong et al., 2014). In some mammals such as the tropical squirrel (which has the least annual variation in photoperiod), seasonal changing photoperiod and MLT levels have been shown to modulate MT_1 expression dynamics in the SCN, alter its functional state, and

gate SCN clock gene profiles by modulating PER1 and CRY1 expression, important for photo-physiological adaptation in seasonal breeders with implication in reproduction and immunity (Gupta et al., 2013). In vitro, the MT_1 receptor has been found to be required for the differential regulatory actions of MLT on neuronal clock gene expression in striatal neurons. The clock genes are proposed regulatory factors in forming dopamine-related behaviours and mood, and MLT is thought to regulate these processes, and MT_1 knockout mice showed a reversal of the MLT-induced decrease of clock gene expression, implicating MT_1 as a target for treating some pathologies of dopamine related behaviours and mood (Imbesi et al., 2009).

MT_1 in neurological and psychiatric conditions

Recent studies have implicated MT_1 as a key player in many neurological and psychiatric conditions. Research into neuroprotection has discovered that MT_1 is neuroprotective in several experimental models of neurodegeneration.

MLT stimulation of MT_1 (but not MT_2) was revealed to increase cell proliferation and survival rate while enhancing neuronal differentiation of cultured amniotic epithelial cells, which, together with VEGF upregulation, were neuroprotective against experimental *in vitro* models of ischemic and oxidative stress injury (Kaneko et al., 2011).

MLT was found to delay disease onset and mortality in a transgenic mouse model of Huntington's disease, and mutant huntingtin (htt)-mediated toxicity was associated with the loss of MT_1; the MLT-mediated protection was found to depend on the presence and activation of MT_1, suggesting that the pathological process involves mutant htt inducing the loss of MT_1, thereby enhancing neuronal vulnerability and accelerating the neurodegenerative process (Wang et al., 2011).

The regional expression of the MLT receptors was studied in Parkinson's disease (PD) (Adi et al., 2010), where the antioxidant properties of MLT were thought to influence the oxidative stress and apoptotic mechanisms resulting from mitochondrial dysfunction in the dopaminergic system. MT_1 expression was found to be significantly decreased in the substantia nigra and amygdala of whole-brain post-portem tissue vs controls; MT_2 was also found to be decreased. This downregulation of the receptors in

regions affected by PD suggests possible involvement in the disease process (Adi et al., 2010). Also in PD, MLT was found to ameliorate depressive symptoms and serve a neuroprotective role in animal models of PD (Bassani et al., 2014). Furthermore, REM-associated sleep behaviour disorder (RBD) can be a preclinical marker of PD, and some trials suggest that MLT may be useful in treating sleep disorders of PD, especially RBD (Srinivasan et al., 2005). Given the findings that MT_1 knockout mice display defecits in REM sleep, the treatment using MLT for RBD and PD sleep disorders likely involves an MT_1 mediated mechanism.

In schizophrenia, antipsychotics-induced tardive dyskinesia (TD) is suggested to be related to altered neurotransmission in the striatum, and since MLT modulates dopaminergic neurotransmission, researchers examined an association between MLT receptor genes and antipsychotics-induced TD and found a significant association between haplotype ATG (a single-nucleotide polymorphism) in the gene encoding MT_1 and non-TD (Lai et al., 2011).

In a transgenic mouse model (SOD1) of amyotrophic lateral sclerosis (ALS), caspase-mediated cell death was found to contribute to pathogenesis of the motor neuron degeneration, in addition to other factors such as inflammation and oxidative damage. Furthermore, disease progression was associated with both the loss of MLT and the receptor MT_1 in the spinal cord of SOD1 mice, and MLT was found to be neuroprotective by inhibiting the caspase-1 cytochrome c-caspase 3 cell death pathway, inhibiting MT_1 receptor loss and delaying disease progression (Zhang et al., 2013).

The MT_1 receptor has been studied for its involvement in Alzheimer's disease (AD) and dementia. MLT receptors were found be decreased in pinealocytes and cortical pyramidal and non-pyramidal cells in AD (Brunner et al., 2006). The levels of MT_1 receptor decreased in the SCN in aging and late state AD, and this is thought to contribute to the clinical circadian disorders and the efficacy of therapeutic MLT admin in AD (Wu et al., 2007). MT_1 receptor levels increased in the hippocampus in AD, indicating upregulation of receptor possibly compensating for impaired MLT levels to augment MLT neuroprotection (Savaskan et al., 2002a). MT_1 is known to cause vasoconstriction in blood vessels, and the finding that cerebrovascular MT_1 levels were increased in AD, especially in the adventitia of superficial and intrahippocampal arteries

may indicate a regulatory response to impaired MLT levels in those patients, as regional blood flow impairments contribute to the neurodegenerative progression (Savaskan et al., 2001). In the human retina, which produces MLT in small amounts, MT_1 receptor was found in ganglion, amacrine, photoreceptor and blood vessels, and were increased in ganglion and amacrine cells and in vessels of AD patients, whose photoreceptors were degenerated and showed low MT_1 expression (Savaskan et al., 2002b).

Further evidence for the function of MT_1 in the eye, $MT_{1/2}$ heteromers were found to play an essential role in retinal function by mediating the light sensitivity of rods in mice (Baba et al., 2013). Using MT_1 knockout mice, the same group found that, via MT_1, MLT modulates visual function and cell viability in the retina and may be of therapeutic use in the future for treating retinal degeneration (Baba et al., 2009).

Researchers have also examined the role of MT_1 receptors in the development of dopaminergic behaviours, such addiction and drug seeking. The development and expression of methamphetamine (METH)-induced locomotor sensitization during the day and the night were differentially affected in MT_1 knockout mice; exposure to a novel environment during the dark but not the light phase facilitated expression of sensitization to METH challenge in a manner dependent on the activation of MT_1 by endogenous MLT (Hutchinson et al., 2012). Furthermore, the deletion of MT_1 was found to abolish the locomotor sensitization induced by a single METH pre-treatment, and the magnitude is altered by time of day and contextual cues (Hutchinson et al., 2014). Studies investigating the drug and region specific effects of protracted antidepressant (fluoxetine, desipramine, clomipramime) and cocaine treatment on MT_1 and MT_2 mRNA expression in the brains of mice found that in the hippocampus, whereas single injections of the drugs did not alter mRNA expression, protracted treatment with most antidepressants but not cocaine or fluoxetine increased the levels of MT_1 mRNA while decreasing MT_2 mRNA, while in the striatum, antidepressants and cocaine decreased striatal MT_1 mRNA but did not significantly alter MT_2 mRNA; these findings indicate that long-term use of these drugs induces alterations in MLT receptor in specific regions of the brain and may cause neuroplastic effects (Imbesi et al., 2006). Weil et al. (2006) reported that MT_1 knockout mice displayed deficits in sensorimotor gating, related to dopaminergic functioning, in addition to depressive-like behaviour. It has also been reported that MLT co-treatment

results in anxiolytic-like effects during cocaine withdrawal and attenuates cAMP levels induced by cocaine in the nucleus accumbens (Uz et al., 2005). The localization of MT_1 to areas related to dopaminergic behaviours such as prefrontal cortex, putamen, caudate nucleus, nucleus accumbens, substantia nigra, amygdala and hippocampus, as well as diurnal variations of MT_1 expression in the nucleus accumbens and ventral tegmental area further substantiate the role of MT_1 in the central dopaminergic system (Uz et al., 2005).

MT_1 in depression

Experiments from our laboratory as well as others employing selective MT_1 and MT_2 receptor ligands as well as MT_1 and MT_2 receptor knockout mice strongly suggest that the MT_1 receptor, but not the MT_2 receptor, is very likely implicated in the antidepressant effects of MLT and MLT analogs.

Weil et al. (2006) found a depressive-like phenotype and altered sensorimotor gating in MT_1 knockout mice. Wu et al. recently demonstrated that MT_1 but not the MT_2 receptors are increased in the SCN of depressed patients (2013).

Previous research has found links between the melatonergic system and melancholic type depression, a specific mood disorder with symptoms that include disturbed mood with a diurnal variation (generally worsened in the mornings), anhedonia, psychomotor retardation and agitation, cognitive impairment, as well as somatic symptoms including weight loss, hypercortisolemia, and sleep disturbances, especially in REMS. These patients respond better to tricyclic antidepressants than other narrower-acting drugs (e.g. SSRIs) and respond poorly to other non-pharmacological types of interventions. Research demonstrating links between melancholic depression and the melatonergic system has identified lower nocturnal MLT levels in melancholic depressed patients versus controls (Brown et al., 1985) and lower 2300h plasma levels of MLT versus patients with other depressive subtypes (Fountoulakis et al., 2001). 5-HT and NE neurotransmission was found to be impaired in melancholic depression (Pier et al., 2004, Malhi et al., 2005). MT_1 knockout mice displayed altered REMS and sleep architecture (Comai et al., 2013) in addition to the depressive-like phenotype and sensorimotor gating deficits reported by Weil et al. (2006).

Our lab recently discovered remarkable similarities between MT_1 knockout mice and humans with melancholic depression (Comai et al., 2015). For the first time, we demonstrated an animal model of melancholic depression that reproduces a great number of the hallmarks of this disease, including most importantly diurnal variations in affect, which differentiates melancholic from non-melancholic depression. Behavioural and electrophysiological experiments were performed to characterize the phenotype of MT_1 knockout mice. MT_1 knockout mice displayed decreased mobility in the FST and TST and decreased sucrose consumption (indicating depressive-like phenotype and anhedonia) and these were reversed by chronic treatment with desipramine. They also found these mice to share other similarities with human melancholic depressed patients, including psychomotor disturbances, and higher serum levels of corticosterone, which also demonstrated a blunted diurnal variation. All experiments were conducted in both the light and the dark phases, and many of the effects were more pronounced in the dark (active) phase. Electrophysiological recordings found that the LC decreased its burst firing activity during the dark phase. High firing neuronal populations in the LC and DR were found to display diurnal variations in spontaneous firing activity in wild type mice, but this variation was abolished in MT_1 knockout mice (see figures II-V and table 1, from Comai et al., 2015, presented here with permission from authors). Altogether, these experiments support MT_1 knockout mice as useful models for human melancholic depression, thereby demonstrating the involvement of MT_1 in the pathogenesis of the condition, and validating the MT_1 receptor as a promising target for the development of novel pharmacological therapeutics.

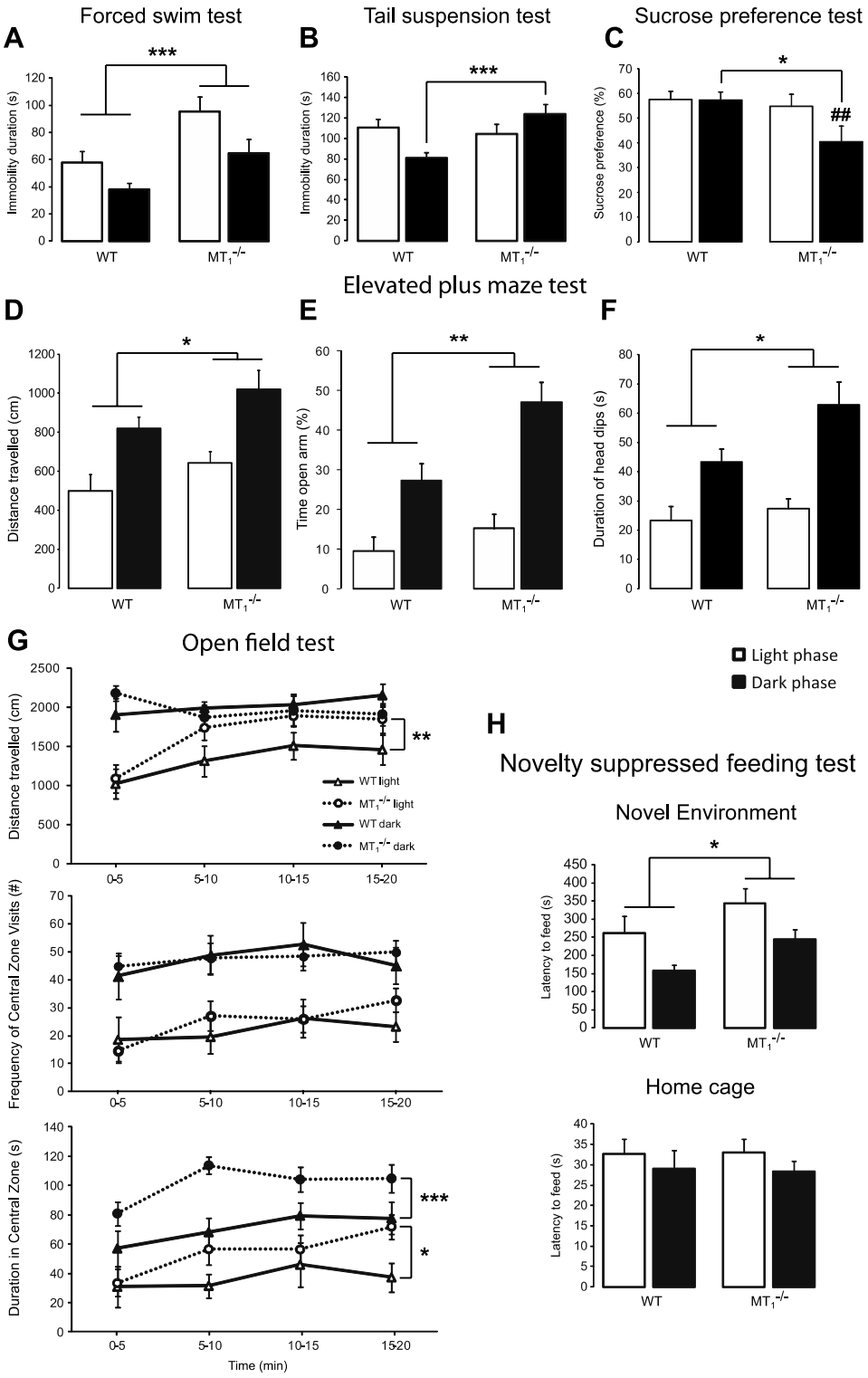


Figure II. Behavioural tests: MT_1 knockout mice demonstrated depressive like behaviour and psychomotor disruption including increased immobility in the FST light and dark phases (A) and TST dark phase (B), decrease in sucrose preference in dark phase (C); in

the EPMT, knockout mice had an increase in total locomotion (D), in the % of time in the open arm (E) and in duration of head dips (F); in the OFT (G), MT_1 knockout mice had increase in total locomotion in the light phase, in time spent in centre of field in both phases, but no changes in the number of central zone visits. In the NSFT (H), MT_1 knockout mice displayed increased latency to eat in the novel environment but not in the home cage (with permission from Comai et al., 2015).

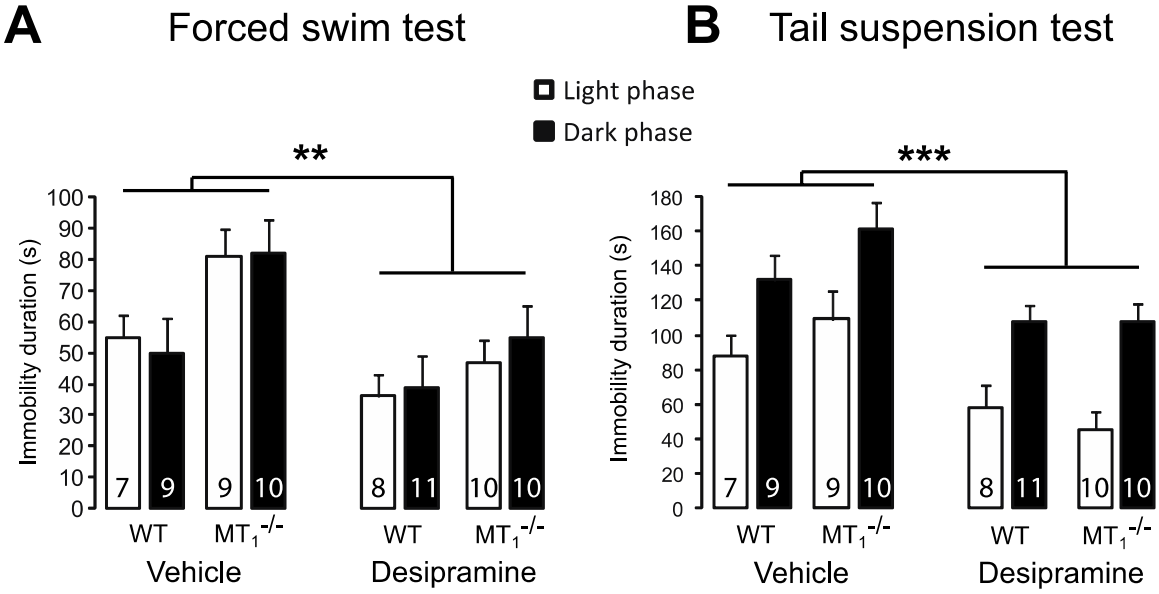


Figure III. Chronic 20-day treatment with the antidepressant desipramine reverses the depressive-like behaviour of MT_1 knockout mice in the FST (A) and TST (B). Desipramine also reduced the duration of immobility in the WT mice after chronic treatment (A and B) (with permission from Comai et al., 2015).

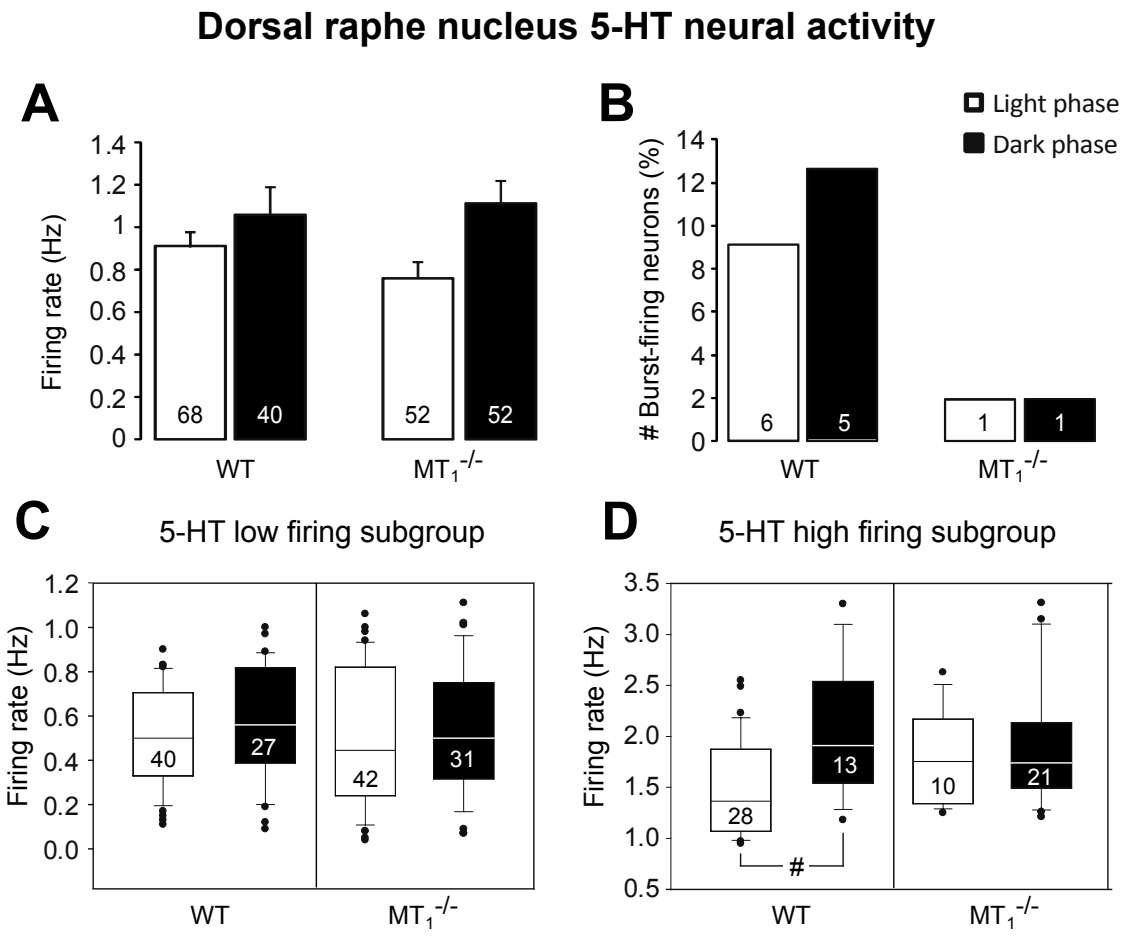


Figure IV. Firing activity of the 5-HT high-firing subgroup in the DR does not change according to phase of day in MT_1 knockout mice. Mean firing rate of all 5-HT neurons in DR did not differ significantly between WT and MT_1 knockout mice in both light and dark phases (A). Number of 5-HT neurons discharging in bursts (too few DR 5-HT neurons displaying burst activity to analyze statistically, but the MT_1 knockout mice appear to follow a trend of decreased burst activity) (B). Analysis of low (C) and high (D) firing 5-HT neuronal subgroups demonstrates the loss of the dark-phase increase in firing of high firing 5-HT subgroup in MT_1 knockout mice (with permission from Comai et al., 2015).

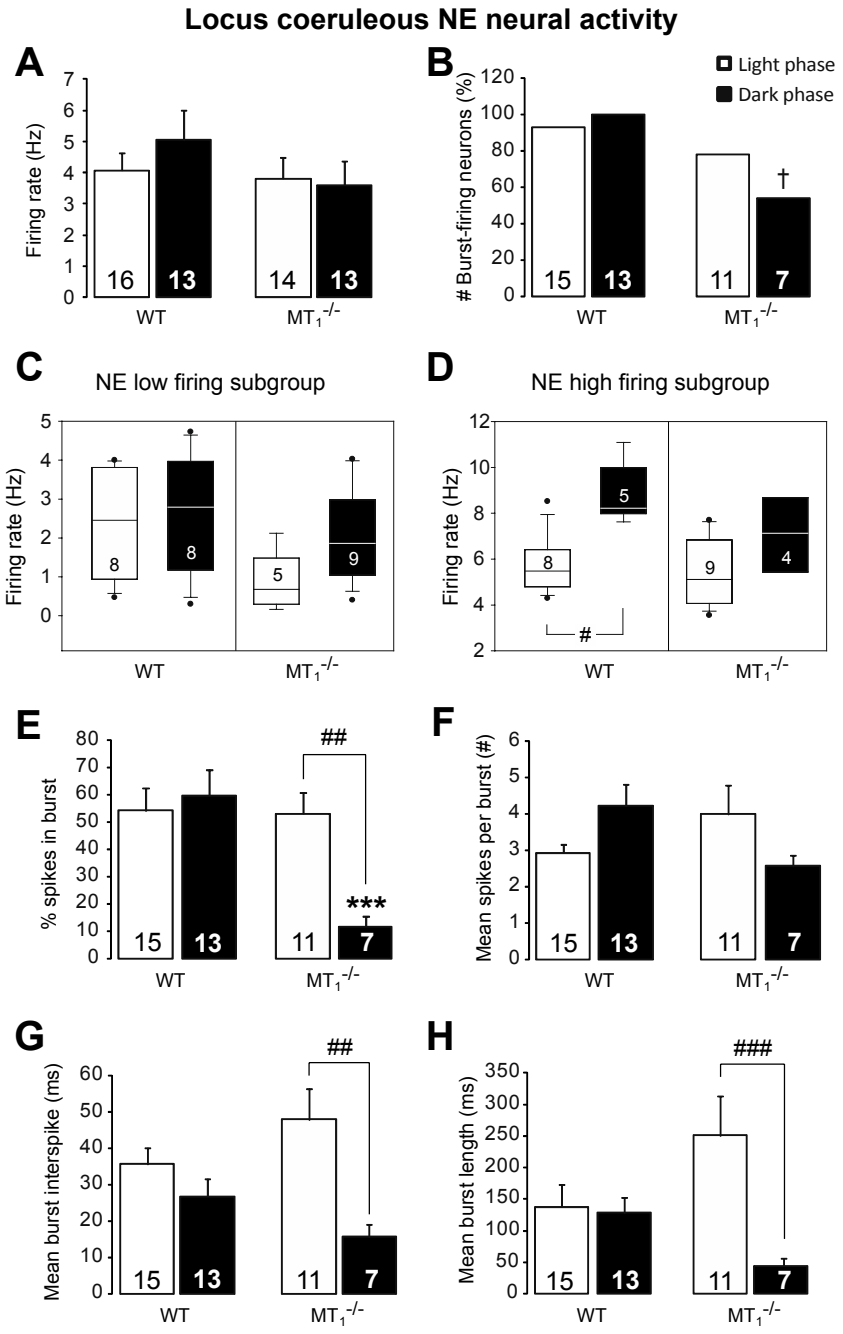


Figure V. LC NE neuronal firing rate is not significantly altered in MT_1 knockout mice (A), but MT_1 knockout mice show decreased number of neurons discharging in bursts in the dark phase (B). Analysis of low (C) and high (D) firing NE neuronal subgroups demonstrates the loss of the dark-phase increase in firing of high firing NE subgroup in MT_1 knockout mice. Analysis of NE neuronal burst firing activity in light and dark phases is shown (E-H). The total % of spikes in bursts (E), the mean burst interspike (G)

and the mean burst length (H) are significantly decreased in the dark phase compared to the light phase in MT_1 knockout mice only (with permission from Comai et al., 2015).

Table 1. A comparison between the psychological and neurobiological symptoms of melancholic depression and the phenotype of MT_1 receptors knockout mice.

Symptoms of melancholic depression (Parker et al., 2010)	MT_1 receptors knockout mice
Anhedonia	Anhedonia (↓ sucrose preference)
Depression	Depression-like behavior (↑ immobility in FST and TST; ↑ latency to eat in NSFT)
Weight loss	↓ Weight
Psychomotor disturbances (agitation or retardation)	Hyperlocomotion (↑ locomotion in OFT), disinhibition (↑ open arm time and entries in EPMT, ↑ head dips in EPMT)
Diurnal variation of mood	behavioral light/dark differences
Hypercortisolemia	↑ corticosterone serum levels during the dark phase, no light/dark differences in corticosterone levels
Disturbance in sleep architecture especially at the level of REMS	↓ REMS duration, ↓ NREMS EEG delta power and REMS EEG theta power (36)
Monoamine activity alterations	↓ DRN 5-HT and LC NE neuronal bursts activity; altered light/dark firing pattern of DRN 5-HT and LC NE neurons
Genetic causes	Genetic inactivation of MT_1 receptors

Table 1. with permission from Comai et al., 2015

Preliminary data from HPLC analyses of brain homogenates (unpublished) found that the levels of 5-HIAA, the primary metabolite of 5-HT, were elevated in the brains of MT_1 knockout mice compared to wild type brains, indicating altered metabolism of 5-HT in these mice that likely relates to the depressive-like behaviours observed. Our laboratory recently published that MT_1 knockout mice display impairment in rapid eye movement sleep and have an impaired light-dark cycle (Comai et al., 2013); this mirrors the disordered sleep seen in individuals with depressive disorders (Reynolds et al., 1985). This evidence emphasizes the need for the development and study of novel selective MT_1

and MT_2 receptor agonists and antagonists in order to elucidate the role of each receptor in mood disorders.

UCM871, a novel selective MT_1 receptor partial agonist

Our collaborators Drs Spadoni and Tarzia have recently synthesized a selective MT_1 receptor partial agonist, UCM871 (N-(2-{Methyl-[3-(4-phenylbutoxy)phenyl]amino}ethyl)acetamide), referred to as molecule #4a in Rivara et al. (2012), a compound belonging to the class of anilinoalkyl-amides (see **Figure VI** for structural comparison with MLT). This class of compounds are rapidly metabolized, have high plasma protein binding and low blood-brain barrier passage. UCM871 is the first MT_1 selective ligand available in mood disorder preclinical therapeutic research, and as such, has a high impact for translational research development. Our data show that UCM871 has a relatively short half life of approximately 47 min, an area under curve (AUC) of 68978 min*ng/ml, a Cmax of 2590 ng/ml and partition coefficient (LogP), a measure of lipophilicity, of 4.01.

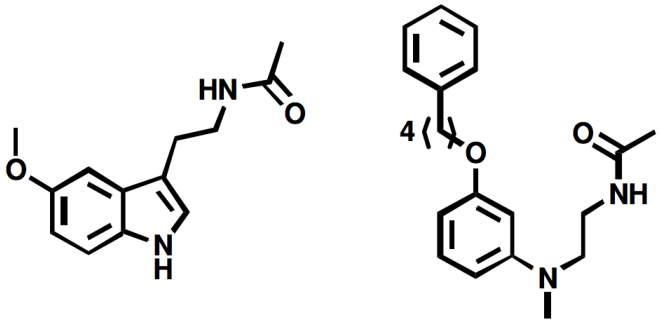


Figure VI. Structural comparison of MLT and the novel selective MT_1 receptor partial agonist, UCM871.

UCM871 displays high MT_1 receptor binding affinity, with a pKi of 8.93 and a 78-fold selectivity for the MT_1 receptor versus the MT_2 receptor, for which it has a pKi of 7.04. Tested in a NIH3T3 cell line expressing human MT_1 and MT_2 receptors, UCM871

was found to behave as a MT_1 partial agonist, with IA = 0.68. The receptor binding affinity of UCM871 has not yet been assessed in rats.

Partial agonists are unique; they bind and activate their given receptor but only have partial efficacy at the receptor relative to a full agonist, and may also be thought of as ligands that display both agonistic and antagonistic effects. They are therefore sometimes refered to as "intelligent drugs" (Calvey and Williams, 2009). They are particularly useful due to their flexible properties; clinically, when inadequate amounts of the endogenous ligand are present, partial agonists can activate receptors to yield the desired submaximal response, and, when excess levels of the endogenous ligand are present, they act as competitive antagonists, reducing the over-stimulation of their receptors. Their adaptable properties relative to full agonists make partial agonists an important and useful option in the development of novel therapeutics.

Using the novel selective partial agonist UCM871, we are able to dissect the function of the MT_1 receptor in depression in a way that was, until recently, not possible and provides tremendous potential for the future of this field of research.

Hypothesis and objectives

As discussed above, previous research has suggested that the genetic blockade of MT_1 receptor induces depressive-like phenotype; from this we hypothesized (and demonstrated in preliminary experiments) that MLT can modulate mood through MT_1 receptor-mediated pathways, and furthermore, that MT_1 receptor agonism induces antidepressant-like effects. This supports the study of the MT_1 receptor as a novel target for the development of antidepressant therapeutics, and we therefore aimed to assess whether the novel MT_1 receptor ligand UCM871 displays antidepressant-like properties in animal models. It should be noted that while the main focus of these experiments was the study of antidepressant potential of UCM871, considering the current knowledge of the bidirectional relationship between depression and anxiety, the anxiolytic-like activity of UCM871 was also considered in parallel.

First, classical behavioural paradigms of depression- and anxiety-like behaviours, the Forced Swim Test (FST) (Porsolt et al., 1977) and the Open Field Test (OFT) (Denenberg, 1969) respectively, were used to determine the effect of UCM871 in depression and anxiety. The FST is sensitive for clinically effective antidepressants and has been repeatedly validated (Porsolt et al., 1977). Several doses of UCM871 (dose-response experiments) were compared, and tested alongside the antidepressant desipramine as a positive control. In the FST, duration of immobility, swimming and climbing behaviours were analyzed. In the OFT, number of entries to the central area and time spent in the central area as well as total distance travelled were analyzed to determine whether 1) UCM871 had an effect on locomotor activity, which might be a confounding factor in the measurement of immobility in the FST, and 2) had an effect on anxiety.

Second, using *in vivo* single-unit extracellular electrophysiological experiments, the effect of UCM871 on monoaminergic neurotransmission was investigated in two key brain regions implicated in depression, the DR and LC, to determine whether, similar to common antidepressants, any alterations of 5-HT and NE neuronal activity were responsible for its antidepressant effects. With this, we aimed to elucidate the mechanism of action of MT_1 in depression. As mentioned previously, all antidepressants currently on the market modulate monoamine neurotransmission as a final outcome (albeit through

different pathways); SSRIs block 5-HT reuptake, activating 5-HT_{1A} autoreceptors in the DR and inducing a decrease in 5-HT firing, which recovers to baseline after 2-3 weeks when 5-HT_{1A} autoreceptors become desensitized; NSRIs such as venlafaxine decrease NE firing, and similarly to SSRIs, desensitize the 5-HT_{1A} autoreceptors (Béïque et al., 2000a, Szabo et al., 1999). Importantly, SSRIs decrease NE firing after chronic treatment. Based on preliminary results, which found UCM871 to induce a fast decrease in NE firing activity, we hypothesized that, UCM871 and other MT_1 agonists may display a faster onset of action compared to other types of antidepressants. In MT_1 knockout mice, we saw a trend of decreased 5-HT burst activity, so we hypothesized that UCM871 would increase burst activity, a finding that is associated with an increase of 5-HT release in post-synaptic targets (Gobbi et al., 2005). Based on our research that found MT_1 receptors to be expressed at high levels in the DR (unpublished), we also hypothesized that UCM871 will activate 5-HT neurons directly.

These experiments represent a valid framework for assessing the potential antidepressant activity of this novel ligand, and validate the role of MT_1 receptors in depression.

Materials & Methods

Maintenance and preparation of animals

All experiments were carried out on male adult Sprague-Dawley rats (Charles River, Ste. Constant, Quebec, Canada), weighing approximately 300 g, housed 2 per standard polycarbonate cage and housed in a facility with standard conditions (12:12 light/dark cycle, lights on at 07:00; temperature approximately 20˚C; 50-60% relative humidity; ad libitum access to food and water). All experiments were initiated after 1 week of acclimatization and animals were left to habituate in the testing laboratory for 30-60 minutes prior to each experiment. All procedures were undertaken in compliance to the standards and ethical guidelines mandated by the Canadian Institutes for Health Research, the Canadian Council on Animal Care, and the McGill Comparative Medicine and Animal Resources Centre.

Drugs

UCM871 (N-(2-{Methyl-[3-(4-phenylbutoxy)phenyl]amino}ethyl)acetamide, referred to as **4a** in Rivara et al., 2012) was synthesized and contributed generously by our collaborators Drs Spadoni and Tarzia. The dosing of UCM871 was chosen on the basis of initial acute intravenous administration results in electrophysiological experiments. For electrophysiological experiments, UCM871 was dissolved in 70% dimethylsulfoxide (because of the hydrophobicity of UCM871) 30% physiological saline, and was injected, i.v, into the lateral tail vein 0.1 ml at a time with each 0.1 ml equivalent to 3.5 mg/kg, up to a maximum dose of 14 mg/kg. Intravenous injection of 0.1 ml of vehicle (VEH, 70% DMSO: 30% saline) preceded injections of UCM871. In experiments in the dorsal raphe, 8-OH-DPAT [(±)-8-Hydroxy-2-dipropylaminotetralin hydrobromide, Sigma-Aldrich], a standard selective 5-HT_{1A} full agonist (De Vry et al., 1998, Arvidsson et al., 1981, Middlemiss and Fozard, 1983) with moderate affinity to 5-HT_7 receptors (pK_i = 6.6 at the human 5-HT_7 receptor expressed in HEK 293 cells), was dissolved in 0.9% saline and injected, i.v., (0.1 ml at a time, with each 0.1 ml equivalent to 1 µg/kg, up to a maximum dose of 3 µg/kg) after UCM871 (or vehicle) to measure the effect of UCM871 on the sensitivity of 5-HT_{1A} receptors (8-OH-DPAT inhibits spontaneous firing

of 5-HT neurons in the DR). In experiments in the Locus Coeruleus, clonidine (*N*-(2,6-dichlorophenyl)-4,5-dihydro-1*H*-imidazol-2-amine,
Sigma-Aldrich), an α2 adrenergic agonist that inhibits NE firing, was dissolved in saline and injected (1 μg/kg per 0.1 ml, i.v. up to a maximum dose of 4 μg/kg) after UCM871 (or vehicle) to measure the effect of UCM871 on the sensitivity of α2 adrenergic autoreceptors (clonidine inhibits spontaneous firing of NE neurons in the LC).

Chloral hydrate (400 mg/kg, i.p., Sigma-Aldrich) was used to anaesthetize the animals in all electrophysiological experiments.

Desipramine hydrochloride (3-(10,11-dihydro-5*H*-dibenzo[*b*,*f*]azepin-5-yl)-*N*-methylpropan-1-amine hydrochloride, Sigma-Aldrich), a tricyclic antidepressant (TCA) that predominantly inhibits NE reuptake but also 5-HT reuptake to a lesser degree, was used as a positive control in behavioural experiments, was dissolved in saline and prepared at a dose of 10 mg/kg. The drugs used did not produce any pain to the animals.

Behavioural assays

Forced Swim Test (FST, Porsolt)

The FST is a well validated behavioural test that is sensitive and selective for detecting antidepressant activity and is popular due to its relative simplicity and reliability, as well as having high predictive validity for antidepressant activity (Porsolt et al., 1977, Lucki, 1997, Cryan et al., 2005). The FST measures the rodent's behavioural transition from active (swimming, climbing) to passive (immobility) coping modes when placed in a deep water-filled cylinder from which it cannot escape. Immobility in the FST is a well-validated paradigm of behavioural despair. Antidepressants with serotonergic-specific action have been shown to selectively increase the duration of swimming and those with noradrenergic-specific activity selectively increase the duration of climbing (Page et al., 1999).

Pre-exposure to the test normally results in an enhancement of immobility, and this phenomenon reflect the depressive-like behaviour of learned behavioural despair, which is prevented by antidepressant treatment (Porsolt et al., 1977). The methodological details were adapted from our recent papers (Bambico et al., 2012). The animals were habituated to the testing room and to the experimenters every day for 1 week prior to the

start of the experiments. To induce this learned behavioural despair, rats were subjected to a 15 minute pre-test; rats were placed individually in Plexiglas cylindrical bins (20 cm in diameter, 50 cm in height) filled with water (25-27˚C) to a depth that did not allow them to touch their tail or hind paws to the bottom of the bin, approximately 30 cm. The FST itself was conducted 24 h later, when rats were re-introduced to the bins under identical conditions to the pretest and left to swim for 5 minutes. During this period their behaviour was captured with CCD cameras fitted with infra-red light-sensitive filters and recorded using an automated behavioural tracking system (Videotrack system, View Point Life Sciences, Montreal, Quebec, Canada). Custom made plates lined with infrared light-emitting diodes were positioned above the bins. Rats received 3 injections of drug or vehicle at 23.5 hours, 5 hours and 45 minutes before the start of the FST (modified from Page et al., 1999), with volumes of 0.2 ml per injection and administered subcutaneously.

Dose response of UCM was measured with experimental groups vehicle (70% DMSO: 30% saline) vs 3 x UCM doses of 3.5, 7 and 14 mg/kg (in 70% DMSO: 30% saline); then UCM871 was compared to a positive control, the antidepressant desipramine HCl (10 mg/kg in saline), and saline (control for desipramine). All tests were conducted toward the end of the light phase in a dark room lit by only red lights and under minimally anxiogenic conditions (Kelliher et al., 2000). After the recording, the rats were retrieved from the bins using a plastic lattice, dried with a towel and housed near a heat source. Between experiments, bins were cleaned and water was replaced with clean water of the appropriate temperature and depth. The video files were randomized and the frequency and duration of behavioural endpoints were analyzed and categorized into periods of immobility, swimming and/or climbing by 3 independent experimenters, blind to the experimental group. A rat was considered immobile when making movements necessary to keep its head above the surface of the water, swimming when making moderate movements with all limbs to propel itself horizontally around the water, and climbing when the rat engaged in forceful thrashing and struggling against the walls of the cylinder, actively attempting to escape.

Open Field Test

To measure changes to anxiety-like behaviours, and to control for false positive results in the OFT (decreased immobility/increased swimming in the FST may be due to non-antidepressant mechanisms, e.g. increased locomotion or hyperactivity), a short (5 min) OFT was conducted immediately preceding each FST to assess baseline locomotor activity, since coping activity in the FST could be influenced by changes in overall locomotion. Briefly, the experiment took place in a dark room lit only by red lamps suspended above the field (the same room as the FST) rats were placed individually at the corner of an open field arena measuring 80 x 80 cm and painted black, and were left to explore the arena for a 5 minute period, as previously described (Bambico et al., 2007). Videotrack software was used to track total locomotion (represented by movement velocity – distance travelled in cm per minute) as well as entries and duration spent in the central area. Thigomatic (anxiety-like) behaviour such as "wall-following" was measured by the number of entries and time spent in the central area; a greater number of entries and longer time in the centre signify anxiolytic-like effect of treatment, whereas anxiogenics decrease these entries and central time. Locomotion was assessed to identify any locomotor effects of treatment. Between experiments, arenas were cleaned with a dilute solution of ethanol and towel dried.

In Vivo Electrophysiology

In vivo extracellular single-unit recordings of presumed DR 5-HT and LC NE neurons were performed to characterize the capacity of the novel MT_1 selective partial agonist UCM871 to modulate 5-HT and NE neurotransmission. All stereotaxic coordinates used in the experiments were based on the stereotaxic atlas of Paxinos and Watson (2006). The following methods were adapted from Bambico et al., 2007, and Gobbi et al., 2005.

Preparation for electrophysiological procedures

Adult male Sprague-Dawley rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and mounted in a stereotaxic frame (Stoelting Instruments) with the rat's skull positioned horizontally with the incisor bar at -3.3. A full anesthetic state was

confirmed by the absence of a nociceptive reflex reaction to a paw or tail pinch and of eyeblink response to applied pressure. To maintain anaesthetic depth, animals were continuously monitored and supplemental chloral hydrate injections (100 mg/kg, i.p.) were administered as needed (approximately 1x/hour). Rat body temperature was maintained at approximately 37˚C throughout the experiment using a heating lamp.

Before recordings, a catheter was inserted into the lateral tail vein to facilitate systemic administration of drugs. Single-barrelled glass micropipettes were pulled from borosilicate Clark capillary glass (1.5 mm O.D. x 0.86 mm I.D., 100 mm L, Harvard Apparatus, OR, USA) on a Narashige (Tokyo, Japan) PE-2 pipette puller and preloaded with fiberglass strands to promote filling with 2% Pontamine Sky Blue dye in 2 M NaCl solution, and the tips were broken down to diameters of 1–3 μm. Electrode impedances ranged from 5–8 MΩ. At the end of experiments, the recording site was marked by iontophoretic ejection (27 μA, negative current applied for 5-10 minutes) of Pontamine Sky Blue for histological verification. Single-unit activity was recorded as discriminated action potentials from filtered electrode signal amplified by a Bak Electronics Model RP-I amplifier (Sanford, Florida, USA), fed to an oscilloscope (BK precision; 20 MHz, 1522) and an audiomonitor, post amplified and band-pass filtered by a Realistic 10 band frequency equalizer, and spike shapes were digitalized by an interface system (CED1401, Cambridge Electronic Design, Cambridge, UK), processed on-line and analyzed off-line by Spike 2 software for Windows PC (Microsoft, Seattle, WA). The spontaneous single-spike activity of neurons was recorded for at least 3 minutes, but the first 30 seconds immediately after detecting the neuron was not considered so as to minimize artefacts due to electrode movement. Changes in neuronal firing activity and pattern resulting from drug injections were monitored continuously (200s intervals were analyzed) but the first 30s following injections were not considered to minimize artefacts caused by the injection.

Single-unit extracellular recordings of DR 5-HT neurons

The DR nucleus is the principal source of 5-HT innervation in the brain. To record from the DR, an incision was made in the scalp and the periosteum was removed. A burr hole was drilled through the cranial midline at stereotaxic coordinates 1.2 mm

anterior to the intra-aural line. A hydraulic micropositioner (model 650; David Kopf Instruments) was used to lower the electrode into the DR to a target depth of 5.0 and 6.5 mm ventral to the dura mater, immediately below the ventral border of the Sylvian aqueduct. The electrode was slowly advanced (approximately 0.15 mm/min) until a clear neuronal signal was isolated. Though some neuronal firing criteria may vary under various pharmacological or environmental conditions (Bambico et al., 2009), under physiological conditions, 5-HT neurons display unique properties such as waveform and spike duration, which are stable across manipulations and make them distinguishable from other neuronal types. 5-HT neurons are characterized as displaying a slow and prominently regular firing rate (0.1-4 Hz, coefficient of variation ranges from 0.12 to 0.87) and possessing a broad biphasic (positive-negative) or triphasic action potential waveforms (0.8-3.5 ms, 1.4 ms first positive and negative deflections) (Baraban and Aghajanian, 1980, Allers and Sharp, 2003). An inhibitory response to the injection of 8-OH-DPAT, a 5-HT_{1A} full agonist (injected after UCM871 or vehicle), also identified that recorded neurons were 5-HT. 3-5 electrode descents were carried out in order to achieve maximum sampling of the DR rostrocaudal medial extent presumed to be richest in 5-HT neurons, without introducing significant tissue damage (Descarries et al., 1982).

5-HT burst activity

Based on Gobbi et al. (2005), burst activity was categorized and analyzed using the following criteria: a train of at least two spikes with a burst onset defined by a maximum initial interspike interval of 20 ms, with the longest interspike interval allowed within bursts being 40ms, within a regular low-frequency firing pattern.

Single-unit extracellular recordings of LC NE neurons

A burr hole was drilled and the micropipette lowered into the LC at the following stereotaxic coordinates: 1.0-1.2 mm posterior to intra-aural line, 1.0-1.3 mm lateral to midline, and 5.0–6.5 mm ventral to dura mater. Spontaneously active NE neurons were identified by the following criteria: a regular firing rate (0.5–5.0 Hz), a positive action potential (0.8–1.2 ms duration), and a brief excitation followed by period of silence in

response to a nociceptive pinch of the contralateral hindpaw (Aghajanian and Vandermaelen, 1982).

NE burst activity

Based on Bambico et al. (2010), NE burst activity was categorized and analyzed using the following criteria: a train of at least two spikes with a burst onset defined by a maximum initial interspike interval of 80 ms, with the longest interspike interval allowed within bursts being 160 ms, within a regular low-frequency firing pattern.

Statistical analysis

All data were organized and analyzed using SPSS version 22 (SPSS Inc., Chicago, Illinois, USA), Sigma Plot version 12.0 (Systat Software Inc., San Jose California, USA) and Excel 2007 (Microsoft Office), and were first tested for assumptions of normality and homogeneity of variance. Student Newman-Keuls multiple post hoc comparisons were used to decompose significant ANOVA results.

In the FST and OFT, desipramine versus saline in all tests were compared using unpaired t-test. Subsequent dose-response FST (immobility, swimming, climbing activities) and OFT (locomotion, entries, time in centre) experiments were analyzed using 1-way between subjects ANOVAs, followed by SNK multiple comparisons to decompose significant ANOVA results.

Electrophysiological data were obtained from 6-8 rats per experimental group. Once a stable neuron was identified, baseline activity was recorded for 3-5 min and then vehicle was injected, and the neuronal activity was recorded for an additional 3-5 min. All subsequent injections of drugs occurred at 3-5 minute intervals, recording the neuronal firing in the intervening periods. Raw data (average neuronal firing rate in Hz over each specific period examined) were converted into percentage change from baseline (spontaneously active neuronal, pre-injection) firing rate. To analyze the effect of UCM871 on spontaneous 5-HT neuron firing in the DR, a 1-way ANOVA was used on converted data. To analyze the effect of 7 mg/kg UCM871 on 5-HT burst activity in the DR, burst parameters were converted to percentage of vehicle, and then subjected to paired t-tests. To analyze the effect of treatment with UCM871 on the sensitivity of DR

5-HT neurons to 8-OH-DPAT, a 2-way mixed design (treatment group x dose 8-OH-DPAT) was conducted on converted data (percentage change from post-UCM871/vehicle-treatment, pre-8-OH-DPAT baseline). To analyze the effect of UCM871 on spontaneous NE neuron firing in the LC, a 1-way repeated measures ANOVA was used. To analyze the effect of UCM871 on burst activity in the LC, burst parameters over different doses were converted to percentage of vehicle and then subjected to 1-way repeated measures ANOVA. To analyze the effect of treatment with UCM871 on the sensitivity of LC NE neurons to clonidine, a 2-way mixed design ANOVA was conducted on converted data (percentage change from post-UCM871/vehicle-treatment, pre-clonidine baseline). All data are expressed as mean ± standard error of the mean (SEM). Statistical significance was taken as probability value of $p \leq 0.05$.

Results

Electrophysiological experiments

UCM871 displayed a bell-shaped excitatory effect on 5-HT firing in the DR

To analyze the effect of increasing doses of UCM871 on spontaneous firing rate of 5-HT neurons in the DR, a one-way repeated measures ANOVA for factor dose revealed a significant effect of treatment ($F(4,18)=8.96$, $p < 0.001$) (**Figures 1** and **2**). SNK post hoc tests indicated that acute i.v. injection of UCM871 at 7 mg/kg significantly increased the firing rate of DR 5-HT neurons (116.2 ± 29.7 % change from baseline) compared to the injection of vehicle (-3.0 ± 3.4 %) ($p<0.001$). While higher and lower doses of UCM871 were not statistically different from vehicle (ps >0.05), they displayed a bell-shaped trend, with lower doses increasing firing rate (3.5 mg/kg: 35.5 ± 14.9 %) until the maximum dose of 7 mg/kg, and then decreasing at higher doses (10.5 mg/kg: 41.5 ± 5.0 %; 14 mg/kg: 32.4 ± 12.3 %).

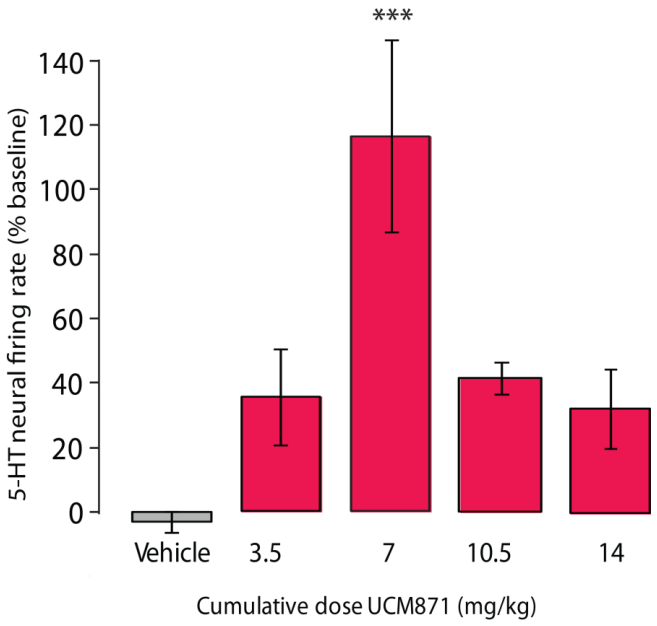


Figure 1. Acute UCM871 administration increased 5-HT neuronal firing activity in the DR. Dose of 7 mg/kg induced a maximum increase in 5-HT firing rate, while higher and lower doses of UCM871 were not statistically different from vehicle. This dose response effect of UCM871 displayed a typical bell-shaped curve. Data are expressed as percentage of baseline firing rate and presented as mean ± SEM. Data from 3-8 neurons per dose. ***p<0.001, 1-way repeated measures ANOVA for factor dose UCM871, followed by SNK multiple comparisons.

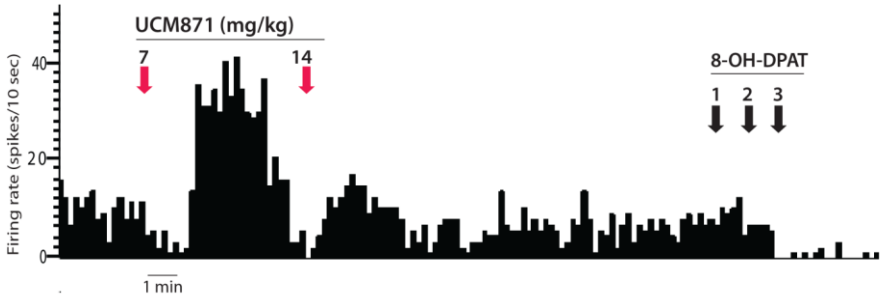


Figure 2. Sample histogram depicting changes in DR 5-HT firing rate in response to UCM871 followed by 8-OH-DPAT. UCM871 increased spontaneous firing rate and 8-OH-DPAT decreases firing rate in DR 5-HT neurons.

UCM871 and 5-HT burst activity in the DR

In the DR, a low % of recorded neurons were found to spontaneously discharge in bursts (~33% of neurons recorded were determined to be discharging in bursts at baseline). Given this low number of cases (bursting neurons), overall ANOVA could not be performed. However, we analyzed the effects on burst activity resulting from 7 mg/kg, (**Figure 3**) which was the dose we previously found to increase DR 5-HT spontaneous firing activity. Expressed as a percentage of vehicle (vehicle = 100%), at 7 mg/kg UCM871, the number of bursts per 200s significantly increased to 241 ± 42.71%, $t(3)=-3.314$, $p=0.045$. The baseline percentage of spontaneous 5-HT neurons firing in bursts of 33.3% increased at 7 mg/kg UCM871 to 45.5%. The results for other doses are however summarized in the Appendix (**Appendix, Figure i**).

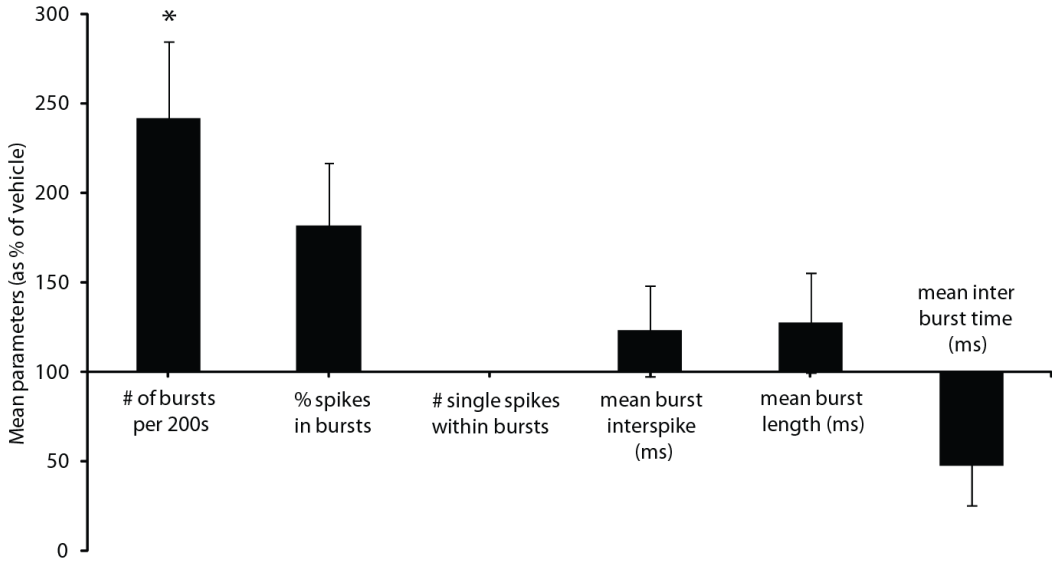
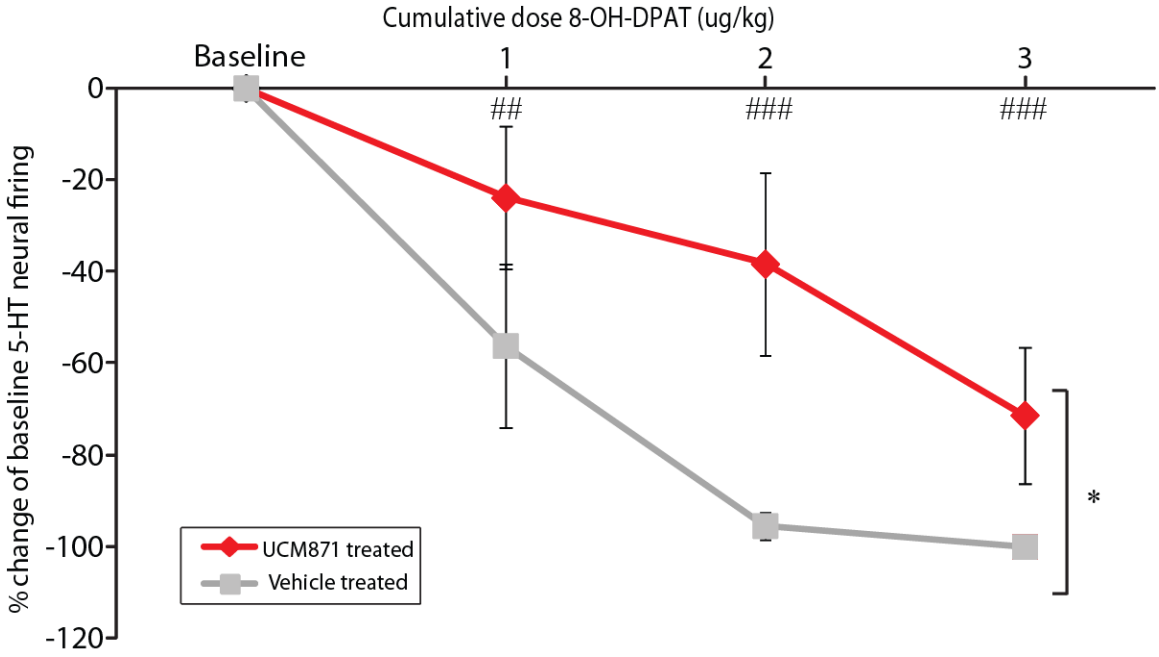


Figure 3. UCM871 modulates DR 5-HT burst activity. At 7 mg/kg UCM871, the number of bursts per 200s significantly increased. No other burst parameters differed significantly from vehicle. Data are expressed a percentage of vehicle and presented as mean ± SEM. Data from 4 bursting neurons. *p<0.05 vs. vehicle, paired samples t-test.

UCM871 attenuates response of 5-HT$_{1A}$ receptors

To analyze the effect of treatment with UCM871 on the response of DR 5-HT neurons to the inhibitory effect of the 5-HT$_{1A}$ agonist 8-OH-DPAT, increasing doses of 8-OH-DPAT were injected after UCM871. A two-way mixed design ANOVA (with treatment as a between subjects factor and dose of 8-OH-DPAT as a within subjects factor) revealed that the decrease on firing rate in DR neurons produced by 8-OH-DPAT after UCM871 treatment was significantly lower than after vehicle (effect of treatment: $F(1,11)=6.77$, $p=0.025$). We also found a significant effect of dose 8-OH-DPAT ($F(3,33)=24.61$, $p<0.001$), and a tendency for interaction ($F(3,33=2.65$, $p=0.06$) (see **Figures 2** and **4**, **Appendix Table i**). SNK multiple comparisons revealed that all 3 doses of 8-OH-DPAT significantly decreased the firing rate of DR 5-HT neurons compared to baseline ($p<0.01$), and the decrease produced by the dose of 3 and 2 was significantly greater than the decrease from the dose of 1 µg/kg ($p<0.001$, $p=0.004$, respectively).

Figure 4. UCM871 administration attenuated the response of 5-HT_{1A} receptors to the inhibitory effect of 8-OH-DPAT. Two-way mixed design ANOVA, followed by SNK multiple comparisons. *p<0.05 (UCM871 treatment vs. vehicle), ##p<0.01, ###p<0.001 dose 8-OH-DPAT vs. baseline. Data are expressed as percentage of baseline (post-treatment, pre-8-OH-DPAT) firing rate and presented as mean ± SEM. Data from 6-9 neurons per group.



UCM871 inhibited NE neuronal firing in the LC

To analyze the effect of increasing doses of UCM871 on spontaneous firing rate of NE neurons in the LC, doses ranging from 3.5 to 14 mg/kg were injected i.v. A one-way repeated measures ANOVA revealed a significant effect of treatment (**Figures 5** and **6**) ($F(4,26)=4.38$, $p=0.008$). SNK multiple comparisons test indicated that acute i.v. injection of UCM871 at 10.5 ($p=0.007$) and 14 ($p=0.037$) mg/kg significantly decreased the firing rate of NE neurons (10.5: -57.8 ± 11.5 %; 14: -45 ± 17.1 %) compared to the injection of vehicle (-8.4 ± 5.5 %). Lower doses of UCM871 had no effect compared to vehicle (3.5: -23.9 ± 14.3 %; 7: -34.3 ± 9.6 %).

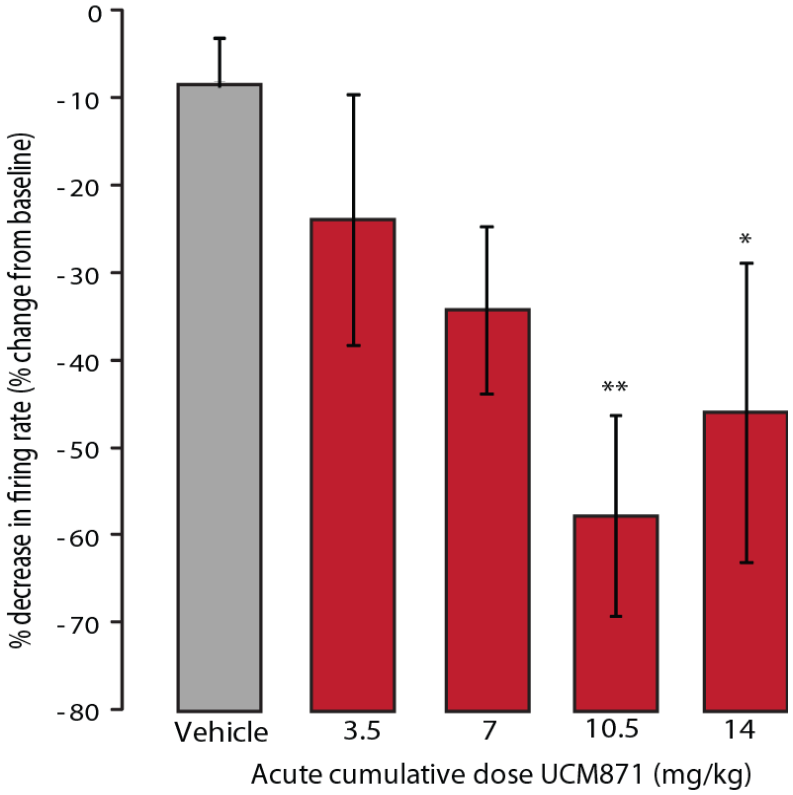


Figure 5. Acute UCM871 administration decreased LC NE neuronal firing activity. Acute i.v. injection of UCM871 at 10.5 and 14 mg/kg significantly decreased the firing rate of NE neurons from baseline compared to the injection of vehicle. Lower doses of UCM871 were not statistically different from vehicle. Data are expressed as percentage of baseline firing rate and presented as mean ± SEM. Data from 6-8 neurons per dose. 1-way repeated measures ANOVA for factor dose UCM871, followed by SNK multiple comparisons. *$p<0.05$ **$p<0.01$ vs. vehicle.

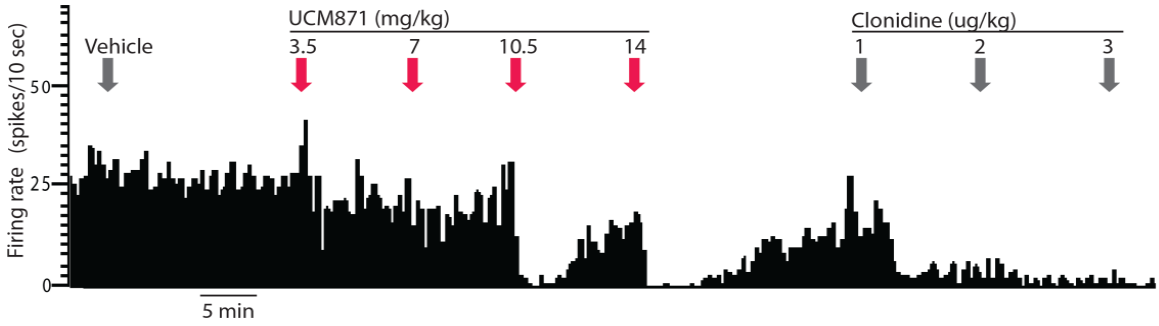


Figure 6. Sample histogram depicting changes in LC NE neuronal firing in response to UCM871 followed by clonidine. UCM871 transiently decreased spontaneous firing rate and clonidine further inhibited firing in LC NE neurons.

UCM871 decreased NE burst activity in the LC

Spontaneous firing LC NE neurons, at baseline, showed burst firing activity, discharging in bursts.

All burst parameters for the UCM871 dose response (**Figure 7, A-F**) were analyzed as percentage of vehicle response, where vehicle is 100% (raw data, untransformed into percentage from vehicle, is included in **Appendix Table iii**).

As the UCM871 dose increased, the mean number of bursts in 200s (**Figure 7A**) for increasing doses of UCM871 were as follows: 111.14 ± 27.5% at 3.5 mg/kg, 84.1 ± 9.3% at 7 mg/kg, 44.4 ± 13.8% at 10.5 mg/kg and 60.2 ± 22.8% at 14 mg/kg. A one-way repeated measures ANOVA revealed a significant effect of dose, $F(4,23)=4.01$, $p=0.013$. SNK post hoc tests showed that the dose of 10.5 mg/kg significantly decreased the number of bursts compared to the doses of 3.5 mg/kg ($p=0.013$) and vehicle ($p=0.033$). No other comparisons were significant.

As the UCM871 dose increased, the mean percent of spikes in bursts (**Figure 7B**) for increasing doses of UCM871 were as follows: 102.0 ± 16.5% at 3.5 mg/kg, 92.0 ± 7.8% at 7 mg/kg, 60.6 ± 12.2% at 10.5 mg/kg and 60.7 ± 20.7% at 14 mg/kg. A one-way repeated measures ANOVA revealed an overall effect of UCM871, $F(4,23)=3.77$, $p=0.017$. SNK post hoc tests showed that none of the doses significantly decreased the percentage of spikes in burst compared to baseline.

As the UCM871 dose increased, the mean number of single spikes within bursts (**Figure 7C**) for increasing doses of UCM871 were as follows: 98.8 ± 10.0% at 3.5 mg/kg, 95.2 ± 10.6% at 7 mg/kg, 84.5 ± 7.8% at 10.5 mg/kg and 106.3 ± 15.7% at 14 mg/kg. A one-way repeated measures ANOVA indicated no effects of UCM871 on the number of single spikes within bursts, $F(4,21)=0.94$, $p=0.46$.

As the UCM871 dose increased, the mean burst interspike (**Figure 7D**) for increasing doses of UCM871 were as follows: 100.4 ± 6.6% at 3.5 mg/kg, 100.6 ± 3.7% at 7 mg/kg, 90,3 ± 9.9% at 10.5 mg/kg and 104.7± 9.3% at 14 mg/kg. A one-way repeated measures ANOVA was conducted and indicated no effects of UCM871 on the mean burst interspike, $F(4,21)=0.63$, $p=0.65$.

As the UCM871 dose increased, the mean burst length (**Figure 7E**) for increasing doses of UCM871 was as follows: 98.6 ± 11.6% at 3.5 mg/kg, 96.7 ± 7.4% at 7 mg/kg, 83.1 ± 12.2% at 10.5 mg/kg and 103.3± 12.9% at 14 mg/kg. A one-way repeated measures ANOVA indicated no effects of UCM871 on the mean burst length, $F(4,21)=0.84$, $p=0.513$.

As the UCM871 dose increased, the mean inter burst time (**Figure 7F**) for increasing doses of UCM871 was as follows: 130.5 ± 30.9% at 3.5 mg/kg, 123.2 ± 15.3% at 7 mg/kg, 278.5 ± 85.2% at 10.5 mg/kg and 120.6± 19.6% at 14 mg/kg. A one-way repeated measures ANOVA revealed a significant effect of UCM871 treatment, $F(4,20)=3.41$, $p=0.028$. SNK post hoc tests found that the dose of 10.5 significantly increased the inter burst time compared to vehicle ($p=0.023$) as well as the doses of 7 ($p=0.036$), and 3.5 ($p=0.027$). No effects of 14 mg/kg were observed.

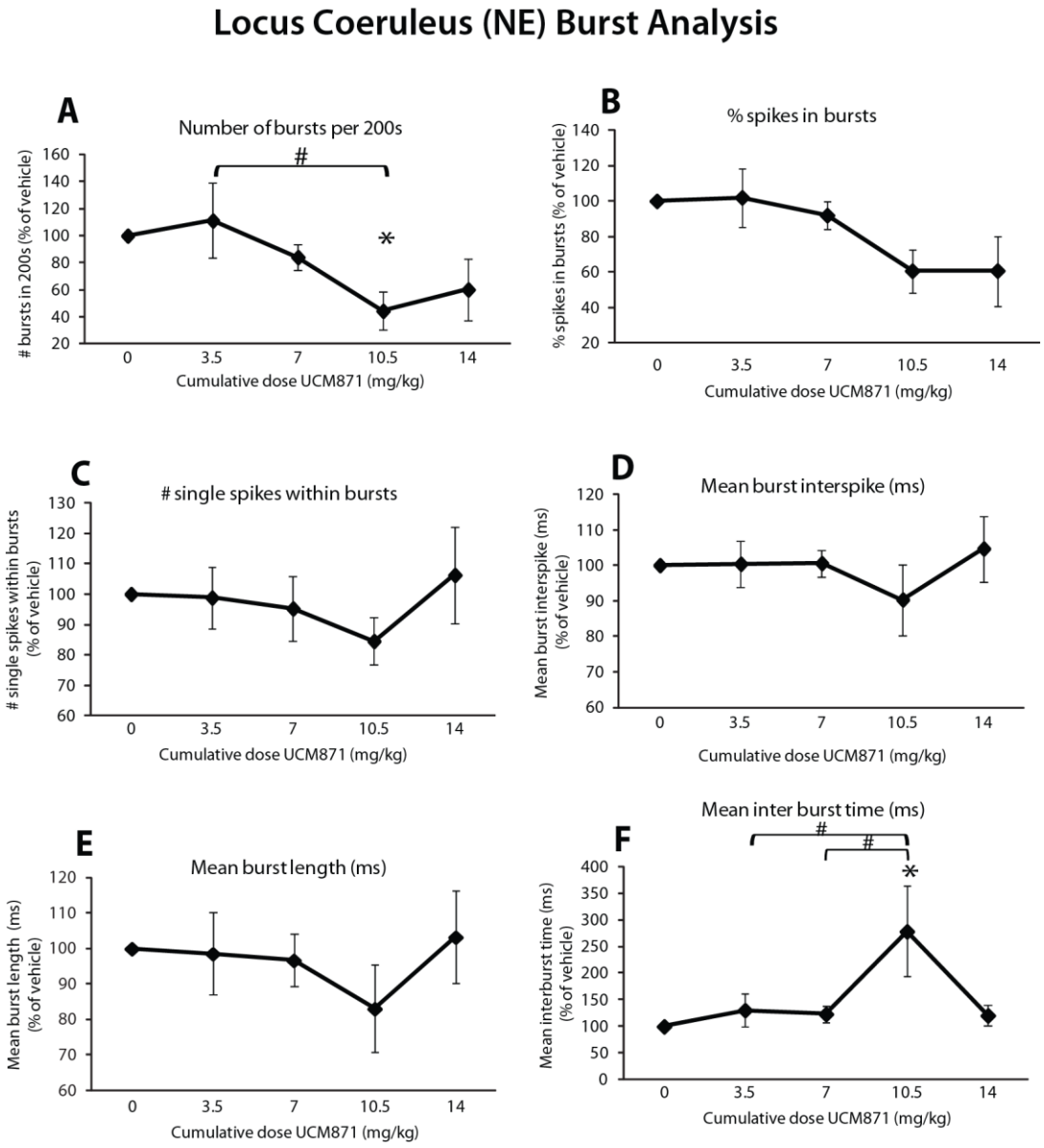


Figure 7 (A-F). UCM871 decreased LC NE neuronal burst activity at the dose of 10.5 mg/kg. Number of bursts in 200s (A) decreased significantly from vehicle at the dose of 10.5 mg/kg UCM871. % of spikes in bursts (B), number of single spikes within bursts (C), mean burst interspike (D), and mean burst length (E) did not display significant changes from vehicle across varying doses of UCM871, but all appeared to reach their lowest point at 10.5 mg/kg. Mean inter burst time (F) increased significantly at 10.5 mg/kg compared to vehicle, 3.5 and 7 mg/kg doses. Data are expressed as percentage of vehicle and presented as mean ± SEM. Data from 4-7 neurons per dose. *$p<0.05$ vs. vehicle, #$p<0.05$ between doses, 1-way repeated measures ANOVA for factor dose UCM871, followed by SNK multiple comparisons.

UCM871 increased response of NE alpha-2 autoreceptors of the LC

To analyze if UCM871 increased the response of alpha-2 autoreceptors, we injected increasing doses of the alpha-2 agonist clonidine. A two-way mixed design ANOVA (with treatment as a between subjects factor and dose of clonidine as a within subjects factor) revealed a significant interaction between treatment and dose of clonidine ($F(5,51)=9.05$, $p<0.001$), treatment ($F(1,13)=17.57$, $p<0.001$), dose clonidine $F(4,51)=17.71$, $p <0.001$ (see **Figures 6** and **8**, and **Appendix Table ii**). In neurons treated with UCM871, all doses of clonidine decreased firing rate compared to baseline ($p<0.001$ for all doses), and in neurons treated with vehicle, only the dose of 4 µg/kg decreased LC NE firing rate compared to baseline ($p=0.006$). Notably, the firing rate of NE neurons was lower in UCM871 vs. vehicle treated neurons after 1 µg/kg ($p=0.002$), 2 µg/kg ($p<0.001$), 3 µg/kg ($p<0.001$), 4 µg/kg ($p=0.011$).

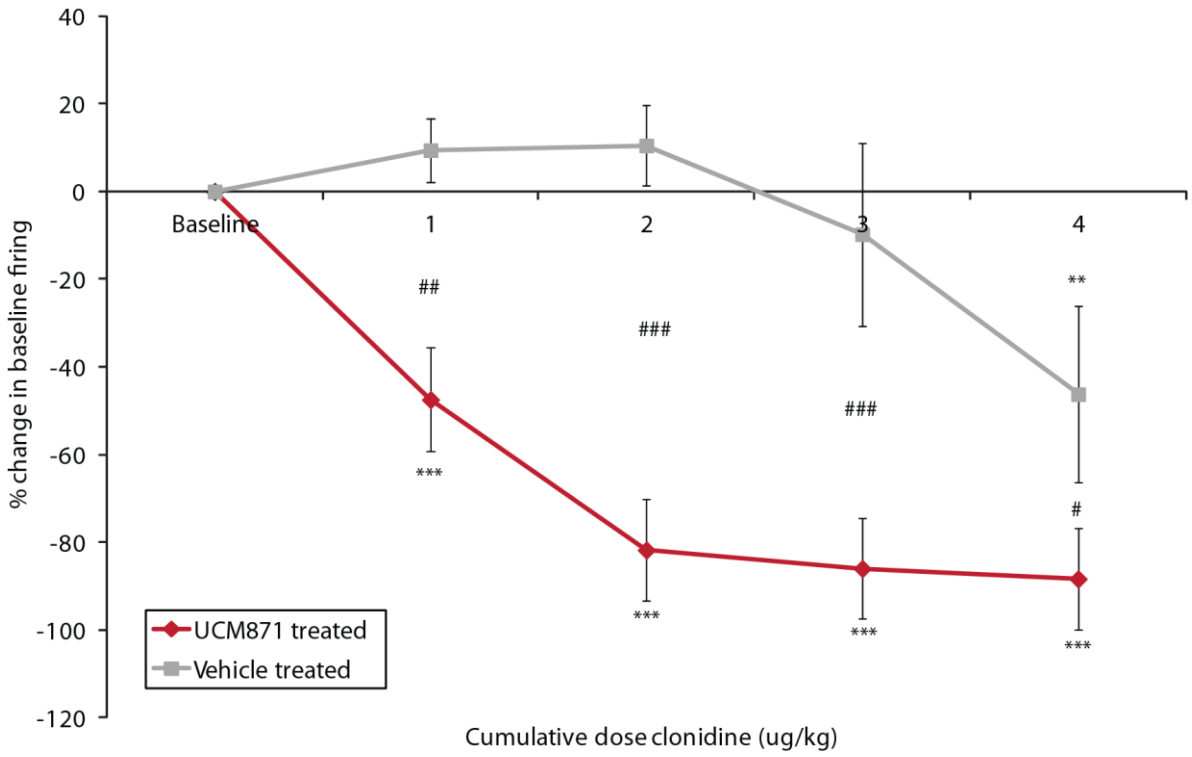


Figure 8. UCM871 increased the response of alpha-2 autoreceptors to the inhibitory effect of clonidine. * denotes a significant difference within a treatment group between indicated dose of clonidine and baseline. *p<0.05, **p<0.01, ***p<0.001. # denotes a significant difference between vehicle and UCM871 groups within a dose of clonidine where #p<0.05, ##p<0.01, ###p<0.001. 2-way mixed design ANOVA followed by SNK post hoc tests. Data from 7-8 neurons per group. Data are expressed as percentage of baseline (post-treatment, pre-clonidine) firing rate and presented as mean ± SEM.

Behavioural experiments

Effect of UCM871 in the Open Field Test

To assess the effect of UCM871 on locomotion and anxiety-like behaviours, the OFT was performed. From preliminary electrophysiological experiments, the active dose was in the range of 7-10.5 mg/kg, so included one dose lower (3.5) and one dose higher (14 mg/kg) in the dose response experiments.

Locomotion (Figure 9A)

To assess the effect of UCM871 on locomotion, we performed the OFT. A one-way between subjects ANOVA revealed a significant effect of dose, F(3, 46)=2.80,

p=0.05. SNK post hoc tests revealed that locomotion among group that received 7 mg/kg was significantly increased compared to the vehicle group (p=0.043), while higher and lower doses did not differ significantly from vehicle nor from each other.

As a positive control for experimental technique and conditions, the OFT and FST were conducted on groups receiving desipramine (10 mg/kg) and its control (saline), and a two-tailed independent samples t-test (**Figure 9A)** revealed that desipramine significantly decreased locomotion compared to saline, t(22)=2.88, p=0.009.

Number of entries and time in central area (anxiety-related) (Figure 9B,C)

We assessed the effect of UCM871 on the number of entries to the centre of the OFT (**Figure 9B**). A one-way between subjects ANOVA indicated no effect of treatment, F(3, 46)=1.72, p=0.18.

We assessed the effect of UCM871 on the time spent in the central area (**Figure 9C**). A one-way between subjects ANOVA showed that a tendency for an effect of UCM871 treatment was present, F(3,46)=2.56, p=0.066. Indeed, a two-tailed independent samples t-test between UCM871 (7 mg/kg) and vehicle revealed that UCM871 (13.8 ± 3.4) significantly increased the time spent in the centre compared to vehicle (6.3 ± 1.4), t(22)=-2.12, p=0.045.

A two-tailed independent samples t-test between desipramine and saline found no significant difference in number of entries (in fact the means were identical; saline 3.7 ± 0.8; desipramine 3.7 ± 0.9), t(22)=0, p=1 (**Figure 9B)**. A two-tailed independent samples t-test on the time spent in centre found no significant difference between desipramine (8.1 ± 2.2) and saline (4.8 ± 1.1), t(22)=-1.35, p=0.19 (**Figure 9C).**

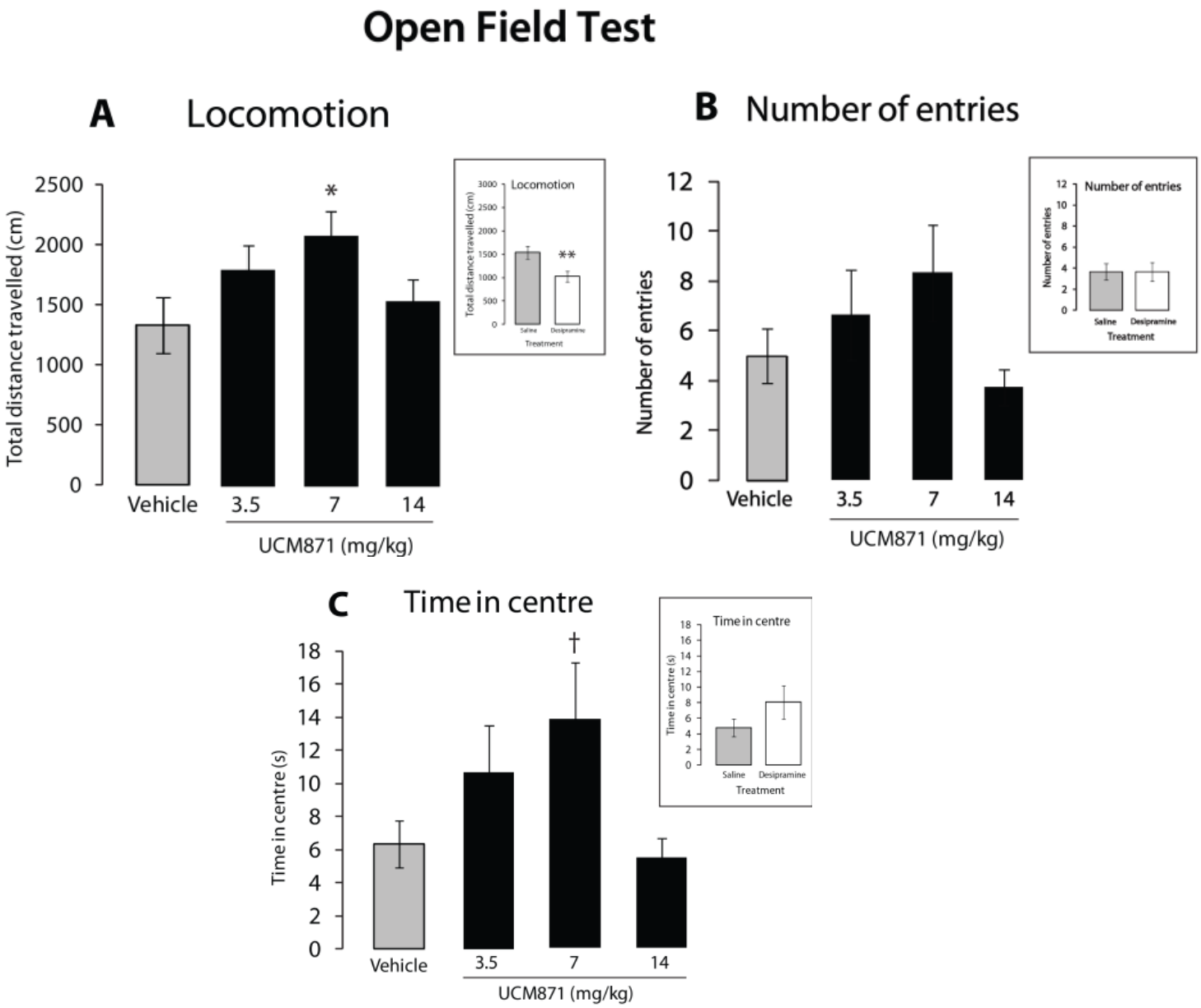


Figure 9 (A-C). UCM871 increased time in the centre and locomotion in the OFT. At the dose of 7 mg/kg, UCM871 increased locomotion (A) and time in the central area (C); additionally, the mean number of entries to the central zone was increased in the UCM871 treated group (B) but this did not reach statistical significance. In contrast, the positive control desipramine significantly decreased locomotion compared to saline (A) but had no effect on number of entries and time in centre (B and C). Dose-response produces a bell-shaped trend in the effect of increasing doses of UCM871, wherein it exerts its maximum effect on behaviour at 7 mg/kg (in agreement with preliminary electrophysiological data) and has no effect on behavioural parameters at the lower and higher doses of 3.5 and 14 mg/kg respectively. One-way between subjects ANOVA was used for dose response of UCM871, followed by SNK multiple comparisons. Independent samples t-tests were used to compare desipramine versus control. Data are presented as mean ± SEM. 11-14 animals per group. *$p<0.05$, **$p<0.01$. †$p=0.06$ (ANOVA), $p<0.05$ (t-test).

Effect of UCM871 in the Forced Swim Test (Figure 10)

To assess the effect of UCM871 in depressive like behaviour, the FST was conducted.

Immobility (Figure 10A)

We assessed the effect of UCM871 on immobility in the FST (**Figure 10A**). A one-way between subjects ANOVA revealed a significant effect of dose, $F(3, 34)=5.07$, $p=0.005$. SNK post hoc tests revealed that immobility among the group that received 3.5 mg/kg (212.2 ± 10.5 s) was significantly increased compared to the vehicle group ($p=0.012$), and also was increased compared to the 7 mg/kg group ($p=0.019$). The 14 mg/kg group (197.8 ± 8.6) did not differ from any other group.

A two-tailed independent samples t-test (**Figure 10A**) revealed that desipramine (137.5 ± 17.2) significantly decreased immobility in the FST compared to saline (197.6 ± 12.4), $t(14)=2.68$, $p=0.018$.

Swimming (Figure 10B)

We assessed the effect of UCM871 on swimming in the FST. A one-way between subjects ANOVA revealed a significant effect of dose, $F(3,34)=3.95$, $p=0.016$. SNK post hoc tests revealed that UCM871 doses of 3.5 (65.7 ± 9.7) and 14 (72.3 ± 6.7) mg/kg significantly decreased the amount of swimming ($p=0.022$ and $p=0.03$, respectively) compared to vehicle. No other comparisons were significantly different.

A two-tailed independent samples t-test revealed that desipramine significantly increased swimming (125.6 ± 14.1) compared to saline (81.0 ± 8.9), $t(14)= -2.50$, $p=0.026$ (**Figure 10B**).

Climbing (Figure 10C)

We assessed the effect of UCM871 on climbing in the FST. A one-way between subjects ANOVA revealed a significant effect of dose, $F(3,34)=6.15$, $p=0.002$. SNK post hoc tests revealed significant differences between 7 mg/kg dose and 3.5 (23.5 ± 3.5; $p=0.002$), vehicle ($p=0.007$), and 14 (34.0 ± 5.4; $p=0.021$) mg/kg doses. No other comparisons were significant.

A two-tailed independent samples t-test was conducted and revealed that desipramine (60.7 ± 10.2) significantly increased the amount of climbing (**Figure 10C**) compared to saline (24.7 ± 7.0), $t(14)=-2.72$, $p=0.017$.

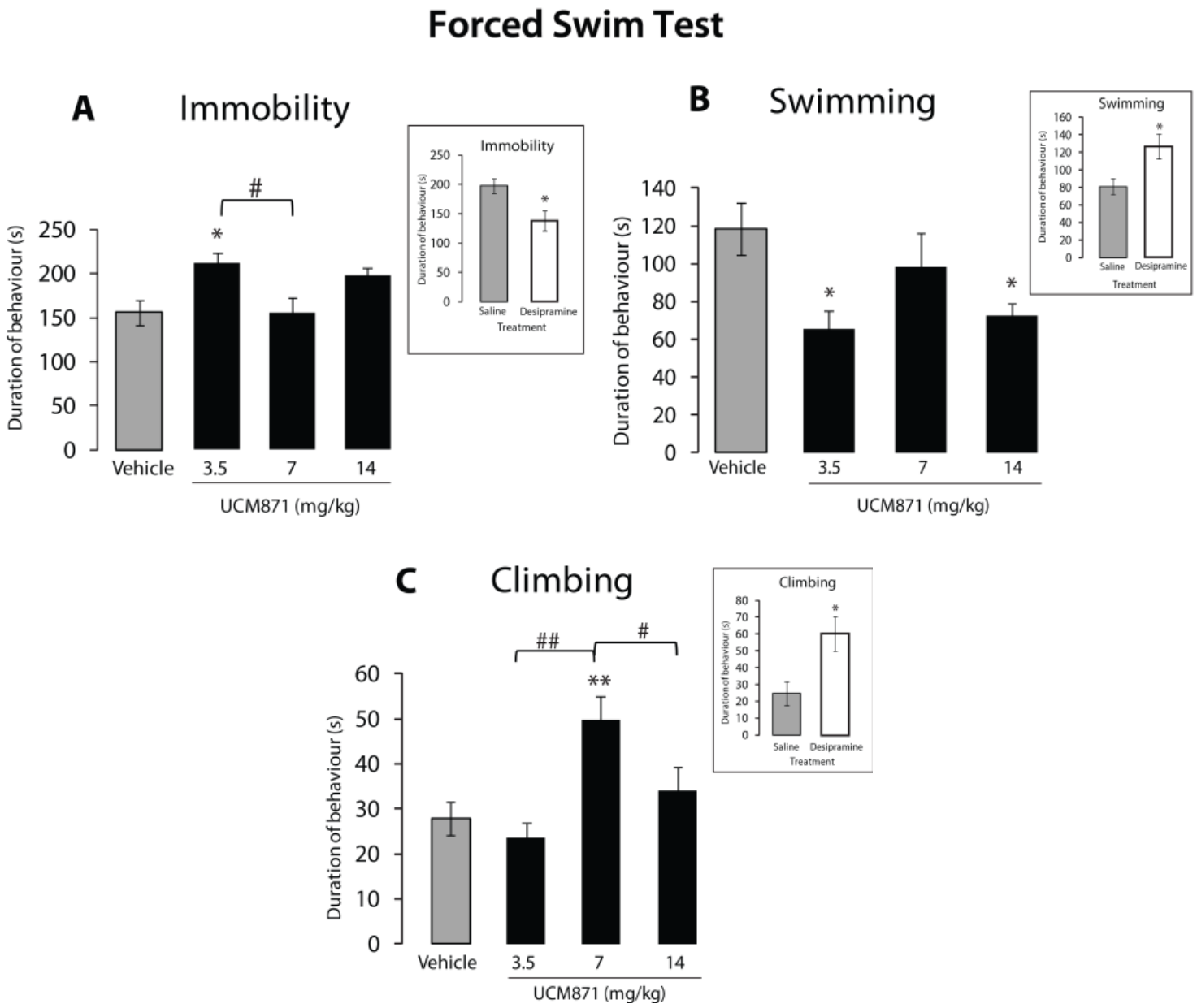


Figure 10 (A-F). UCM871 increased climbing in the FST but displayed a narrow therapeutic window. The dose of 7 mg/kg UCM871 that was active in electrophysiological experiments significantly increased climbing activity vs. control (C) but had no effect on immobility (A) or swimming (B) behaviours. The positive control desipramine decreased immobility (A), and increased both swimming (B) and climbing (C) in the FST. At the dose of 3.5 mg/kg, UCM871 increased immobility (A) compared to vehicle and 7 mg/kg, both 3.5 and 14 mg/kg decreased swimming (B) compared to vehicle, and decreased climbing (C) compared to 7 mg/kg but not compared to vehicle. Independent samples t-tests were used for comparisons between desipramine and control, and 1-way between subjects ANOVA was used for dose response experiments, followed by SNK multiple comparisons. Data are presented as mean ± SEM. Data from 7-10 animals per group. # denotes significant difference between doses of UCM871, # $p<0.05$, ##$p<0.01$. * denotes significant difference in effect from control, *$p<0.05$, *$p<0.01$.

Discussion

In this thesis we have explored the involvement of the melatonin MT_1 receptor in depression by examining the putative antidepressant-like properties of the novel selective MT_1 receptor partial agonist UCM871 in behavioural paradigms of depression and in the electrical activities of 5-HT and NE neurons. Overall, UCM871 at the dose of 7-10.5 mg/kg displays antidepressant-like properties through the modulation of 5-HT and NE neural activities. UCM871 increases climbing in the FST, has anxiolytic-like properties in the OFT, and increases DR 5-HT neural firing, paralleled by a decrease in LC NE neural firing. In addition, UCM871 prevents the activation of $5\text{-}HT_{1A}$ inhibitory autoreceptors, and increases the response of α-2 adrenergic autoreceptors. However, UCM871 dose-response curves in both FST and 5-HT firing activity show a bell curve response, with a depressive-like effect at lower and higher doses. Altogether, these results suggest that UCM871 at the therapeutic dose of 7 mg/kg displays similarities to current available antidepressant drugs, and therefore MT_1 receptor may represent a promising target for the development of novel antidepressant therapeutics.

Comparison of MT1 agonism vs. MT1 knockout mice

These results should be considered in the context of previous studies with MT_1 knockout mice, which have displayed a depressive-like phenotype and monoaminergic abnormalities at the level of 5-HT and NE neurotransmission. In particular, there are some important parallels between genetic blockage and pharmacological stimulation of MT_1 receptors.

First, we will compare the behavioural outcomes of genetic deletion of the MT_1 receptor versus MT_1 agonism.

Weil et al. (2006) showed that MT_1 knockout mice displayed increased immobility in the FST, and consequently, a decrease in the amount of active behaviours (combination of swimming and climbing). Comai et al. (2015) also confirmed the depressive-like phenotype of these mice in the TST and sucrose preference test. It should be noted that Comai et al. (2015) performed FST and TST in both light and dark phases, and found the significant depressogenic behaviour in MT_1 knockout mice was more pronounced in the dark phase. Therefore, further experiments in the dark phase should be

performed to examine whether UCM871 has stronger effects during the dark/active phase.

Very importantly, the depressive-like phenotype in MT_1 knockout mice was reversed by a chronic treatment (20 days) with desipramine, a tricyclic antidepressant acting mostly on the re-uptake of NE. In our experiments, we found that the pharmacological stimulation of MT_1 receptors by UCM871 (7 mg/kg) in rats resulted in an increase in climbing, the more vigorous of the two active behaviours and a function linked to NE activity (Page et al., 1999), though we did not observe a significant decrease in immobility in the FST at the doses of UCM871 we examined. These results suggest that the antidepressant-like effects of UCM871 are mostly mediated by NE, as suggested by the strong effects of UCM871 in noradrenergic firing activity.

Regarding anxiety, MT_1 knockout mice showed a mixed phenotype. In the novelty suppressed feeding test (NSFT), one of the more sensitive tests of anxiety measures, the knockout mice displayed increased latency to feed, representing an anxiogenic phenotype (Comai et al., 2015), while on the other hand MT_1 knockout mice showed increased time spent in the central zone of the the OFT, as well as increased time spent in the open arms in the elevated plus maze test (EPMT), representing decreased anxiety (Comai et al., 2015). However, it must be noted that in both the OFT and EPMT, the mice also displayed significantly increased locomotor activity, and this may partly account for the apparent "anxiety-resistant" phenotype. Moreover, in the OFT, no change in the number of entries to the central zone was found in KO mice, indicating that the increased time in the central zone was due to an increased locomotion and not to a real anti-anxiety effect.

After acute UCM871 (7 mg/kg), we found increased locomotion, paralleled by an increase in the time spent in the central area and a tendency of an increased number of entries to the central quadrant, suggesting potential anxiolytic effects. However, to further establish this property, the NSFT and the EPMT will help elucidate its putative anxiolytic activity.

Comai et al. (2015) studied DR 5-HT neural activity in MT_1 knockout mice and found no differences in firing rate compared to wild type mice, in neither light nor dark phases. Dividing the total population of 5-HT neurons into low and high firing subgroups

revealed that, in the high firing subgroup, whereas an increased firing rate was observed in wild type mice in the dark phase, MT_1 knockout mice showed no such increase, indicating an impaired circadian rhythm of 5-HT neurons induced by MT_1 receptor deletion. Importantly, DR 5-HT burst activity is likely reduced in these knockout mice.

We studied the effect of acute UCM871 administration on 5-HT neuronal activity in the DR during the light phase. We observed that UCM871 produces a bell-shaped dose-response curve in the firing activity of 5-HT neurons, with a peak excitatory dose of 7 mg/kg.

According to the literature (Bambico et al., 2009), only 15-20% of 5-HT neurons recorded spontaneously discharge in bursts, in our sample we confirmed that only 25% discharged in bursts. We thus analyzed the effect of UCM871 at 7 mg/kg on burst activity and found an increased number of bursts compared to vehicle. In parallel, the genetic deletion of MT_1 receptors (Comai et al., 2015) reduced 5-HT burst activity. While the MT_1 knockout mice did not display markedly lower firing rate compared to wild type mice, they showed a blunted diurnal variation in the firing rate of the high firing neuronal subgroup. UCM871 significantly increased 5-HT neuronal firing rate during the light phase; experiments in the dark phase should be performed to examine whether 5-HT neurons respond differently to acute challenge with UCM871 during the dark phase.

In the LC, MT_1 knockout mice (Comai et al., 2015) displayed no change to the overall NE neuronal firing rate, but, as was observed among DR 5-HT neurons, in the high-firing subgroup, MT_1 knockout mice displayed a blunted diurnal rhythm of LC NE firing activity, without the dark-phase increase in firing that was observed in wild type mice. Strikingly, MT_1 knockout mice displayed altered LC NE burst activity: the percentage of spikes in bursts, the mean burst interspike and mean burst length were lower during the dark phase, and the light/dark pattern of percentage of spikes in burst was different than that of WT controls. Here, we studied the effect of acute UCM871 administration on LC NE neuronal firing and burst activity. We observed a decrease in LC NE neuronal firing in response to increasing doses of the MT_1 receptor partial agonist UCM871. As firing decreased, we observed a parallel decrease in burst activity, in number of bursts, % of spikes in bursts, and an increase in mean interburst time. Again,

experiments in the dark phase should be performed to examine whether these changes in NE firing and burst activity in response to UCM871 change according to phase of day.

Comparing results from MT_1 agonism against the results from MT_1 genetic deletion (Comai et al., 2015), we notice several opposing effects, as might be expected when comparing the pharmacological agonism vs. the genetic antagonism (**Table 1**)

For example, MT_1 knockout mice display increased immobility in the FST i.e. a decrease in active behaviours whereas UCM871-treated rats display an increase in climbing behaviour, and MT_1 knockout mice show a decrease in burst activity in DR 5-HT neurons whereas UCM871 treatment results in an increase in burst activity.

	MT_1 Knockout mice	UCM871, acute
Depressive-like behaviour	Depression during the dark in TST, sucrose consumption, during both phases in FST	Antidepressant (?) activity (increased climbing, no effect on immobility) with the dose of 7mg/kg in FST
Anxiety-like behaviour	Anxiogenic in NSFT, anxiolytic in EPM and OFT (likely due to increase locomotion)	Anxiolytic in OFT, increased time in centre and locomotion with a tendency to increase central entries
5-HT firing activity	Impaired circadian rhythm in high firing neurons and a trend for decreased burst activity.	Increase 5-HT firing and burst activity at 7mg/kg, with attenuated $5\text{-}HT_{1A}$ receptor response
NE firing activity	Decreased burst in dark phase, impaired circadian rhythm in high firing subgroup	Decreased firing and burst activity, with increased response of alpha-2 autoreceptors

Table 1. Comparison of depressive and anxiety like behaviour and 5-HT and NE firing activity between MT_1 deletion and acute UCM871 treatment.

However, there are also cases where they show similar effects (**Table 1**). For example, both knockout mice and UCM871-treated rats increase locomotion and time spent in the centre of the OFT, and both display a decrease in burst activity in the LC. These similarities may result from differences in the experimental model; UCM871 is

administered acutely and thus activates MT_1 receptors acutely, while the MT_1 knockout mice represent a more "chronic" model of MT_1 receptor inhibition. Experiments aimed at assessing chronic effects of UCM871 may help to better explain these partial discrepancies. However, as many knockout strains are developmentally lethal, these adult MT_1 knockout mice may have adapted compensatory mechanisms in their development to accommodate for the absence of MT_1 that might make them less suitable for comparison to pharmacologically altered rodents in some ways. One solution for this difference in models might be employing a conditional MT_1 receptor knockout strain so as to induce the deletion of MT_1 receptor only in adult mice, thus minimizing the possibility that other compensatory systems might become involved earlier in development.

Comparison of UCM871 and other classes of antidepressants in the electrophysiology responses of 5-HT and NE neurons

Next, it is important to examine our findings of acute changes to DR 5-HT and LC NE neurotransmission in the context of the current understanding of the relationships of the serotonergic and noradrenergic systems to each other as well as to MLT, and to compare our findings with mechanisms of antidepressant action.

MLT regulates SCN activity via its two receptors, MT_1 and MT_2. Liu et al. (1997) found that MT_1 mediates the acute inhibitory effect of MLT on SCN firing rate. Validating that MT_1 is involved in regulating the activity of the SCN, Wu et al. (2007) found that MT_1 levels decrease in the SCN in aging and in late stage Alzheimer's disease. Furthermore, Wu et al. (2013) in a post mortem study found that expression levels of MT_1 (but not MT_2) were increased in the SCN of depressed patients. Importantly, the SCN is known to project to both DR and LC (Deurveilher and Semba, 2005, Aston-Jones et al., 2001), influencing the activity of the neurotransmitters 5-HT and NE, respectively, which have long been implicated in the pathophysiology of depressive disorders (Gobbi and Blier, 2005). The neuromodulatory effects we observed from UCM871 may result from stimulation of MT_1 receptors in the SCN, direct stimulation of the DR/LC, activity at other sites, and/or likely a combination of all these.

In our experiments, we found that acute administration of UCM871 increases 5-HT activity in the DR. SSRIs are considered to be the gold standard first-line treatment for depressive and also anxiety disorders. They exert therapeutic effects by blocking 5-HT reuptake, thereby increasing the net output of 5-HT neurons (Gobbi and Blier, 2005).

Acute SSRI administration initially enhances extracellular 5-HT resulting in a decrease in firing rate of 5-HT neurons due to negative feedback on 5-HT_{1A} autoreceptors, however, after chronic treatment, the autoreceptors attenuate their response and 5-HT firing rate returns to normal levels, thus potentiating the 5-HT neurotransmission as a final outcome (**Figure 11 and Table 2**) (Manta et al., 2009, Blier and de Montigny, 1999). This mechanism underlies the delay in antidepressants response observed in humans.

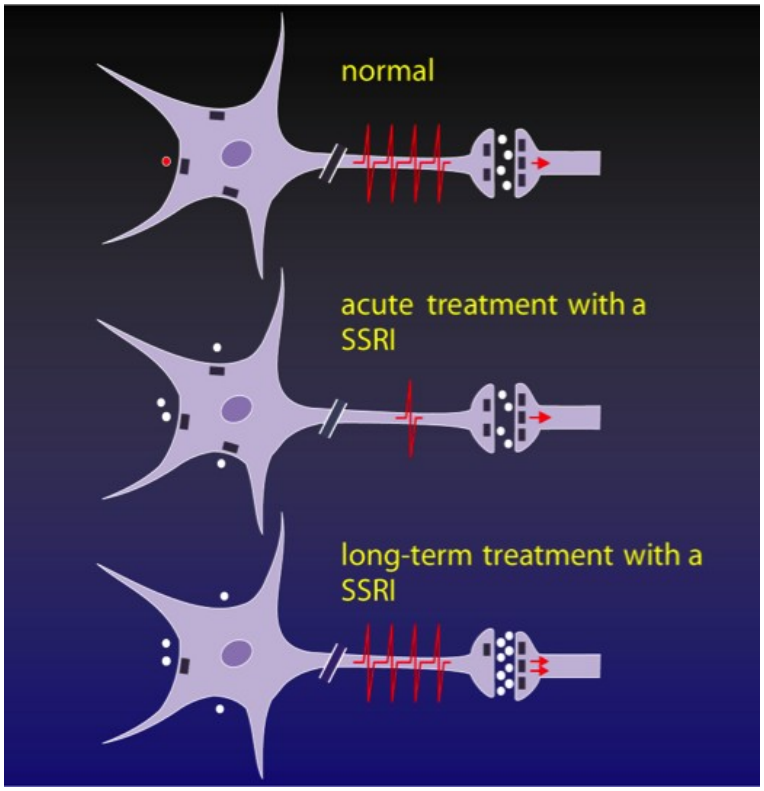


Figure 11. Effect of acute and long-term treatment with SSRI on 5-HT neurons. *Image courtesy of Dr. P. Blier.*

Here, we reported that acute UCM871 administration increases 5-HT neuronal firing and burst activity up to a peak activity around 7 mg/kg (i.v.), and then, at higher doses, begins to decrease firing back to near basal levels. Remarkably, using the 5-HT_{1A}

agonist, 8-OH-DPAT, we found that, like other classes of antidepressants (**Table 2**), UCM871 attenuated the response of 5-HT_{1A} receptors and increased 5-HT firing rate and burst activity, but unlike other antidepressants, it did so after acute treatment. This important difference suggests that UCM871 can be considered a rapid antidepressant, similarly to the recent rapid effects observed with ketamine (Machado-Vieira et al., 2009). Moreover, the rapid increase in 5-HT firing rate following UCM871 administration (7mg/kg) could be compared to the effects of the antidepressant mirtazapine (Haddjeri et al., 1998), NK1 antagonists (Santarelli et al., 2001), and vagus nerve stimulation (Manta et al., 2009) (**Table 2**).

The return of 5-HT firing rate to near baseline at higher doses of UCM871 may reflect the negative feedback activity of 5-HT_{1A} autoreceptors on DR 5-HT firing, or may reflect the partial agonist properties of UCM871, wherein at higher doses it could indeed act as an antagonist. The acute use of a selective MT_1 receptor antagonist may aid in elucidating this mechanism in the future. Chronic experiments with UCM871 will be critical in determining the effect of UCM871 on DR 5-HT firing and burst activity and on 5-HT_{1A} autoreceptors after prolonged treatment.

	Responsiveness of somatodendritic 5-HT_{1A} autoreceptors	Function of terminal 5-HT_{1B} autoreceptors	Function of terminal a_2-adrenergic heteroreceptors	Responsiveness of postsynaptic 5-HT_{1A} receptors	Net effect on 5-HT neurotransmission
SSRI	↓	↓	O	O	↑
MAOI	↓	O	↓	O or ↓	↑
5-HT_{1A} agonists	↓	O	n.d.	O	↑
TCA	O	O	↓	↑	↑
ECS	O	O	O	↑	↑
Mirtazapine	↓	O	↓	O	↑
APP	O	?	?	↑	↑

Table 2. Effects of long-term administration of antidepressant treatments on 5-HT neurotransmission. *Modified after Gobbi and Blier, 2005.*

The majority of NE innervation in the forebrain derives from projections of the LC, and the LC projects to the DR. Inhibitory somatodendritic and terminal alpha-2 adrenergic autoreceptors regulate NE firing and release, respectively (Svensson et al., 1975, Curet and de Montigny, 1989). NRIs result in increased extracellular levels of NE, which overactivate the alpha-2 receptors and decrease LC NE firing (Lacroix et al., 1991, Kasamo et al., 1996, Mongeau et al., 1998, Béïque et al., 2000a, Szabo and Blier, 2001). Chronic administration of NRIs desensitizes only the terminal alpha-2 autoreceptors but not the somatodendritic receptors, and, despite decreased firing activity, this results in an overall increase in forebrain NE levels and thus antidepressant action (Manta et al., 2009). The LC NE neurons project to the DR, where they release NE, activating excitatory somatodendritic alpha-1 adrenoreceptors on 5-HT neurons to increase neuronal firing (Baraban and Aghajanian, 1980). The NE neurons in the LC are themselves regulated by 5-HT projections from the DR (Kaehler et al., 1999, Aston-Jones et al., 1991a) and also from pericoerulear 5-HT neurons. 5-HT primarily serves a modulatory function on LC neurons, selectively filtering NE afferents, and regulating their response to other inputs rather than directly increasing or decreasing their firing themselves (Aston-Jones et al., 1991b). More recent studies have indicated that the DR can regulate NE neurons in the LC through 5-HT_{2A} receptors located in the GABAergic interneurons within LC (Blier et al., 2004) (**Figure 12**).

Very importantly, the decrease in LC NE activity we observed after acute treatment with UCM871 is similar to a decrease in NE activity that has been well characterized in other classes of antidepressants including SSRIs, e.g. paroxetine (Szabo et al., 2000), SNRIs, e.g. venlafaxine (Béïque et al., 2000b), and NRIs, e.g. reboxetine (with acute and chronic administration in reboxetine) (Szabo and Blier, 2001, Gobbi and Blier, 2005), suggesting that UCM871 likely shares pharmacological characteristics with other antidepressants (**Table 3**). High doses of the SNRI venlafaxine acutely (2 day treatment) reduces DR 5-HT firing activity, which is restored back to control levels after 21 days of treatment, and also decreases LC NE firing activity, but this does not recover after sustained treatment (Béïque et al., 2000b); importantly, at lower doses, venlafaxine appears to induce predominantly 5-HT related, rather than both 5-HT and NE related effects, and thus is similar to classical SSRIs in the long-term. SSRIs such as citalopram

and paroxetine have been shown to tonically inhibit LC NE firing but only after chronic treatment (Szabo et al., 1999, Szabo et al., 2000). Similarly, we found that acute UCM871 administration dose-dependently decreased NE firing in the LC.

ANTIDEPRESSANT CLASS	ACUTE	LONG-TERM
MAOI	↓	↓
TCA	↓	↓
NE REUPTAKE INHIBITOR	↓	↓
α-2 ADRENERGIC ANTAGONIST (e.g. mirtazapine)	↑	↑
DUAL NE/5-HT REUPTAKE INHIBITORS	↓	↓
SSRIs	=	↓
ATYPICAL ANTIPSYCHOTIC (AAP)	↑	↑
NE RELEASER	↓	ND

Table 3. Effects of long-term administration of antidepressants on NE neurotransmission. *Modified after Gobbi and Blier, 2005*

Nevertheless, unlike the majority of established antidepressants, UCM871 induced neuromodulatory effects (as well as behavioural changes) after acute administration (almost immediately in electrophysiological experiments, and in less than an hour in behavioural experiments). These data again, suggest the potential rapid antidepressant effect mediated by MT_1 receptor ligands.

Lacroix et al. (1991) demonstrated that chronic (14 day, 10 mg/kg per day) desipramine administration yielded a significant shift to the right of the dose-response curve of clonidine, an alpha-2 adrenoreceptor agonist, in suppressing LC firing rate i.e. the inhibitory effects of clonidine were reduced following chronic desipramine treatment. This effect was not found after chronic (21 day) reboxetine treatment (Szabo and Blier,

2001), wherein the inhibition of clonidine on LC NE neurons was similar between chronic reboxetine vs. saline treatment, therefore reboxetine did not lose inhibitory effects in LC NE neurons in the long-term; acute (2d) reboxetine also decreased LC NE firing. Mirtazapine is a unique antidepressant, acting by blocking the alpha-2 adrenergic auto and heteroreceptors responsible for NE and 5-HT release, as well as having a low affinity for 5-HT_{1A} receptors and blocking 5-HT_2 and 5-HT_3 receptors rather than blocking NE or 5-HT reuptake (de Boer, 1995). Chronic treatment inactivates alpha-2 heteroreceptors and enhances the tonic activation of postsynaptic 5-HT receptors due to the sustained increase in 5-HT activity (Haddjeri et al., 1995). Mirtazapine is also unique in that it acutely increases both LC NE and DR 5-HT neurotransmission, by activating noradrenergic pathways and indirectly enhancing tonic activation of 5-HT receptors, and is associated with anxiolytic and sleep-improving effects (de Boer, 1995) (**Table 3**).

In our study, we observed that UCM871 acutely decreased LC NE neuronal firing and burst activity. UCM871 therefore displayed pharmacological similarities to antidepressants such as SSRIs, reboxetine and venlafaxine in its acute ability to modulate NE-neurotransmission, and, like mirtazapine, in its acute 5-HT neuromodulatory effects. Moreover, we observed that acute UCM871 shifted the clonidine dose-response curve significantly to the left, indicating a significant increase in the responsiveness of inhibitory alpha-2 adrenoreceptors. The mechanism by which the increased response to clonidine occurs may be due to the decrease in NE release, making alpha-2 receptors immediately available to activation by the agonist clonidine, and/or due to the upregulation of the receptor in response to the decrease in NE; at present, the affinity of UCM871 for alpha-2 adrenoreceptors is not known. Chronic experiments will examine the long-term effects of MT_1 receptor agonism by UCM871 on LC NE firing rate and alpha-2 receptor activity and sensitivity.

In summary, UCM871, differently from most other classes of antidepressants, acutely increased DR 5-HT activity, but decreased LC NE activity, additionally increasing responsiveness of alpha-2 autoreceptors and resulting in anxiolytic-like effects and an increase in climbing in the FST. Overall, UCM871 did display many pharmacological similarities to antidepressants such as SSRIs, reboxetine and

venlafaxine, and mirtazapine in its ability to acutely modulate NE and 5-HT neuronal activity.

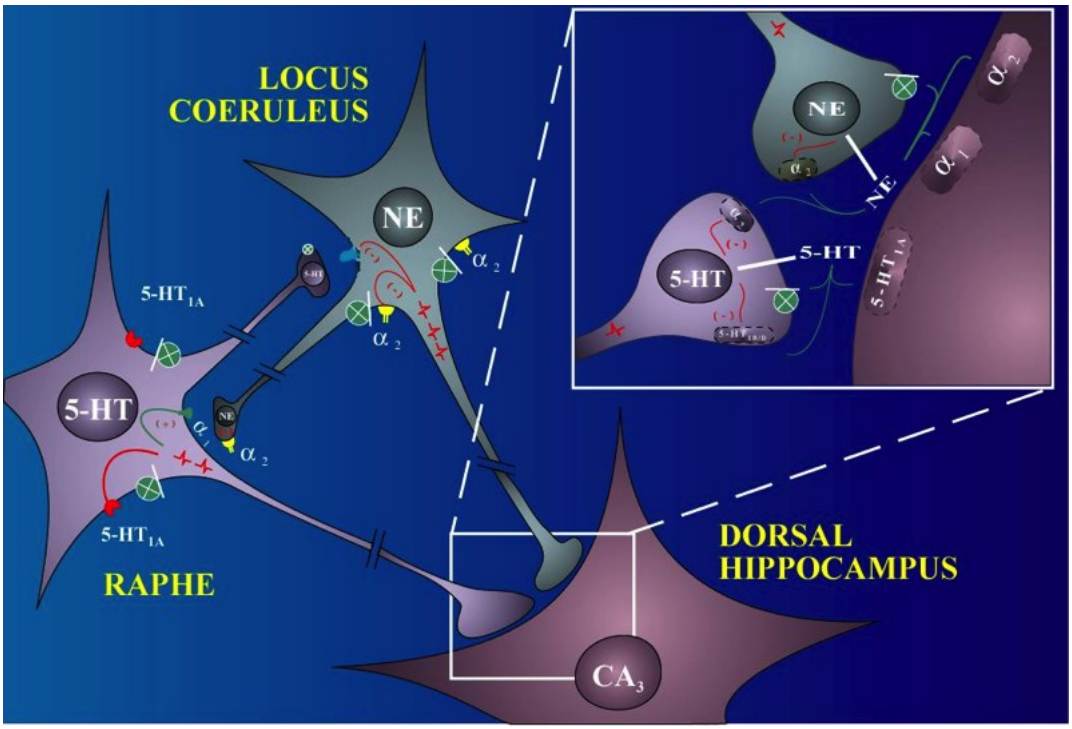


Figure 12. Relationship of 5-HT and NE neurons in the DR and LC.
Image courtesy of Dr. P. Blier.

One may also hypothesize a novel mechanism for UCM871: it may directly activate MT_1 receptors on 5-HT neurons, enhancing 5-HT neuronal firing and release, which, as with chronic SSRIs, may in turn lead to reduced LC NE firing, albeit much more rapidly than is seen with SSRIs.

Unpublished data from our lab showed that the selective MT_2 receptor partial agonist UCM765 had no effect in the FST, nor did it modify DR 5-HT firing activity after a single injection in electrophysiological experiments. This further supports the specific role of MT_1 in mood disorders and validates it as a target for development of novel antidepressant therapeutics in the future.

Comparison of UCM871 vs. agomelatine

Finally, we aim to examine our results in the context of agomelatine, the only commercially available MLT-based antidepressant. The MT_1/MT_2 receptor agonist and 5-

HT_{2C} receptor antagonist agomelatine (Valdoxan®, Servier) is the first melatonergic acting compound to be marketed for the treatment of MDD. The development of agomelatine was based on the evidence that prolonged treatment with antidepressants including desipramine, clomipramine and fluoxetine modify the distribution of MLT receptor mRNA in the CNS. Most of the antidepressants examined were revealed to specifically increase the amount of MT_1 but decrease the amount of MT_2 receptor mRNA in the hippocampus (Larson et al., 2006, Hirsch-Rodriguez et al., 2007). Agomelatine is a naphthalene derivative; the naphthalene ring is more lipophilic than the indole of MLT, and in its development, researchers aimed to achieve improvement in penetration to the brain (de Bodinat et al., 2010). Remarkably, the antidepressant agomelatine has higher affinity for MT_1 (K_i=6.15x10^{-11} M) than for MT_2 (2.68x10^{-10} M) or 5-HT_{2c} (IC_{50}=2.7x10^{-7} M) receptors (de Bodinat et al., 2010), even though its antidepressant activity seems related to its multi-activity on MT_1, MT_2, 5-HT_{2C} and 5-HT_{2B} receptors (Chenu et al., 2014a). It has no detectable affinity to adrenergic, dopaminergic or other receptor types (Rouillon, 2006). Like MLT itself, agomelatine acts as an agonist to the MLT receptors, suppressing cAMP formation, and it dose dependently inhibits SCN firing, thereby exerting its chronobiological effects (de Bodinat et al., 2010). In behavioural tests, both acute and chronic agomelatine administration was shown to induce antidepressant-like effects in rats, but in mice, only long term administration yielded a significant antidepressant like effect in the FST (Bourin et al., 2004). In the learned helplessness behavioural model of depression, agomelatine only showed significant antidepressant-like effects after long-term administration (Bertaina-Anglade et al., 2006). The antidepressant capacity of agomelatine was shown to be blocked by a single administration of the $MT_{1/2}$ antagonist S22153, demonstrating that the melatonergic activity contributes to these antidepressant-like effects (Chenu et al., 2013). In humans, clinical trials comparing agomelatine against placebo (Kennedy and Emsley, 2006) and active antidepressants such as venlafaxine (Lemoine et al., 2007), sertraline (Kasper et al., 2010), and fluoxetine (Hale et al., 2010) found it to display comparable antidepressant efficacy. There is evidence to suggest that the overall antidepressant effects are mediated via its combined functions as both a MLT receptor agonist and as a 5-HT_{2C} antagonist. While MLT, the endogenous MT_1 and MT_2 agonist, itself has not been shown to have

antidepressant properties in human MDD patients, some antidepressant capabilities have been observed in rodents (Dalton et al., 2000, Dolberg et al., 1998). Until recently, a 5-HT_{2C} antagonist, while having shown the ability to increase NE and DA in rodent prefrontal cortex (Gobert et al., 2000), had not independently been demonstrated to elicit measureable antidepressant-like effects; however, one very recent study found that 5-HT_{2C} antagonists did in fact exert antidepressant actions with a fast (5 day) onset, acting through enhancing mesocortical dopamine signaling (Opal et al., 2014); future work examining specific activity in dopaminergic areas including the VTA will be valuable in understanding these putative antidepressant pathways. In addition, some antagonist activity at the 5-HT_{2B} receptor has been observed, wherein blocking only 5-HT_{2C} has not been observed to effect LC NE firing rate, but blocking both 5-HT subtypes dose-dependently increases LC neuronal firing (Chenu et al., 2013). The proposed putative mechanism of agomelatine action involves a dose dependent extracellular enhancement of DA and NE in the prefrontal cortex, and indirect action on 5-HT activity. Importantly, acute administration of agomelatine was shown to dose dependently increase the firing rate of NE neurons (Millan et al., 2003). The combination of melatonergic and other monoaminergic pathways therefore contribute to the efficacy of agomelatine as an antidepressant.

In detail, agomelatine was found to exert direct and indirect modulation of monoaminergic neuronal activity; after short term (2 day) treatment, agomelatine increased DA activity in the ventral tegmental area (VTA) (measured as an increase in the number of spontaneously active neurons), and also increased LC NE firing rate, while having no acute effect on DR 5-HT neuronal activity; after chronic (14 day) treatment however, agomelatine caused an increase in the number and burst activity of spontaneously active VTA DA neurons, increased DR 5-HT firing through a dopamine D2 receptor mechanism (as well as excitatory inputs from NE neurons via alpha-1 adrenoreceptors), and dampened LC NE firing back to regular levels due to the enhanced negative feedback of upregulated 5-HT neurotransmission (Chenu et al., 2013).

We found that acute UCM871 administration increased DR 5-HT firing and bursting activity, and in parallel decreased LC NE firing and bursting activity. Even though chronic experiments with UCM871 still need to be performed in order to have a

clearer neurobiological framework of the mechanism through which MT_1 receptor agonism modulates monoaminergic activity, several similarities between the effects of UCM871 and agomelatine have already been established. However, the robust suppression of LC NE firing by UCM871 was not observed with agomelatine, likely due to the higher and selective affinity of UCM871 for MT_1 receptors compared to agomelatine.

Some antidepressant drugs have also been shown to modulate DA neurotransmission (Blier, 2013), and therefore the finding that the 5-HT component of agomelatine action is mediated via DA neurons emphasizes the need for acute and chronic experiments examining the effect of UCM871 also on DA neurons in the VTA.

Summary and Conclusions

Here, we have investigated the putative effects of the MT_1 receptor on mood regulation using the novel selective MT_1 receptor partial agonist, UCM871 in electrophysiological and behavioural paradigms. Remarkably, we found that acute intravenous administration of UCM871 induced potent 5-HT and LC neuromodulatory effects. Increasing doses of acute UCM871 treatment showed a bell-curve effect on firing of 5-HT DR neurons, increasing firing at lower doses and decreasing firing at higher doses, and also increased the number of bursts at its active dose (7 mg/kg, i.v.). We found that treatment with UCM871 attenuated the response of $5\text{-}HT_{1A}$ receptors to the inhibitory effect of 8-OH-DPAT. In NE LC neurons, we found that increasing doses of UCM871 decreased firing rate and burst activity in a dose-dependent manner. We observed that UCM871 rapidly decreases LC NE neuronal firing via increasing the responsiveness of alpha-2 adrenergic autoreceptors. The dose of UCM871 that produced the peak of 5-HT firing (7 mg/kg) also was shown to increase the amount of climbing activity in the FST with no effect on immobility. Contrastingly, doses of UCM871 that did not induce changes in 5-HT firing produced depressive-like effects in FST (decreased immobility). In the OFT, UCM871 at 7 mg/kg showed an anxiolytic-like effect, increasing the time in the centre and also increasing locomotion.

To further elucidate the effects of UCM871 in mood regulation, future electrophysiological experiments will examine the effects of UCM871 on DA VTA neurons, and chronic experiments will be performed in behaviour as well as in electrophysiological effects on the neural activity of DR, LC and VTA. Additionally, due to the changes in the MLT system according to the circadian rhythm, these experiments should be performed in the dark phase to determine whether the effects of UCM871 differ according to phase of day. Future behavioural experiments will examine anxiety (elevated plus maze test, novelty suppressed feeding test), cognition (object recognition, water maze), anhedonia (sucrose preference test) and other behaviours involved in the complex depressive phenotype to further elucidate the precise role of MT_1 receptors. Additionally, we will examine the effects of UCM871 on cell signalling pathways to better understand its biochemical activity.

Overall our data shows that the selective melatonin MT_1 ligand UCM871 shares neurobiological features with other classes of antidepressants including increased 5-HT activity, decreased NE activity, increased climbing in FST and an increased time spent in the central area in the OFT. However, we have also found that UCM871 possesses a narrow therapeutic window, as high and lower doses resulted in neutral or opposite activity. This effect may in part derive from the partial agonism activity of UCM871. For the first time, these results demonstrate that the MT_1 receptor is involved in the pathophysiology of depression by modulating 5-HT and NE activity, and may be considered a target for the development of novel antidepressant drugs. Further research is needed to better understand the effects of UCM871 and to develop novel melatonin MT1 ligands with larger therapeutic window allowing a safer profile for human antidepressant therapeutics.

Despite these open questions and more research to be done, this work represents the first preclinical study with a novel selective MT_1 receptor partial agonist and forms the basis for the development of a novel class of drugs for the treatment of psychiatric disorders.

References

AARON, B. L., JAMES, D. C. & RICHARD, V. H. 1959. STRUCTURE OF MELATONIN 1. *Journal of the American Chemical Society,* 81.

AARON, B. L., JAMES, D. C., YOSHIYATA, T., TEH, H. L. & WATARU, M. 1958. ISOLATION OF MELATONIN, THE PINEAL GLAND FACTOR THAT LIGHTENS MELANOCYTES 1. *Journal of the American Chemical Society,* 80.

ADAMAH-BIASSI, E., ZHANG, Y., JUNG, H., VISSAPRAGADA, S., MILLER, R. & DUBOCOVICH, M. 2014. Distribution of MT1 melatonin receptor promoter-driven RFP expression in the brains of BAC C3H/HeN transgenic mice. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society,* 62**,** 70-84.

ADI, N., MASH, D., ALI, Y., SINGER, C., SHEHADEH, L. & PAPAPETROPOULOS, S. 2010. Melatonin MT1 and MT2 receptor expression in Parkinson's disease. *Medical science monitor : international medical journal of experimental and clinical research,* 16**,** 7.

AGHAJANIAN, G. & VANDERMAELEN, C. 1982. Intracellular identification of central noradrenergic and serotonergic neurons by a new double labeling procedure. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 2**,** 1786-1792.

ALLERS, K. & SHARP, T. 2003. Neurochemical and anatomical identification of fast- and slow-firing neurones in the rat dorsal raphe nucleus using juxtacellular labelling methods in vivo. *Neuroscience,* 122**,** 193-204.

ANGELONI, D., LONGHI, R. & FRASCHINI, F. 2000. Production and characterization of antibodies directed against the human melatonin receptors Mel-1a (mt1) and Mel-1b (MT2). *European journal of histochemistry : EJH,* 44**,** 199-204.

ARVIDSSON, L. E., HACKSELL, U., NILSSON, J. L., HJORTH, S., CARLSSON, A., LINDBERG, P., SANCHEZ, D. & WIKSTROM, H. 1981. 8-Hydroxy-2-(di-n-propylamino)tetralin, a new centrally acting 5-hydroxytryptamine receptor agonist. *J Med Chem,* 24**,** 921-3.

ASTON-JONES, G., AKAOKA, H., CHARLETY, P. & CHOUVET, G. 1991a. Serotonin selectively attenuates glutamate-evoked activation of noradrenergic locus coeruleus neurons. *J Neurosci,* 11**,** 760-9.

ASTON-JONES, G., CHEN, S., ZHU, Y. & OSHINSKY, M. L. 2001. A neural circuit for circadian regulation of arousal. *Nat Neurosci,* 4**,** 732-8.

ASTON-JONES, G., SHIPLEY, M. T., CHOUVET, G., ENNIS, M., VAN BOCKSTAELE, E., PIERIBONE, V., SHIEKHATTAR, R., AKAOKA, H., DROLET, G., ASTIER, B. & ET AL. 1991b. Afferent regulation of locus coeruleus neurons: anatomy, physiology and pharmacology. *Prog Brain Res,* 88**,** 47-75.

BABA, K., BENLEULMI-CHAACHOUA, A., JOURNÉ, A.-S., KAMAL, M., GUILLAUME, J.-L., DUSSAUD, S., GBAHOU, F., YETTOU, K., LIU, C., CONTRERAS-ALCANTARA, S., JOCKERS, R. & TOSINI, G. 2013. Heteromeric MT1/MT2 melatonin receptors modulate photoreceptor function. *Science signaling,* 6.

BABA, K., POZDEYEV, N., MAZZONI, F., CONTRERAS-ALCANTARA, S., LIU, C., KASAMATSU, M., MARTINEZ-MERLOS, T., STRETTOI, E., IUVONE, P. & TOSINI, G. 2009. Melatonin modulates visual function and cell viability in the mouse retina via the MT1 melatonin receptor. *Proceedings of the National Academy of Sciences of the United States of America,* 106**,** 15043-15048.

BAMBICO, F. & GOBBI, G. 2008. The cannabinoid CB1 receptor and the endocannabinoid anandamide: possible antidepressant targets. *Expert opinion on therapeutic targets,* 12**,** 1347-1366.

BAMBICO, F., HATTAN, P., GARANT, J.-P. & GOBBI, G. 2012. Effect of delta-9-tetrahydrocannabinol on behavioral despair and on pre- and postsynaptic serotonergic transmission. *Progress in neuro-psychopharmacology & biological psychiatry,* 38**,** 88-96.

BAMBICO, F., KATZ, N., DEBONNEL, G. & GOBBI, G. 2007. Cannabinoids elicit antidepressant-like behavior and activate serotonergic neurons through the medial prefrontal cortex. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 27**,** 11700-11711.

BAMBICO, F., NGUYEN, N.-T. & GOBBI, G. 2009. Decline in serotonergic firing activity and desensitization of 5-HT1A autoreceptors after chronic unpredictable stress. *European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology,* 19**,** 215-228.

BAMBICO, F., NGUYEN, N.-T., KATZ, N. & GOBBI, G. 2010. Chronic exposure to cannabinoids during adolescence but not during adulthood impairs emotional behaviour and monoaminergic neurotransmission. *Neurobiology of disease,* 37**,** 641-655.

BARABAN, J. & AGHAJANIAN, G. 1980. Suppression of serotonergic neuronal firing by alpha-adrenoceptor antagonists: evidence against GABA mediation. *European Journal of Pharmacology,* 66**,** 287-294.

BASSANI, T., GRADOWSKI, R., ZAMINELLI, T., BARBIERO, J., SANTIAGO, R., BOSCHEN, S., DA CUNHA, C., LIMA, M., ANDREATINI, R. & VITAL, M. 2014. Neuroprotective and antidepressant-like effects of melatonin in a rotenone-induced Parkinson's disease model in rats. *Brain Research,* 1593**,** 95-105.

BECK-FRIIS, J., VON ROSEN, D., KJELLMAN, B., LJUNGGREN, J. & WETTERBERG, L. 1984. Melatonin in relation to body measures, sex, age, season and the use of drugs in patients with major affective disorders and healthy subjects. *Psychoneuroendocrinology,* 9**,** 261-277.

BÉÏQUE, J., DE MONTIGNY, C., BLIER, P. & DEBONNEL, G. 2000a. Effects of sustained administration of the serotonin and norepinephrine reuptake inhibitor venlafaxine: I. in vivo electrophysiological studies in the rat. *Neuropharmacology,* 39**,** 1800-1812.

BÉÏQUE, J., DE MONTIGNY, C., BLIER, P. & DEBONNEL, G. 2000b. Effects of sustained administration of the serotonin and norepinephrine reuptake inhibitor venlafaxine: II. In vitro studies in the rat. *Neuropharmacology,* 39**,** 1813-1822.

BENARROCH, E. 2008. Suprachiasmatic nucleus and melatonin: reciprocal interactions and clinical correlations. *Neurology,* 71**,** 594-598.

BERNARD, M., GUERLOTTÉ, J., GRÈVE, P., GRÉCHEZ-CASSIAU, A., IUVONE, M., ZATZ, M., CHONG, N., KLEIN, D. & VOISIN, P. 1999. Melatonin synthesis pathway: circadian regulation of the genes encoding the key enzymes in

the chicken pineal gland and retina. *Reproduction, nutrition, development,* 39**,** 325-334.

BERTAINA-ANGLADE, V., LA ROCHELLE, C., BOYER, P.-A. & MOCAËR, E. 2006. Antidepressant-like effects of agomelatine (S 20098) in the learned helplessness model. *Behavioural pharmacology,* 17**,** 703-713.

BINFARÉ, R., MANTOVANI, M., BUDNI, J., SANTOS, A. & RODRIGUES, A. 2010. Involvement of dopamine receptors in the antidepressant-like effect of melatonin in the tail suspension test. *European Journal of Pharmacology,* 638**,** 78-83.

BLIER, P. 2013. Neurotransmitter targeting in the treatment of depression. *The Journal of clinical psychiatry,* 74 Suppl 2**,** 19-24.

BLIER, P. & DE MONTIGNY, C. 1994a. Current advances and trends in the treatment of depression. *Trends Pharmacol Sci,* 15**,** 220-6.

BLIER, P. & DE MONTIGNY, C. 1994b. Current advances and trends in the treatment of depression. *Trends in pharmacological sciences,* 15**,** 220-226.

BLIER, P. & DE MONTIGNY, C. 1999. Serotonin and drug-induced therapeutic responses in major depression, obsessive-compulsive and panic disorders. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology,* 21.

BLIER, P., GOBBI, G., HADDJERI, N., SANTARELLI, L., MATHEW, G. & HEN, R. 2004. Impact of substance P receptor antagonism on the serotonin and norepinephrine systems: relevance to the antidepressant/anxiolytic response. *Journal of psychiatry & neuroscience : JPN,* 29**,** 208-218.

BOURIN, M., MOCAËR, E. & PORSOLT, R. 2004. Antidepressant-like activity of S 20098 (agomelatine) in the forced swimming test in rodents: involvement of melatonin and serotonin receptors. *Journal of psychiatry & neuroscience : JPN,* 29**,** 126-133.

BROWN, R., KOCSIS, J., CAROFF, S., AMSTERDAM, J., WINOKUR, A., STOKES, P. & FRAZER, A. 1985. Differences in nocturnal melatonin secretion between melancholic depressed patients and control subjects. *The American Journal of Psychiatry,* 142**,** 811-816.

BRUNNER, P., SÖZER-TOPCULAR, N., JOCKERS, R., RAVID, R., ANGELONI, D., FRASCHINI, F., ECKERT, A., MÜLLER-SPAHN, F. & SAVASKAN, E. 2006. Pineal and cortical melatonin receptors MT1 and MT2 are decreased in Alzheimer's disease. *European journal of histochemistry : EJH,* 50**,** 311-316.

CALVEY, N. & WILLIAMS, N. 2009. Principles and practice of pharmacology for anaesthetists. *John Wiley & Sons*.

CHENU, F., EL MANSARI, M. & BLIER, P. 2013. Electrophysiological effects of repeated administration of agomelatine on the dopamine, norepinephrine, and serotonin systems in the rat brain. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology,* 38**,** 275-284.

CHENU, F., SHIM, S., EL MANSARI, M. & BLIER, P. 2014a. Role of melatonin, serotonin 2B, and serotonin 2C receptors in modulating the firing activity of rat dopamine neurons. *Journal of psychopharmacology (Oxford, England),* 28**,** 162-167.

CHENU, F., SHIM, S., EL MANSARI, M. & BLIER, P. 2014b. Role of melatonin, serotonin 2B, and serotonin 2C receptors in modulating the firing activity of rat dopamine neurons. *J Psychopharmacol,* 28**,** 162-7.

CLAUSTRAT, B., BRUN, J. & CHAZOT, G. 2005. The basic physiology and pathophysiology of melatonin. *Sleep medicine reviews,* 9**,** 11-24.

COMAI, S., OCHOA-SANCHEZ, R., DOMINGUEZ-LOPEZ, S., BAMBICO, F. R. & GOBBI, G. 2015. Melancholic-Like Behaviors and Circadian Neurobiological Abnormalities in Melatonin MT1 Receptor Knockout Mice. *Int J Neuropsychopharmacol,* 18.

COMAI, S., OCHOA-SANCHEZ, R. & GOBBI, G. 2013. Sleep-wake characterization of double MT_1/MT_2 receptor knockout mice and comparison with MT_1 and MT_2 receptor knockout mice. *Behavioural brain research,* 243**,** 231-238.

CRUPI, R., MAZZON, E., MARINO, A., LA SPADA, G., BRAMANTI, P., CUZZOCREA, S. & SPINA, E. 2010. Melatonin treatment mimics the antidepressant action in chronic corticosterone-treated mice. *Journal of Pineal Research,* 49**,** 123-129.

CRYAN, J., VALENTINO, R. & LUCKI, I. 2005. Assessing substrates underlying the behavioral effects of antidepressants using the modified rat forced swimming test. *Neuroscience and biobehavioral reviews,* 29**,** 547-569.

CURET, O. & DE MONTIGNY, C. 1989. Electrophysiological characterization of adrenoceptors in the rat dorsal hippocampus. III. Evidence for the physiological role of terminal alpha 2-adrenergic autoreceptors. *Brain Research,* 499**,** 18-26.

DALTON, E., ROTONDI, D., LEVITAN, R., KENNEDY, S. & BROWN, G. 2000. Use of slow-release melatonin in treatment-resistant depression. *Journal of psychiatry & neuroscience : JPN,* 25**,** 48-52.

DE BODINAT, C., GUARDIOLA-LEMAITRE, B., MOCAËR, E., RENARD, P., MUÑOZ, C. & MILLAN, M. 2010. Agomelatine, the first melatonergic antidepressant: discovery, characterization and development. *Nature reviews. Drug discovery,* 9**,** 628-642.

DE BOER, T. 1995. The effects of mirtazapine on central noradrenergic and serotonergic neurotransmission. *International clinical psychopharmacology,* 10 Suppl 4**,** 19-23.

DE VRY, J., SCHOHE-LOOP, R., HEINE, H. G., GREUEL, J. M., MAULER, F., SCHMIDT, B., SOMMERMEYER, H. & GLASER, T. 1998. Characterization of the aminomethylchroman derivative BAY x 3702 as a highly potent 5-hydroxytryptamine1A receptor agonist. *J Pharmacol Exp Ther,* 284**,** 1082-94.

DENENBERG, V. 1969. Open-field bheavior in the rat: what does it mean? *Annals of the New York Academy of Sciences,* 159**,** 852-859.

DESCARRIES, L., WATKINS, K., GARCIA, S. & BEAUDET, A. 1982. The serotonin neurons in nucleus raphe dorsalis of adult rat: a light and electron microscope radioautographic study. *The Journal of comparative neurology,* 207**,** 239-254.

DEURVEILHER, S. & SEMBA, K. 2005. Indirect projections from the suprachiasmatic nucleus to major arousal-promoting cell groups in rat: implications for the circadian control of behavioural state. *Neuroscience,* 130**,** 165-83.

DOLBERG, O., HIRSCHMANN, S. & GRUNHAUS, L. 1998. Melatonin for the treatment of sleep disturbances in major depressive disorder. *The American Journal of Psychiatry,* 155**,** 1119-1121.

DOOLEN, S., KRAUSE, D., DUBOCOVICH, M. & DUCKLES, S. 1998. Melatonin mediates two distinct responses in vascular smooth muscle. *European Journal of Pharmacology,* 345**,** 67-69.

DUBOCOVICH, M. & MARKOWSKA, M. 2005. Functional MT1 and MT2 melatonin receptors in mammals. *Endocrine,* 27**,** 101-110.

DUBOCOVICH, M. & TAKAHASHI, J. 1987. Use of 2-[125I]iodomelatonin to characterize melatonin binding sites in chicken retina. *Proceedings of the National Academy of Sciences of the United States of America,* 84**,** 3916-3920.

EKMEKCIOGLU, C. 2006. Melatonin receptors in humans: biological role and clinical relevance. *Biomedicine & pharmacotherapy = Biomédecine & pharmacothérapie,* 60**,** 97-108.

FAVA, M., TARGUM, S., NIERENBERG, A., BLEICHER, L., CARTER, T., WEDEL, P., HEN, R., GAGE, F. & BARLOW, C. 2012. An exploratory study of combination buspirone and melatonin SR in major depressive disorder (MDD): a possible role for neurogenesis in drug discovery. *Journal of Psychiatric Research,* 46**,** 1553-1563.

FOUNTOULAKIS, K., KARAMOUZIS, M., IACOVIDES, A., NIMATOUDIS, J., DIAKOGIANNIS, J., KAPRINIS, G., DEMITRIADOU, A. & BECH, P. 2001. Morning and evening plasma melatonin and dexamethasone suppression test in patients with nonseasonal major depressive disorder from northern Greece (latitude 40-41.5 degrees). *Neuropsychobiology,* 44**,** 113-117.

GARFINKEL, D., ZISAPEL, N., WAINSTEIN, J. & LAUDON, M. 1999. Facilitation of benzodiazepine discontinuation by melatonin: a new clinical approach. *Archives of internal medicine,* 159**,** 2456-2460.

GEOFFRIAU, M., CLAUSTRAT, B. & VELDHUIS, J. 1999. Estimation of frequently sampled nocturnal melatonin production in humans by deconvolution analysis: evidence for episodic or ultradian secretion. *Journal of Pineal Research,* 27**,** 139-144.

GOBBI, G., BAMBICO, F., MANGIERI, R., BORTOLATO, M., CAMPOLONGO, P., SOLINAS, M., CASSANO, T., MORGESE, M., DEBONNEL, G., DURANTI, A., TONTINI, A., TARZIA, G., MOR, M., TREZZA, V., GOLDBERG, S.,

CUOMO, V. & PIOMELLI, D. 2005. Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 18620-18625.

GOBBI, G. & BLIER, P. 2005. Effect of neurokinin-1 receptor antagonists on serotoninergic, noradrenergic and hippocampal neurons: comparison with antidepressant drugs. *Peptides,* 26**,** 1383-1393.

GOBERT, A., RIVET, J., LEJEUNE, F., NEWMAN-TANCREDI, A., ADHUMEAU-AUCLAIR, A., NICOLAS, J., CISTARELLI, L., MELON, C. & MILLAN, M. 2000. Serotonin(2C) receptors tonically suppress the activity of mesocortical dopaminergic and adrenergic, but not serotonergic, pathways: a combined dialysis and electrophysiological analysis in the rat. *Synapse (New York, N.Y.),* 36**,** 205-221.

GOLOMBEK, D., MARTINI, M. & CARDINALI, D. 1993. Melatonin as an anxiolytic in rats: time dependence and interaction with the central GABAergic system. *European Journal of Pharmacology,* 237**,** 231-236.

GOLUS, P. & KING, M. 1981. The effects of melatonin on open field behavior. *Pharmacology, biochemistry, and behavior,* 15**,** 883-885.

GREENBLATT, D. & SHADER, R. 1978. Dependence, tolerance, and addiction to benzodiazepines: clinical and pharmacokinetic considerations. *Drug metabolism reviews,* 8**,** 13-28.

GUPTA, S., HALDAR, C. & SINGH, S. 2013. Daily variations in plasma melatonin and melatonin receptor (MT1), PER1 and CRY1 expression in suprachiasmatic nuclei of tropical squirrel, Funambulus pennanti. *Journal of comparative physiology. A, Neuroethology, sensory, neural, and behavioral physiology,* 199**,** 763-773.

HADDJERI, N., BLIER, P. & DE MONTIGNY, C. 1995. Noradrenergic modulation of central serotonergic neurotransmission: acute and long-term actions of mirtazapine. *International clinical psychopharmacology,* 10 Suppl 4**,** 11-17.

HADDJERI, N., BLIER, P. & DE MONTIGNY, C. 1998. Acute and long-term actions of the antidepressant drug mirtazapine on central 5-HT neurotransmission. *Journal of Affective Disorders,* 51**,** 255-266.

HALE, A., CORRAL, R.-M., MENCACCI, C., RUIZ, J., SEVERO, C. & GENTIL, V. 2010. Superior antidepressant efficacy results of agomelatine versus fluoxetine in severe MDD patients: a randomized, double-blind study. *International clinical psychopharmacology,* 25**,** 305-314.

HARDELAND, R. 2012. Melatonin in aging and disease -multiple consequences of reduced secretion, options and limits of treatment. *Aging and disease,* 3**,** 194-225.

HARDELAND, R. & POEGGELER, B. 2012. Melatonin and synthetic melatonergic agonists: actions and metabolism in the central nervous system. *Central nervous system agents in medicinal chemistry,* 12**,** 189-216.

HARINATH, K., MALHOTRA, A., PAL, K., PRASAD, R., KUMAR, R., KAIN, T., RAI, L. & SAWHNEY, R. 2004. Effects of Hatha yoga and Omkar meditation on cardiorespiratory performance, psychologic profile, and melatonin secretion. *Journal of alternative and complementary medicine (New York, N.Y.),* 10**,** 261-268.

HICKIE, I. & ROGERS, N. 2011. Novel melatonin-based therapies: potential advances in the treatment of major depression. *Lancet,* 378**,** 621-631.

HIRSCH-RODRIGUEZ, E., IMBESI, M., MANEV, R., UZ, T. & MANEV, H. 2007. The pattern of melatonin receptor expression in the brain may influence antidepressant treatment. *Medical hypotheses,* 69**,** 120-124.

HIRSCHFELD, R. 2000. History and evolution of the monoamine hypothesis of depression. *The Journal of clinical psychiatry,* 61 Suppl 6**,** 4-6.

HONG, L., HONG, W., ZHU, W., SHI, Q., YOU, X. & CHEN, S. 2014. Cloning and expression of melatonin receptors in the mudskipper Boleophthalmus pectinirostris: their role in synchronizing its semilunar spawning rhythm. *General and comparative endocrinology,* 195**,** 138-150.

HUNT, A., AL-GHOUL, W., GILLETTE, M. & DUBOCOVICH, M. 2001. Activation of MT(2) melatonin receptors in rat suprachiasmatic nucleus phase advances the circadian clock. *American Journal of Physiology. Cell Physiology,* 280**,** 8.

HUTCHINSON, A., HUDSON, R. & DUBOCOVICH, M. 2012. Genetic deletion of MT(1) and MT(2) melatonin receptors differentially abrogates the development

and expression of methamphetamine-induced locomotor sensitization during the day and the night in C3H/HeN mice. *Journal of Pineal Research,* 53**,** 399-409.

HUTCHINSON, A., MA, J., LIU, J., HUDSON, R. & DUBOCOVICH, M. 2014. Role of MT1 melatonin receptors in methamphetamine-induced locomotor sensitization in C57BL/6 mice. *Psychopharmacology,* 231**,** 257-267.

IMBESI, M., ARSLAN, A., YILDIZ, S., SHARMA, R., GAVIN, D., TUN, N., MANEV, H. & UZ, T. 2009. The melatonin receptor MT1 is required for the differential regulatory actions of melatonin on neuronal 'clock' gene expression in striatal neurons in vitro. *Journal of Pineal Research,* 46**,** 87-94.

IMBESI, M., UZ, T., YILDIZ, S., ARSLAN, A. & MANEV, H. 2006. Drug- and region-specific effects of protracted antidepressant and cocaine treatment on the content of melatonin MT(1) and MT(2) receptor mRNA in the mouse brain. *International journal of neuroprotection and neuroregeneration,* 2**,** 185-189.

JIN, X., VON GALL, C., PIESCHL, R., GRIBKOFF, V., STEHLE, J., REPPERT, S. & WEAVER, D. 2003. Targeted disruption of the mouse Mel(1b) melatonin receptor. *Molecular and cellular biology,* 23**,** 1054-1060.

KAEHLER, S., SINGEWALD, N. & PHILIPPU, A. 1999. Dependence of serotonin release in the locus coeruleus on dorsal raphe neuronal activity. *Naunyn-Schmiedeberg's archives of pharmacology,* 359**,** 386-393.

KAMAL, M., GBAHOU, F., GUILLAUME, J. L., DAULAT, A. M., BENLEULMI-CHAACHOUA, A., LUKA, M., CHEN, P., KALBASI ANARAKI, D., BARONCINI, M., MANNOURY LA COUR, C., MILLAN, M. J., PREVOT, V., DELAGRANGE, P. & JOCKERS, R. 2015. Convergence of Melatonin and 5-HT Signaling at MT2/5-HT2C Receptor Heteromers. *J Biol Chem*.

KANEKO, Y., HAYASHI, T., YU, S., TAJIRI, N., BAE, E., SOLOMITA, M., CHHEDA, S., WEINBREN, N., PAROLINI, O. & BORLONGAN, C. 2011. Human amniotic epithelial cells express melatonin receptor MT1, but not melatonin receptor MT2: a new perspective to neuroprotection. *Journal of Pineal Research,* 50**,** 272-280.

KARASEK, M. 2007. Does melatonin play a role in aging processes? *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society,* 58 Suppl 6**,** 105-113.

KASAMO, K., BLIER, P. & DE MONTIGNY, C. 1996. Blockade of the serotonin and norepinephrine uptake processes by duloxetine: in vitro and in vivo studies in the rat brain. *J Pharmacol Exp Ther,* 277**,** 278-86.

KASPER, S., HAJAK, G., WULFF, K., HOOGENDIJK, W., MONTEJO, A., SMERALDI, E., RYBAKOWSKI, J., QUERA-SALVA, M.-A., WIRZ-JUSTICE, A., PICAREL-BLANCHOT, F. & BAYLÉ, F. 2010. Efficacy of the novel antidepressant agomelatine on the circadian rest-activity cycle and depressive and anxiety symptoms in patients with major depressive disorder: a randomized, double-blind comparison with sertraline. *The Journal of clinical psychiatry,* 71**,** 109-120.

KELLIHER, P., CONNOR, T., HARKIN, A., SANCHEZ, C., KELLY, J. & LEONARD, B. 2000. Varying responses to the rat forced-swim test under diurnal and nocturnal conditions. *Physiology & behavior,* 69**,** 531-539.

KENNEDY, S. & EMSLEY, R. 2006. Placebo-controlled trial of agomelatine in the treatment of major depressive disorder. *European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology,* 16**,** 93-100.

LACOSTE, B., ANGELONI, D., DOMINGUEZ-LOPEZ, S., CALDERONI, S., MAURO, A., FRASCHINI, F., DESCARRIES, L. & GOBBI, G. 2015. Anatomical and cellular localization of melatonin MT and MT receptors in the adult rat brain. *J Pineal Res*.

LACROIX, D., BLIER, P., CURET, O. & DE MONTIGNY, C. 1991. Effects of long-term desipramine administration on noradrenergic neurotransmission: electrophysiological studies in the rat brain. *The Journal of pharmacology and experimental therapeutics,* 257**,** 1081-1090.

LAI, I. C., CHEN, M.-L., WANG, Y.-C., CHEN, J.-Y., LIAO, D.-L., BAI, Y.-M., LIN, C.-C., CHEN, T.-T. & LIOU, Y.-J. 2011. Analysis of genetic variations in the human melatonin receptor (MTNR1A, MTNR1B) genes and antipsychotics-induced tardive dyskinesia in schizophrenia. *The world journal of biological*

psychiatry : the official journal of the World Federation of Societies of Biological Psychiatry, 12**,** 143-148.

LARSON, J., JESSEN, R., UZ, T., ARSLAN, A., KURTUNCU, M., IMBESI, M. & MANEV, H. 2006. Impaired hippocampal long-term potentiation in melatonin MT2 receptor-deficient mice. *Neuroscience letters,* 393**,** 23-26.

LEMOINE, P., GUILLEMINAULT, C. & ALVAREZ, E. 2007. Improvement in subjective sleep in major depressive disorder with a novel antidepressant, agomelatine: randomized, double-blind comparison with venlafaxine. *The Journal of clinical psychiatry,* 68**,** 1723-1732.

LEWY, A., AHMED, S., JACKSON, J. & SACK, R. 1992. Melatonin shifts human circadian rhythms according to a phase-response curve. *Chronobiology international,* 9**,** 380-392.

LEWY, A., WEHR, T., GOODWIN, F., NEWSOME, D. & MARKEY, S. 1980. Light suppresses melatonin secretion in humans. *Science (New York, N.Y.),* 210**,** 1267-1269.

LI, N., LEE, B., LIU, R.-J., BANASR, M., DWYER, J., IWATA, M., LI, X.-Y., AGHAJANIAN, G. & DUMAN, R. 2010. mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. *Science (New York, N.Y.),* 329**,** 959-964.

LIU, C., WEAVER, D., JIN, X., SHEARMAN, L., PIESCHL, R., GRIBKOFF, V. & REPPERT, S. 1997. Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. *Neuron,* 19**,** 91-102.

LOISEAU, F., LE BIHAN, C., HAMON, M. & THIÉBOT, M.-H. 2006. Effects of melatonin and agomelatine in anxiety-related procedures in rats: interaction with diazepam. *European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology,* 16**,** 417-428.

LONGATTI, P., PERIN, A., RIZZO, V., COMAI, S., GIUSTI, P. & COSTA, C. 2007. Ventricular cerebrospinal fluid melatonin concentrations investigated with an endoscopic technique. *Journal of Pineal Research,* 42**,** 113-118.

LUCKI, I. 1997. The forced swimming test as a model for core and component behavioral effects of antidepressant drugs. *Behavioural pharmacology,* 8**,** 523-532.

MACHADO-VIEIRA, R., SALVADORE, G., DIAZGRANADOS, N. & ZARATE, C. 2009. Ketamine and the next generation of antidepressants with a rapid onset of action. *Pharmacology & therapeutics,* 123**,** 143-150.

MAGNUSSON, A. & BOIVIN, D. 2003. Seasonal affective disorder: an overview. *Chronobiology international,* 20**,** 189-207.

MALHI, G., PARKER, G. & GREENWOOD, J. 2005. Structural and functional models of depression: from sub-types to substrates. *Acta psychiatrica Scandinavica,* 111**,** 94-105.

MANTA, S., DONG, J., DEBONNEL, G. & BLIER, P. 2009. Enhancement of the function of rat serotonin and norepinephrine neurons by sustained vagus nerve stimulation. *Journal of psychiatry & neuroscience : JPN,* 34**,** 272-280.

MASANA, M. & DUBOCOVICH, M. 2001. Melatonin receptor signaling: finding the path through the dark. *Science's STKE : signal transduction knowledge environment,* 2001.

MCCLUNG, C. 2013. How might circadian rhythms control mood? Let me count the ways. *Biological Psychiatry,* 74**,** 242-249.

MIDDLEMISS, D. N. & FOZARD, J. R. 1983. 8-Hydroxy-2-(di-n-propylamino)-tetralin discriminates between subtypes of the 5-HT1 recognition site. *Eur J Pharmacol,* 90**,** 151-3.

MILLAN, M., GOBERT, A., LEJEUNE, F., DEKEYNE, A., NEWMAN-TANCREDI, A., PASTEAU, V., RIVET, J. M. & CUSSAC, D. 2003. The novel melatonin agonist agomelatine (S20098) is an antagonist at 5-hydroxytryptamine2C receptors, blockade of which enhances the activity of frontocortical dopaminergic and adrenergic pathways. *The Journal of pharmacology and experimental therapeutics,* 306**,** 954-964.

MILOYAN, B., BYRNE, G. & PACHANA, N. 2014. Threshold and Subthreshold Generalized Anxiety Disorder in Later Life. *The American journal of geriatric psychiatry : official journal of the American Association for Geriatric Psychiatry*.

MISHIMA, K., TOZAWA, T., SATOH, K., MATSUMOTO, Y., HISHIKAWA, Y. & OKAWA, M. 1999. Melatonin secretion rhythm disorders in patients with senile dementia of Alzheimer's type with disturbed sleep-waking. *Biological Psychiatry,* 45**,** 417-421.

MONGEAU, R., WEISS, M., DE MONTIGNY, C. & BLIER, P. 1998. Effect of acute, short- and long-term milnacipran administration on rat locus coeruleus noradrenergic and dorsal raphe serotonergic neurons. *Neuropharmacology,* 37**,** 905-18.

NILES, L., ARMSTRONG, K., RINCÓN CASTRO, L., DAO, C., SHARMA, R., MCMILLAN, C., DOERING, L. & KIRKHAM, D. 2004. Neural stem cells express melatonin receptors and neurotrophic factors: colocalization of the MT1 receptor with neuronal and glial markers. *BMC Neuroscience,* 5**,** 41.

NUTT, D., WILSON, S. & PATERSON, L. 2008. Sleep disorders as core symptoms of depression. *Dialogues in clinical neuroscience,* 10**,** 329-336.

OCHOA-SANCHEZ, R., COMAI, S., LACOSTE, B., BAMBICO, F., DOMINGUEZ-LOPEZ, S., SPADONI, G., RIVARA, S., BEDINI, A., ANGELONI, D., FRASCHINI, F., MOR, M., TARZIA, G., DESCARRIES, L. & GOBBI, G. 2011. Promotion of non-rapid eye movement sleep and activation of reticular thalamic neurons by a novel MT2 melatonin receptor ligand. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 31**,** 18439-18452.

OCHOA-SANCHEZ, R., RAINER, Q., COMAI, S., SPADONI, G., BEDINI, A., RIVARA, S., FRASCHINI, F., MOR, M., TARZIA, G. & GOBBI, G. 2012. Anxiolytic effects of the melatonin MT(2) receptor partial agonist UCM765: comparison with melatonin and diazepam. *Progress in neuro-psychopharmacology & biological psychiatry,* 39**,** 318-325.

OPAL, M. D., KLENOTICH, S. C., MORAIS, M., BESSA, J., WINKLE, J., DOUKAS, D., KAY, L. J., SOUSA, N. & DULAWA, S. M. 2014. Serotonin 2C receptor antagonists induce fast-onset antidepressant effects. *Mol Psychiatry,* 19**,** 1106-14.

PAGE, M., DETKE, M., DALVI, A., KIRBY, L. & LUCKI, I. 1999. Serotonergic mediation of the effects of fluoxetine, but not desipramine, in the rat forced swimming test. *Psychopharmacology,* 147**,** 162-167.

PANDI-PERUMAL, S., SRINIVASAN, V., MAESTRONI, G., CARDINALI, D., POEGGELER, B. & HARDELAND, R. 2006. Melatonin: Nature's most versatile biological signal? *The FEBS journal,* 273**,** 2813-2838.

PAPP, M., LITWA, E., GRUCA, P. & MOCAËR, E. 2006. Anxiolytic-like activity of agomelatine and melatonin in three animal models of anxiety. *Behavioural pharmacology,* 17**,** 9-18.

PARDRIDGE, W. & MIETUS, L. 1980. Transport of albumin-bound melatonin through the blood-brain barrier. *Journal of Neurochemistry,* 34**,** 1761-1763.

PARK, Y.-J., PARK, J.-G., TAKEUCHI, Y., HUR, S.-P., LEE, Y.-D., KIM, S.-J. & TAKEMURA, A. 2014. Influence of moonlight on mRNA expression patterns of melatonin receptor subtypes in the pineal organ of a tropical fish. *Marine Genomics,* 14**,** 67-70.

PAXINOS, G. & WATSON, C. 2006. The rat brain in stereotaxic coordinates: hard cover edition. *Academic press*.

PERREAU-LENZ, S., KALSBEEK, A., GARIDOU, M.-L., WORTEL, J., VAN DER VLIET, J., VAN HEIJNINGEN, C., SIMONNEAUX, V., PÉVET, P. & BUIJS, R. 2003. Suprachiasmatic control of melatonin synthesis in rats: inhibitory and stimulatory mechanisms. *The European journal of neuroscience,* 17**,** 221-228.

PFEFFER, M., RAUCH, A., KORF, H.-W. & VON GALL, C. 2012. The endogenous melatonin (MT) signal facilitates reentrainment of the circadian system to light-induced phase advances by acting upon MT2 receptors. *Chronobiology international,* 29**,** 415-429.

PIER, M., HULSTIJN, W. & SABBE, B. 2004. Differential patterns of psychomotor functioning in unmedicated melancholic and nonmelancholic depressed patients. *Journal of Psychiatric Research,* 38**,** 425-435.

PORSOLT, R., BERTIN, A. & JALFRE, M. 1977. Behavioral despair in mice: a primary screening test for antidepressants. *Archives internationales de pharmacodynamie et de thérapie,* 229**,** 327-336.

REITER, R. 1991. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. *Endocrine reviews,* 12**,** 151-180.

REYNOLDS, C. F., 3RD, KUPFER, D. J., TASKA, L. S., HOCH, C. C., SPIKER, D. G., SEWITCH, D. E., ZIMMER, B., MARIN, R. S., NELSON, J. P., MARTIN, D. & ET AL. 1985. EEG sleep in elderly depressed, demented, and healthy subjects. *Biol Psychiatry,* 20**,** 431-42.

RIVARA, S., PALA, D., LODOLA, A., MOR, M., LUCINI, V., DUGNANI, S., SCAGLIONE, F., BEDINI, A., LUCARINI, S., TARZIA, G. & SPADONI, G. 2012. MT1-selective melatonin receptor ligands: synthesis, pharmacological evaluation, and molecular dynamics investigation of N-{[(3-O-substituted)anilino]alkyl}amides. *ChemMedChem,* 7**,** 1954-1964.

ROUILLON, F. 2006. Efficacy and tolerance profile of agomelatine and practical use in depressed patients. *International clinical psychopharmacology,* 21 Suppl 1**,** 5.

SAMARKANDI, A., NAGUIB, M., RIAD, W., THALAJ, A., ALOTIBI, W., ALDAMMAS, F. & ALBASSAM, A. 2005. Melatonin vs. midazolam premedication in children: a double-blind, placebo-controlled study. *European Journal of Anaesthesiology,* 22**,** 189-196.

SANTARELLI, L., GOBBI, G., DEBS, P., SIBILLE, E., BLIER, P., HEN, R. & HEATH, M. 2001. Genetic and pharmacological disruption of neurokinin 1 receptor function decreases anxiety-related behaviors and increases serotonergic function. *Proceedings of the National Academy of Sciences of the United States of America,* 98**,** 1912-1917.

SAVASKAN, E., OLIVIERI, G., BRYDON, L., JOCKERS, R., KRÄUCHI, K., WIRZ-JUSTICE, A. & MÜLLER-SPAHN, F. 2001. Cerebrovascular melatonin MT1-receptor alterations in patients with Alzheimer's disease. *Neuroscience letters,* 308**,** 9-12.

SAVASKAN, E., OLIVIERI, G., MEIER, F., BRYDON, L., JOCKERS, R., RAVID, R., WIRZ-JUSTICE, A. & MÜLLER-SPAHN, F. 2002a. Increased melatonin 1a-receptor immunoreactivity in the hippocampus of Alzheimer's disease patients. *Journal of Pineal Research,* 32**,** 59-62.

SAVASKAN, E., WIRZ-JUSTICE, A., OLIVIERI, G., PACHE, M., KRÄUCHI, K., BRYDON, L., JOCKERS, R., MÜLLER-SPAHN, F. & MEYER, P. 2002b. Distribution of melatonin MT1 receptor immunoreactivity in human retina. *The*

journal of histochemistry and cytochemistry : official journal of the Histochemistry Society, 50**,** 519-526.

SHOPSIN, B., GERSHON, S., GOLDSTEIN, M., FRIEDMAN, E. & WILK, S. 1975. Use of synthesis inhibitors in defining a role for biogenic amines during imipramine treatment in depressed patients. *Psychopharmacology communications,* 1**,** 239-249.

SKENE, D., BOJKOWSKI, C. & ARENDT, J. 1994. Comparison of the effects of acute fluvoxamine and desipramine administration on melatonin and cortisol production in humans. *British journal of clinical pharmacology,* 37**,** 181-186.

SRINIVASAN, V., PANDI-PERUMAL, S., MAESTRONI, G., ESQUIFINO, A., HARDELAND, R. & CARDINALI, D. 2005. Role of melatonin in neurodegenerative diseases. *Neurotoxicity Research,* 7**,** 293-318.

SRINIVASAN, V., PANDI-PERUMAL, S., TRAKHT, I., SPENCE, D., HARDELAND, R., POEGGELER, B. & CARDINALI, D. 2009. Pathophysiology of depression: role of sleep and the melatonergic system. *Psychiatry research,* 165**,** 201-214.

SRINIVASAN, V., SMITS, M., SPENCE, W., LOWE, A., KAYUMOV, L., PANDI-PERUMAL, S., PARRY, B. & CARDINALI, D. 2006. Melatonin in mood disorders. *The world journal of biological psychiatry : the official journal of the World Federation of Societies of Biological Psychiatry,* 7**,** 138-151.

STEIN, D., AHOKAS, A. & DE BODINAT, C. 2008. Efficacy of agomelatine in generalized anxiety disorder: a randomized, double-blind, placebo-controlled study. *Journal of clinical psychopharmacology,* 28**,** 561-566.

STEIN, D., PICAREL-BLANCHOT, F. & KENNEDY, S. 2013. Efficacy of the novel antidepressant agomelatine for anxiety symptoms in major depression. *Human Psychopharmacology,* 28**,** 151-159.

STEIN, M. 2009. Neurobiology of generalized anxiety disorder. *The Journal of clinical psychiatry,* 70 Suppl 2**,** 15-19.

STEIN, M., FUETSCH, M., MÜLLER, N., HÖFLER, M., LIEB, R. & WITTCHEN, H. 2001. Social anxiety disorder and the risk of depression: a prospective community study of adolescents and young adults. *Archives of general psychiatry,* 58**,** 251-256.

SVENSSON, T., BUNNEY, B. & AGHAJANIAN, G. 1975. Inhibition of both noradrenergic and serotonergic neurons in brain by the alpha-adrenergic agonist clonidine. *Brain Research,* 92**,** 291-306.

SZABO, S. & BLIER, P. 2001. Effect of the selective noradrenergic reuptake inhibitor reboxetine on the firing activity of noradrenaline and serotonin neurons. *The European journal of neuroscience,* 13**,** 2077-2087.

SZABO, S., DE MONTIGNY, C. & BLIER, P. 1999. Modulation of noradrenergic neuronal firing by selective serotonin reuptake blockers. *British Journal of Pharmacology,* 126**,** 568-571.

SZABO, S., DE MONTIGNY, C. & BLIER, P. 2000. Progressive attenuation of the firing activity of locus coeruleus noradrenergic neurons by sustained administration of selective serotonin reuptake inhibitors. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP),* 3**,** 1-11.

TAN, D.-X., MANCHESTER, L., HARDELAND, R., LOPEZ-BURILLO, S., MAYO, J., SAINZ, R. & REITER, R. 2003. Melatonin: a hormone, a tissue factor, an autocoid, a paracoid, and an antioxidant vitamin. *Journal of Pineal Research,* 34**,** 75-78.

THASE, M. 2003. Achieving remission and managing relapse in depression. *The Journal of clinical psychiatry,* 64 Suppl 18**,** 3-7.

THOMBS, B. & ZIEGELSTEIN, R. 2013. Depression screening in primary care: why the Canadian task force on preventive health care did the right thing. *Canadian journal of psychiatry. Revue canadienne de psychiatrie,* 58**,** 692-696.

TIAN, S.-W., LAUDON, M., HAN, L., GAO, J., HUANG, F.-L., YANG, Y.-F. & DENG, H.-F. 2010. Antidepressant- and anxiolytic effects of the novel melatonin agonist Neu-P11 in rodent models. *Acta Pharmacologica Sinica,* 31**,** 775-783.

TRIVEDI, M., RUSH, A., WISNIEWSKI, S., NIERENBERG, A., WARDEN, D., RITZ, L., NORQUIST, G., HOWLAND, R., LEBOWITZ, B., MCGRATH, P., SHORES-WILSON, K., BIGGS, M., BALASUBRAMANI, G., FAVA, M. & TEAM, S. D. S. 2006. Evaluation of outcomes with citalopram for depression

using measurement-based care in STAR*D: implications for clinical practice. *The American Journal of Psychiatry,* 163**,** 28-40.

UCHIDA, K., OKAMOTO, N., OHARA, K. & MORITA, Y. 1996. Daily rhythm of serum melatonin in patients with dementia of the degenerate type. *Brain Research,* 717**,** 154-159.

UZ, T., ARSLAN, A., KURTUNCU, M., IMBESI, M., AKHISAROGLU, M., DWIVEDI, Y., PANDEY, G. & MANEV, H. 2005. The regional and cellular expression profile of the melatonin receptor MT1 in the central dopaminergic system. *Brain research. Molecular brain research,* 136**,** 45-53.

VANSTEENSEL, M., MICHEL, S. & MEIJER, J. 2008. Organization of cell and tissue circadian pacemakers: a comparison among species. *Brain research reviews,* 58**,** 18-47.

VOGEL, C. & MARCOTTE, E. 2012. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nature reviews. Genetics,* 13**,** 227-232.

VON GALL, C., STEHLE, J. & WEAVER, D. 2002. Mammalian melatonin receptors: molecular biology and signal transduction. *Cell and tissue research,* 309**,** 151-162.

WANG, X., SIRIANNI, A., PEI, Z., CORMIER, K., SMITH, K., JIANG, J., ZHOU, S., WANG, H., ZHAO, R., YANO, H., KIM, J., LI, W., KRISTAL, B., FERRANTE, R. & FRIEDLANDER, R. 2011. The melatonin MT1 receptor axis modulates mutant Huntingtin-mediated toxicity. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 31**,** 14496-14507.

WEHR, T., DUNCAN, W., SHER, L., AESCHBACH, D., SCHWARTZ, P., TURNER, E., POSTOLACHE, T. & ROSENTHAL, N. 2001. A circadian signal of change of season in patients with seasonal affective disorder. *Archives of general psychiatry,* 58**,** 1108-1114.

WEIL, Z., HOTCHKISS, A., GATIEN, M., PIEKE-DAHL, S. & NELSON, R. 2006. Melatonin receptor (MT1) knockout mice display depression-like behaviors and deficits in sensorimotor gating. *Brain research bulletin,* 68**,** 425-429.

WEST, E. & DALLY, P. 1959. Effects of iproniazid in depressive syndromes. *British medical journal,* 1**,** 1491-1494.

WITT-ENDERBY, P., BENNETT, J., JARZYNKA, M., FIRESTINE, S. & MELAN, M. 2003. Melatonin receptors and their regulation: biochemical and structural mechanisms. *Life sciences,* 72**,** 2183-2198.

WU, Y.-H., URSINUS, J., ZHOU, J.-N., SCHEER, F., AI-MIN, B., JOCKERS, R., VAN HEERIKHUIZE, J. & SWAAB, D. 2013. Alterations of melatonin receptors MT1 and MT2 in the hypothalamic suprachiasmatic nucleus during depression. *Journal of Affective Disorders,* 148**,** 357-367.

WU, Y.-H., ZHOU, J.-N., BALESAR, R., UNMEHOPA, U., BAO, A., JOCKERS, R., VAN HEERIKHUIZE, J. & SWAAB, D. 2006. Distribution of MT1 melatonin receptor immunoreactivity in the human hypothalamus and pituitary gland: colocalization of MT1 with vasopressin, oxytocin, and corticotropin-releasing hormone. *The Journal of comparative neurology,* 499**,** 897-910.

WU, Y.-H., ZHOU, J.-N., VAN HEERIKHUIZE, J., JOCKERS, R. & SWAAB, D. 2007. Decreased MT1 melatonin receptor expression in the suprachiasmatic nucleus in aging and Alzheimer's disease. *Neurobiology of Aging,* 28**,** 1239-1247.

ZHANG, Y., COOK, A., KIM, J., BARANOV, S., JIANG, J., SMITH, K., CORMIER, K., BENNETT, E., BROWSER, R., DAY, A., CARLISLE, D., FERRANTE, R., WANG, X. & FRIEDLANDER, R. 2013. Melatonin inhibits the caspase-1/cytochrome c/caspase-3 cell death pathway, inhibits MT1 receptor loss and delays disease progression in a mouse model of amyotrophic lateral sclerosis. *Neurobiology of disease,* 55**,** 26-35.

ZLOTOS, D., JOCKERS, R., CECON, E., RIVARA, S. & WITT-ENDERBY, P. 2014. MT1 and MT2 melatonin receptors: ligands, models, oligomers, and therapeutic potential. *Journal of Medicinal Chemistry,* 57**,** 3161-3185.

Appendix

Dorsal Raphe (5-HT)

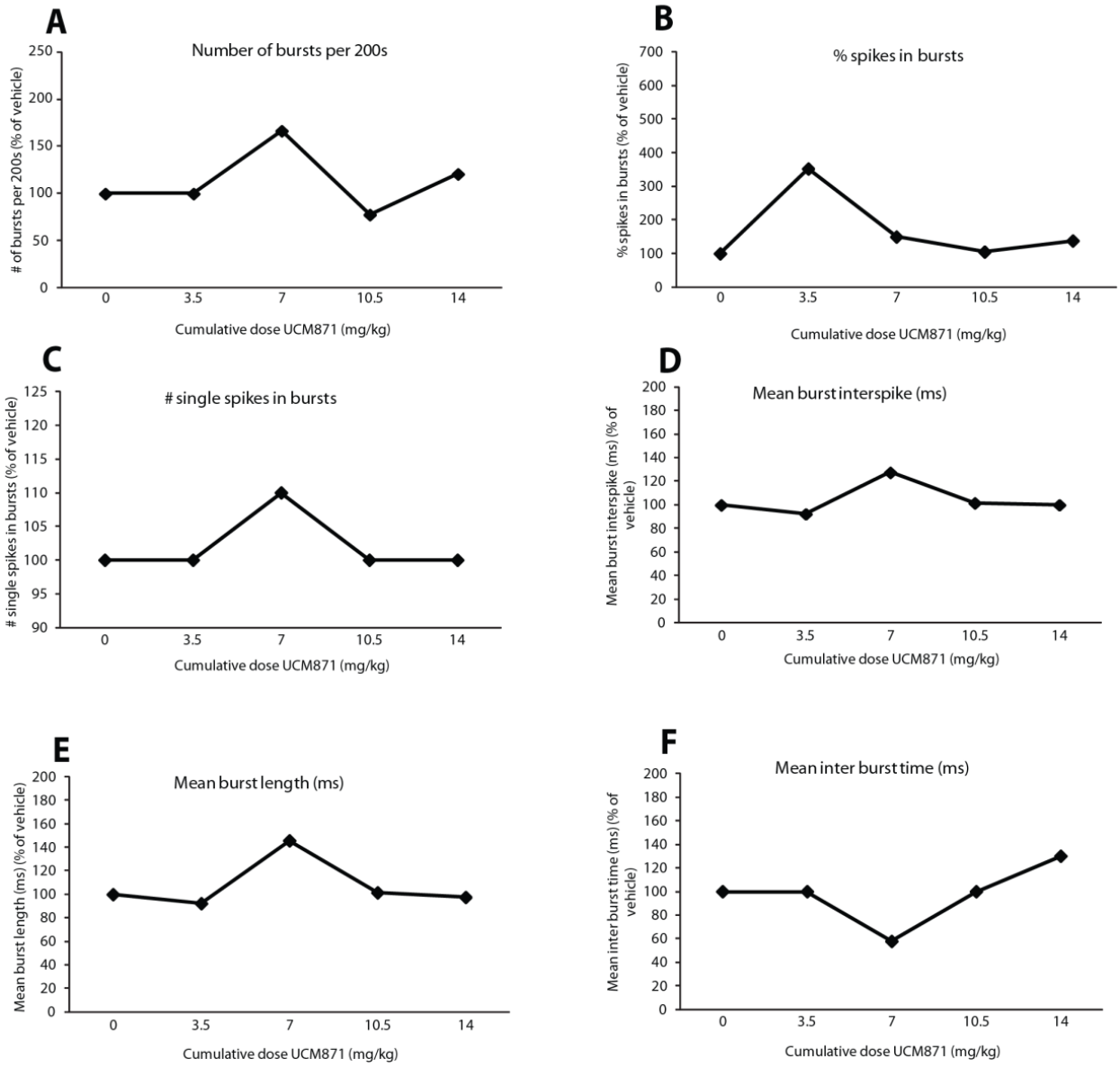


Figure i. UCM871 dose response of DR 5-HT neuron burst activity.

Dose 8-OH-DPAT (µg/kg)	Baseline (0)	1	2	3
UCM871 (n=7)	0	-23.9 ± 15.4	-38.3± 19.9	-71.3 ± 14.9
Vehicle (n=6)	0	-56.2 ± 17.8	-95.4 ± 2.9	-100 ± 0.0

Table i. Effect of UCM871 treatment on 8-OH-DPAT-induced inhibition of DR.

Dose clonidine (µg/kg)	Baseline (0)	1	2	3	4
UCM871 (n=8)	0	-47.5 ± 11.9	-81.8 ± 11.6	-86.0 ± 11.5	-88.3 ± 11.7
Vehicle (n=7)	0	9.4 ± 7.2	10.5 ± 9.1	-9.8 ± 20.8	-46.2 ± 20.0

Table ii. Effect of UCM871 treatment on clonidine-induced inhibition of LC NE neurons.

Dose UCM871	Baseline	Vehicle (0)	3.5	7	10.5	14
%bursting neurons	100	100	100	100	100	57.1
% spikes in bursts	47.9	42.6	38.6	37.12	23.9	23.6
# single spikes within bursts	3.1	2.9	2.7	2.5	2.25	2.75
mean burst interspike (ms)	33.2	31.6	31.2	31.3	27.7	33.5
mean burst length (ms)	99.9	92.5	84.4	82.79	68.1	88.6
mean inter burst time (ms)	2244.7	3223.1	2987.7	3273.5	5779.9	3466.3

Table iii. Summary of LC NE burst activity.