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MOLECULAR PATHWAYS OF DISTURBED SLEEP AND DEPRESSION:

STUDIES ON ADENOSINE AND GENE EXPRESSION PATTERNS

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ACADEMIC DISSERTATION

To be presented,
with the permission of the Faculty of Medicine of the University of Helsinki,
for public examination in Lecture Hall II, Haartman Institute, Haartmaninkatu 3,
on December 10, 2010 at 12 noon.

Helsinki 2010

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ISBN 978-952-10-6643-6 (Paperback)
ISBN 978-952-10-6644-3 (PDF)
http://ethesis.helsinki.fi
Helsinki 2010
Yliopistopaino

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ABBREVIATIONS

A_1R -	A_1 receptors
$A_{2A}R$ -	A_{2A} receptors
aCSF -	artificial cerebrospinal fluid
ACTH -	adrenocorticotropic hormone
ADA -	adenosine deaminase
ADK -	adenosine kinase
AMPA -	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
BDNF -	brain-derived neurotrophic factor
BF -	basal forebrain
CLI -	clomipramine
CPT -	8-cyclopentyl-1,3-dimethylxanthine
CRH -	corticotropin-releasing hormone
DMPX -	3,7-dimethyl-propargylxanthine
DRN -	dorsal raphe nucleus
EEG -	electroencephalographic
EMG -	electromyographic
ENT -	equlibrative nucleoside transporter
ERβ -	estrogen receptor beta
FC –	fold change
GABA -	gamma-aminobutyrate
GC -	glucocorticoid
GHRH -	growth hormone releasing hormone
GO –	gene ontology
HDAC -	histone deacetylase
HDB -	horizontal limb of the diagonal band of Broca
HPA -	hypothalamo-pituitary-adrenal
IL-1β -	interleukin-1β
LC -	locus coeruleus
LDT -	laterodorsal tegmental nucleus
MCPO -	magnocellular preoptic area
MDD -	major depressive disorder
MPN -	median preoptic nucleus
NMDA -	N-methyl-D-aspartate
NREM -	non-rapid eye movement

PCR -	polymerase chain reaction
PFLH -	perifornical-lateral hypothalamic area
PPT -	pedunculopontine tegmentum
PVN -	paraventricular nucleus
REM –	rapid eye movement
SAHH -	S-adenosylhomocysteine hydrolase
SAPE -	streptavidin-phycoerythrin
SCN -	suprachiasmatic nuclei
SD -	sleep deprivation
SFA -	spindle frequency activity
SI -	substantia innominata
SNP -	single nucleotide polymorphism
SON -	supraoptic nucleus
SSRI -	selective serotonin reuptake inhibitors
TMN -	tuberomammillary nucleus
TNF-α -	tumor necrosis factor α
TRH -	thyrotropin-releasing hormone
TSH -	thyroid-stimulating hormone
VLPO -	ventrolateral preoptic area
VP -	vasopressin
VTA -	ventral tegmental area

ABSTRACT

Background: Adenosine is a potent sleep-promoting substance, and one of its targets is the basal forebrain. Fairly little is known about its mechanism of action in the basal forebrain and about the receptor subtype mediating its regulating effects on sleep homeostasis. Homeostatic deficiency might be one of the causes of the profoundly disturbed sleep pattern in major depressive disorder, which could explain the reduced amounts of delta-activity-rich stages 3 and 4. Since major depression has a relatively high heritability, and on the other hand adenosine regulates sleep homeostasis and might also be involved in mood modulation, adenosine-related genes should be considered for their possible contribution to a predisposition for depression and disturbed sleep in humans. Depression is a complex disorder likely involving the abnormal functioning of several genes. Novel target genes which could serve as the possible common substrates for depression and comorbid disturbed sleep should be identified. In this way specific brain areas related to sleep regulation should be studied by using animal model of depression which represents more homogenous phenotype as compared to humans. It is also important to study these brain areas during the development of depressive-like features to understand how early changes could facilitate pathophysiological changes in depression.

Aims and methods: We aimed to find out whether, in the basal forebrain, adenosine induces recovery non-rapid eye movement (NREM) sleep after prolonged waking through the A_1 or/and A_{2A} receptor subtype. A_1 and A_{2A} receptor antagonists were perfused into the rat basal forebrain during 3 h of sleep deprivation, and the amount of NREM sleep and delta power during recovery NREM sleep were analyzed. We then explored whether polymorphisms in genes related to the metabolism, transport and signaling of adenosine could predispose to depression accompanied by signs of disturbed sleep. DNA from 1423 individuals representative of the Finnish population and including controls and cases with depression, depression accompanied by early morning awakenings and depression accompanied by fatigue, was used in the study to investigate the possible association between polymorphisms from adenosine-related genes and cases. Finally to find common molecular substrates of depression and disturbed sleep, gene expression changes were investigated in specific brain areas in the rat clomipramine model of depression. We focused on the basal forebrain of 3-week old clomipramine-treated rats which develop depressive-like symptoms later in adulthood and on the hypothalamus of adult female clomipramine-treated rats.

Results: Blocking of the A_1 receptor during sleep deprivation resulted in a reduction of the recovery NREM sleep amount and delta power, whereas A_{2A} receptor antagonism had no effect. Polymorphisms in adenosine-related genes *SLC29A3*

(equilibrative nucleoside transporter type 3) in women and *SLC28A1* (concentrative nucleoside transporter type 1) in men associated with depression alone as well as when accompanied by early morning awakenings and fatigue. In Study III the basal forebrain of postnatal rats treated with clomipramine displayed disturbances in gamma-aminobutyric acid (GABA) receptor type A signaling, in synaptic transmission and possible epigenetic changes. CREB1 was identified as a common transcription denominator which also mediates epigenetic regulation. In the hypothalamus the major changes included the expression of genes in $GABA_A$ receptor pathway, K^+ channel-related, glutamatergic and mitochondrial genes, as well as an overexpression of genes related to RNA and mRNA processing.

Conclusions: Adenosine plays an important role in sleep homeostasis by promoting recovery NREM sleep via the A_1 receptor subtype in the basal forebrain. Also adenosine levels might contribute to the risk of depression with disturbed sleep, since the genes encoding nucleoside transporters showed the strongest associations with depression alone and when accompanied by signs of disturbed sleep in both women and men. Sleep and mood abnormalities in major depressive disorder could be a consequence of multiple changes at the transcriptional level, $GABA_A$ receptor signaling and synaptic transmission in sleep-related basal forebrain and the hypothalamus.

INTRODUCTION

Major depressive disorder and poor sleep have a reciprocal relationship: depression causes sleep disturbances, and insomnia is an independent risk factor for depression (Ford and Kamerow, 1989; Paffenbarger *et al.*, 1994; Riemann *et al.*, 2001). Depressed patients show decreased amounts of non-rapid-eye movement sleep stages 3 and 4, which have high electroencephalographic delta activity (power density in 1.0-4.5 Hz) (Armitage and Hoffmann, 2001). This activity is one of the main markers of sleep homeostasis, which is defined as an intensified sleep period after prolonged wakefulness (Borbely, 1982; Porkka-Heiskanen *et al.*, 1997; Kalinchuk *et al.*, 2008). Depressed men have an attenuated sleep homeostatic response to sleep deprivation (Armitage, 2007). The homeostatic deficiency might underlie the reduced sleep depth, thus resulting in shallow sleep, fatigue and exacerbation of the already existing depressive symptoms. Several compounds have been nominated as putative homeostatic factors, including cytokines, prostaglandin D_2, brain-derived neurotrophic factor, numerous peptides and adenosine (Borbely and Tobler, 1989; Spier and de Lecea, 2000; Garcia-Garcia *et al.*, 2009). Maintaining wakefulness depletes energy stores, which results in a feeling of tiredness and increased sleepiness. Of the many candidates only the nucleoside adenosine is able to couple energy depletion to sleep induction: it is a by-product of ATP breakdown, its levels rise site-specifically under the conditions of higher metabolic demand during prolonged waking, and it promotes sleep, thus laying the foundation for sleep homeostasis. Disturbances in adenosine signaling could promote the sleep disruptions characteristic for major depression. Apart from its role in sleep, adenosine exerts neuroprotective effects when acting through A_1 and A_{2A} receptors (Aden *et al.*, 2001; Aden *et al.*, 2003). Neuroprotection might be disrupted in depression as inflammatory processes are profoundly expressed in this illness (Maes *et al.*, 2009). In addition, adenosine is also capable of modifying mood by affecting the release of such neurotransmitters as serotonin, dopamine, glutamate, and gamma-aminobutyric acid (Dunwiddie and Masino, 2001). Since major depression has relatively high levels of heritability (42-49% in women and 25-29% in men) ((Kendler *et al.*, 2006; Orstavik *et al.*, 2007), and adenosine could affect mood through its diverse facets, it would be important to know whether adenosine-related genes contribute in any way to a predisposition for depression and disturbed sleep in humans.

One of the areas of increased adenosine release during prolonged waking is the arousal-maintaining basal forebrain where adenosine is able to suppress neuronal activity, thus promoting sleep (Strecker *et al.*, 2000). Four types of adenosine receptors are expressed in the brain: A_1, A_{2A}, A_{2B} and A_3. Two of them, A_1 and A_{2A},

mediate adenosine sleep-inducing effects (Strecker *et al.*, 2000). However, it has been unclear which receptor subtype is responsible for adenosine action in the basal forebrain.

So far there has not been identified any single gene which would be responsible for the whole spectrum of depressive symptoms and pathophysiology and would also invariably cause depression in case of its disrupted function. Therefore, depression most likely constitutes a complex-trait phenomenon and is caused by abnormalities in multiple genes and molecular pathways. Screening the alterations in transcription of large numbers of genes allows to identify candidate genes for further investigation of their possible contribution to the disorder. It also provides a broader view as well as insights into the molecular cascades which might be malfunctioning in depression. In contrast to human studies, animal models utilize more homogenous phenotypes and escape the pitfalls of post-mortem studies. One of the well-established models is the clomipramine model, in which adult rats treated at neonatal age with clomipramine, a serotonin and noradreanline reuptake inhibitor, express depressive-like behavior and the sleep abnormalities characteristic for depression (Vogel *et al.*, 1990a). Exploration of the gene expression patterns in specific brain areas related to sleep regulation by means of the clomipramine model could reveal the possible common substrates for depression and comorbid disturbed sleep and detect new treatment targets. Also an examination of gene expression changes at an early age immediately after clomipramine treatment would disclose molecular pathways which are abnormally functioning at this stage and thus, maybe, lead to the development of a depressive-like phenotype.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I. Gass N, Porkka-Heiskanen T, Kalinchuk AV. **The role of the basal forebrain adenosine receptors in sleep homeostasis.** Neuroreport. 2009 Jul 15, 20 (11):1013-8.

II. Gass N, Ollila HM, Utge S, Partonen T, Kronholm E, Pirkola S, Suhonen J, Silander K, Porkka-Heiskanen T, Paunio T**. Contribution of adenosine related genes to the risk of depression with disturbed sleep.** Journal of Affective Disorders. 2010 Oct, 126(1-2): 134-139.

III. Lagus M, Gass N, Saharinen J, Saarela J, Porkka-Heiskanen T, Paunio T. **Gene expression patterns in a rodent model for depression.** The European Journal of Neuroscience. 2010 Apr, 31 (8):1465-73.

IV. Gass N, Lagus M, Saharinen J, Saarela J, Porkka-Heiskanen T, Paunio T. **Gene expression signature in the female rat hypothalamus in clomipramine model of depression**. Submitted.

1. REVIEW OF THE LITERATURE

1.1. ADENOSINE ROLE IN SLEEP REGULATION

1.1.1. SLEEP HOMEOSTASIS

One of the basic principles of sleep regulation is sleep homeostasis, which is defined as an increase in sleep propensity after prolonged wakefulness (Borbely, 1982). The homeostatic aspect of sleep regulation is especially important in relation to the function of sleep. The markers of sleep homeostasis are delta activity (electroencephalographic (EEG) power density in the 1.0-4.5 Hz range) and spindle frequency activity (SFA) (11-15 Hz) in non-rapid eye movement (NREM) sleep, as well as theta activity (5-9 Hz) in prior waking. The latter increases during prolonged waking and parallels a wake-dependent increase in delta activity in recovery sleep (Cajochen *et al.*, 1995; Vyazovskiy and Tobler, 2005), whereas SFA decreases after sleep deprivation (SD) (Knoblauch *et al.*, 2003). Concerning rapid eye movement (REM) sleep homeostasis, in humans REM sleep rebound is moderate and can last several nights (Borbely, 1982; Tobler and Borbely, 1986). So far, the marker for REM sleep homeostasis remains contradictory. It has been suggested that it might be the alpha activity (8-12 Hz) in REM sleep which is inversely related to REM sleep pressure (Roth *et al.*, 1999). The mechanism of REM sleep homeostasis is also unclear. One of the hypotheses claims that REM sleep pressure accumulates during NREM sleep (Benington and Heller, 1995). Another regulatory mechanism for sleep propensity regulation is a circadian oscillator (Borbely, 1982) which consists of transcriptional/translational feedback loops involving clock-related genes *CLOCK*, *BMAL1*, *PER1-3*, *CRY1-2*, and *TIM* (Barnard and Nolan, 2008). The central circadian pacemaker resides in the suprachiasmatic nuclei (SCN) in the anterior hypothalamus, and furthermore the circadian control can be also carried out in tissues independently from the core circadian oscillator (Panda *et al.*, 2002).

Both the circadian rhythmicity and the homeostatic process are interrelated. Mice lacking clock-controlled gene *Dbp* demonstrate reduced delta power in NREM sleep (Franken *et al.*, 2000), while the reverse is true for *Bmal1/Mop3* knockout mice (Laposky *et al.*, 2005). *Cry1,2*-deficient mice have higher delta power in NREM sleep and a smaller NREM delta power response to SD (Wisor *et al.*, 2002). *Clock* mutant mice have a lower REM sleep rebound after SD (Naylor *et al.*, 2000). In

humans, polymorphism in circadian gene *PER3* affects each state: PER3$^{5/5}$ subjects have more NREM sleep and higher initial delta activity in recovery sleep, as well as an increase in theta activity during prolonged waking and higher alpha activity in recovery REM sleep (Viola *et al.*, 2007).

The homeostatic process exerts a reciprocal impact on the circadian pacemaker. Sleep states affect activity of the SCN neurons (Deboer *et al.*, 2003). SD significantly suppresses electrical activity in the SCN (Deboer *et al.*, 2007) and alters the expression of circadian genes in the forebrain (Wisor *et al.*, 2002; Wisor *et al.*, 2008). The clock genes *Per1-3*, *Clock* and *Bmal1* encode PAS-domain transcription factors, many of which can sense oxygen, redox potential, voltage and light. Therefore these proteins might sense a cellular energy state which would be designated by the homeostatic drive (Franken and Dijk, 2009). The third process underlying sleep regulation is an ultradian process which occurs within the NREM-REM sleep cycle (Borbely and Achermann, 1999).

1.1.2. SLEEP DEPRIVATION

Sleep loss promotes a compensatory increase in sleep amount and sleep intensity. Both 3-h and 6-h SD in rats induces increases in NREM sleep amount and delta activity in recovery NREM sleep (Tobler and Borbely, 1986; Tobler and Borbely, 1990). Long-term 24-h SD in rats has similar effects on recovery NREM sleep parameters: a delta increase in the first 4 h of recovery NREM sleep and an elevated NREM sleep amount during both recovery days (Schwierin *et al.*, 1999). In another study 24-h SD triggered a delta increase in the first hours of recovery NREM sleep, enhanced theta (7.25-10 Hz) in REM sleep and waking, and a reduced NREM sleep amount (Borbely *et al.*, 1984).

1.1.3. ADENOSINE IN SLEEP REGULATION

The neurochemical and neuroanatomical substrates for the homeostatic process are still unclear. As the markers for sleep homeostasis are the delta activity and SFA in NREM sleep and the theta activity in preceding wakefulness, the putative homeostatic factor should meet the following criteria: (1) it should be able to elevate delta activity and suppress SFA in recovery NREM sleep, (2) it should be built up during wakefulness proportionally to activity during this period and reflect an increase in sleep pressure. Sleep is a use-dependent process, and delta activity rises in those brain areas which were mostly active during wakefulness (Vyazovskiy and Tobler, 2008), therefore this putative homeostatic factor should in addition reflect

metabolic use-dependent aspects of delta activity regulation.

Among many candidates for this role, purine nucleoside adenosine appears to be one of the most fitting compounds, since it reflects both metabolic and hypnogenic aspects. First and most importantly, adenosine is an end-product of ATP metabolism; under increased energy demand adenosine levels increase and promote delta activity in NREM sleep (Porkka-Heiskanen *et al.*, 1997; Kalinchuk *et al.*, 2008). Nobody has yet explored the effects of adenosine on SFA; however, studies on caffeine, an adenosine receptor antagonist, indicate that adenosine might have an affect: caffeine enhances SFA power density which serves as an indirect sign that adenosine might have an opposite effect (Landolt *et al.*, 1995; Landolt *et al.*, 2004). In a study with glutamatergic stimulation by N-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and glutamate, only NMDA elevated waking theta power and adenosine levels (Wigren *et al.*, 2007). This finding suggests that adenosine levels might reflect activity during waking.

Adenosine metabolism. Adenosine was first described in 1929 (Drury and Szent-Gyorgyi, 1929). It is an ATP constituent and the end-product of ATP breakdown. In the brain and under physiological conditions, ATP is mostly released by astrocytes into the extracellular space, where it is hydrolysed by nucleoside triposphate diphosphohydrolases and converted to adenosine by ectonucleotidase (Boison *et al.*, 2009). It can be released from astrocyte vesicles by a "kiss-and-run" mechanism, from a subset of lysosomes, through connexin/pannexin "hemi-channels", or via maxi-anion channels (Boison *et al.*, 2009). It may also originate from storage vesicles containing hormones, from transport vesicles delivering proteins to the cell membrane, or be co-released with various neurotransmitters (Richardson and Brown, 1987; Strecker *et al.*, 2000; Fredholm, 2010). As the intracellular ATP concentration is much higher than adenosine levels, even a 1% change in the ATP level could result in 100-fold increase in the adenosine content (Dunwiddie and Masino, 2001).

When energy demands increase as it occurs during prolonged waking, ATP expenditure exceeds ATP synthesis, resulting in elevated adenosine concentrations. In addition to extracellular production, adenosine also forms intracellularly and can be transported from neurons via equlibrative nucleoside transporters (ENT) (Porkka-Heiskanen *et al.*, 2002). Adenosine is generated from AMP by cytosolic 5'-nucleotidase and converted back to AMP by adenosine kinase (ADK) (Porkka-Heiskanen *et al.*, 2002). Since the activity of ADK is usually higher compared to 5'-nucleotidase, intracellular adenosine levels are low; therefore the transport through ENTs is normally inward according to the concentration gradient (Porkka-Heiskanen *et al.*, 2002). Adenosine uptake into the cell is also carried out by concentrative nucleoside transporters. Under the conditions of increased energy demand, though, when neuronal ATP expenditure is high, the direction of adenosine transport through ENTs is outward (Porkka-Heiskanen *et al.*, 2002). Adenosine

can also originate from glial cells, which is supported by data which demonstrate the prevalent association of 5'-ectonucleotidase with glia (Huston *et al.*, 1996) and the absence of sleep-suppressing effects of adenosine A_1 receptor (A_1R) antagonist in mice with inhibited gliotransmission (Halassa *et al.*, 2009). Another source of extracellular adenosine could be cyclic AMP, which released through a probenicide-sensitive transporter, may be converted to adenosine by ecto-phosphodiesterase (Dunwiddie and Masino, 2001).

The half-life of adenosine is only 0.6-1.5 s (Porkka-Heiskanen *et al.*, 2002) as it is rapidly metabolized intra- and extracellularly by ADK and adenosine deaminase (ADA). ADK is mostly expressed in astrocytes, where it serves as a metabolic reuptake mechanism (Boison *et al.*, 2009). ADA dominates under conditions of higher adenosine levels (Porkka-Heiskanen *et al.*, 2002). The third mechanism of adenosine clearance is mediated through S-adenosylhomocysteine hydrolase (SAHH), but this pathway is probably less important in the CNS (Fredholm *et al.*, 2005a).

Adenosine and sleep. The sleep-inducing effects of adenosine were first demonstrated in cats (Feldberg and Sherwood, 1954) and then in dogs (Haulica *et al.*, 1973). Later these effects were repeatedly confirmed by systemic and intracerebral injections of adenosine and adenosine receptor agonists into rodents (Dunwiddie and Worth, 1982; Virus *et al.*, 1983; Radulovacki *et al.*, 1984; Ticho and Radulovacki, 1991). The common-sense knowledge about the sleep-promoting effect of adenosine in humans derives from the fact that the worldwide-consumed stimulant caffeine is an adenosine receptor antagonist (Fredholm, 2010).

There are four different adenosine receptors (A_1, A_{2A}, A_{2B}, A_3) known up to date (Fredholm, 2010). All of them are G-protein-coupled and have seven transmembrane domains. So far only two receptor types, A_1 and A_{2A}, have been shown to mediate adenosine's sleep-inducing effects (Satoh *et al.*, 1998; Strecker *et al.*, 2000). A_1R are coupled to $G_{i/o}$ protein and have widespread distribution in the brain (Kudlacek *et al.*, 2002; Porkka-Heiskanen *et al.*, 2002). Stimulation of A_1R results in adenylate cyclase inhibition and phospholipase C activation (Basheer *et al.*, 2004). Higher levels of A_1R agonist or higher expression of A_1R potentiates the phospholipase C pathway (Biber *et al.*, 1997). When acting through A_1R, adenosine opens K^+ channels and inhibits Q-, P- and N-type Ca^{2+} channels (Gundlfinger *et al.*, 2007). By acting via presynaptic A_1R, adenosine inhibits neurotransmitter release, mostly of excitatory neurotransmitters such as glutamate and acetylcholine. This effect is mainly due to Ca^{2+}-channels inhibition, although Ca^{2+}-independent mechanisms are also possible (Dunwiddie and Masino, 2001).

Systemic administration of selective A_1R agonist elevates NREM sleep amount in rats and induces an increase in NREM delta activity similarly to the SD response (Benington *et al.*, 1995; Schwierin *et al.*, 1996). Mice with CNS A_1R deletion exhibit reduced delta activity in the range 3.0-4.5 Hz during both spontaneous and recovery NREM sleep (Bjorness *et al.*, 2009). However, since multiple processes

are affected by adenosine, such as locomotion and thermoregulation (Basheer *et al.*, 2004; Jinka *et al.*, 2010), data obtained from knockout animals should be interpreted cautiously due to possible confounding behavioral effects.

A_{2A} receptors ($A_{2A}R$) are expressed in a limited number of brain areas such as striatum, nucleus accumbens, and olfactory tubercle (Rosin *et al.*, 1998). They are mostly coupled to G_s protein (G_{olf} in striatum), and their stimulation results in elevated cyclic AMP levels as well as protein kinase C activation via the phospholipase C signaling pathway (Cunha, 2001). The latter probably occurs via presynaptic $A_{2A}R$ activation. Selective $A_{2A}R$ agonist administration into the subarachnoid space adjacent to the basal forebrain (BF) and the lateral preoptic area induces NREM sleep (Satoh *et al.*, 1998; Scammell *et al.*, 2001). $A_{2A}R$ knockout mice show reduced amounts of sleep and no clear recovery NREM sleep after SD (Urade *et al.*, 2003).

To sum up, adenosine is a potent sleep-promoting and sleep-modulating agent with a number of brain-specific effects.

1.1.4. THE BASAL FOREBRAIN AND SLEEP

The neuroanatomy and functions of the BF. Literally the BF is a ventral part of the forebrain, but in sleep literature this term is mostly used to describe the following structures: the medial septum, nuclei of the vertical and horizontal limbs of the diagonal band of Broca (HDB), the ventral pallidum, the magnocellular preoptic area (MCPO), the substantia innominata (SI), and the magnocellular basal nucleus (Semba, 2000). Although cholinergic neurons determine the boundaries of the area, the neuronal population in this region is neurochemically heterogeneous and comprises, along with cholinergic cells, also gamma-aminobutyrate (GABA) and glutamatergic cells (Henny and Jones, 2008). Heterogeneity of the BF is also reflected in the multitude of firing patterns during sleep-wake cycles, differing membrane properties and in responses to neurochemical signals (Semba, 2000; Lee *et al.*, 2004).

The cholinergic cells constitute approximately 5% of all the BF neurons (Lee *et al.*, 2005; Gritti *et al.*, 2006). Recent studies demonstrate that these cells also synthesize glutamate (Gritti *et al.*, 2006), which appears to be a cotransmitter with acetylcholine (Allen *et al.*, 2006). Furthermore, some neuropeptides are colocalized within the cholinergic neurons, of which galanin is the most frequent (Semba, 2000). The cholinergic neurons from the anterior BF primarily project to the hippocampus, while the posterior part of the BF sends cholinergic projections to the neocortex and the amygdala. It was assumed that in the cortex the cholinergic cells make connections to pyramidal cells and to excitatory and inhibitory interneurons, but a recent study in rats demonstrated that the cholinergic cells make synaptic connections only with cortical interneurons (Henny and Jones, 2008). Additionally, the cholinergic neurons project to the olfactory bulb, the preoptic-anterior hypo-

thalamic area (POAH) and the brainstem nuclei (Semba, 2000).

The GABAergic neurons constitute approximately 35% of the BF cells and, similarly to cholinergic cells, are able to synthesize glutamate (Gritti *et al.*, 2006). Some of the GABAergic cells project to the cortex in parallel with cholinergic neurons, where they target pyramidal cells and GABAergic interneurons (Gritti *et al.*, 2003; Henny and Jones, 2008). In addition, the BF sends GABAergic projections to the hippocampus, the olfactory bulb, the thalamus, the hypothalamus and the brainstem wake- and REM sleep related areas (the mesopontine tegmentum, the dorsal raphe nucleus (DRN), the locus coeruleus (LC), the laterodorsal tegmental nucleus (LDT), the pedunculopontine tegmentum (PPT)) (Semba, 2000).

The third neuronal population comprises glutamate-synthesizing cells which overlap with the cholinergic and the GABAergic neurons and represent the vast majority (90%) of the BF cells (Gritti *et al.*, 2006). They project to the cortex where they make synaptic connections with cortical interneurons (Henny and Jones, 2008).

The BF receives input from the brainstem reticular formation through the hypothalamus and sends projections to the cortex, thus constituting a basalo-cortical system which serves as a ventral extrathalamic relay to the cerebral cortex from the brainstem's activating systems (Luppi, 2005). Cortical activation critically depends on the BF inputs to the cortex (Zaborszky and Duque, 2000). The BF plays an important role in cognitive functions such as attention and memory due to its connections with the cortex, the hippocampus and the thalamus (Semba, 2000). It also participates in controlling limbic, motivational, regulatory and motor functions through its projections to the amygdala, the hypothalamus and the brainstem (Semba, 2000).

The BF in sleep regulation. The BF is well-established brain region for sleep regulation. Cholinergic neurons of the BF discharge in rhythmic bursts in association with theta activity, and they promote theta and gamma activity during waking and REM sleep through rhythmic modulation of the cortex (Luppi, 2005). They excite cortical neurons through both muscarinic and nicotinic receptors. Cortical interneurons are activated via fast nicotinic actions which would pace the activity in pyramidal neurons, or via slower muscarinic actions which would excite interneurons and pyramidal cells and consequently stimulate gamma activity that rides upon theta (Luppi, 2005).

Since contrary to the cholinergic cells, the GABAergic BF neurons are physiologically heterogeneous and comprise different types which discharge maximally either with cortical activation or deactivation (Luppi, 2005), it is more difficult to define their role in modulating cortical activity. Moreover, they innervate both cortical interneurons and pyramidal cells, which makes them able to stimulate or pace different EEG activities, whether theta or delta, in cortical networks during various behavioural states (Semba, 2000; Henny and Jones, 2008). 40% of the GABAergic

neurons increase their discharge rate in association with cortical activation, while the majority (60%) decrease their rate (Luppi, 2005). In the latter group there is a part that discharges in an irregular tonic manner during delta activity and is not activated from the cerebral cortex. It presumably affects the posterior hypothalamus, the brainstem and the BF. Another part discharges in bursts of spikes (200 Hz) during delta activity and could be activated from the cortex; thus it probably inhibits cortical neurons during delta activity. To conclude, the GABAergic cells could promote NREM sleep by inhibiting neurons of activating systems in the posterior hypothalamus, the brainstem or the BF and thus attenuating cortical activation, as well as by directly inhibiting cortical neurons (Semba, 2000). They could as well facilitate or inhibit pyramidal cell discharge by inhibiting different subsets of cortical interneurons (Semba, 2000).

Most of the glutamatergic cells discharge in association with cortical activation (Hassani *et al.*, 2009). Since they have synaptic connections with cortical interneurons, they might excite them through NMDA or AMPA receptors in a phasic or tonic manner and this way activate the cortex (Henny and Jones, 2008).

Lesion of the BF results in the EEG slowing in the ipsilateral hemisphere and in reduced arousal (Detari, 2000; Berntson *et al.*, 2002), as well as a decreased amplitude of theta and gamma activities (Henny and Jones, 2008). On the other hand, in an animal model of Alzheimer disease associated with a striking loss of BF cholinergic cells, mice show a blunted delta power response in recovery NREM sleep, which might be partly explained by the cholinergic deficiency (Wisor *et al.*, 2005). Altogether this emphasizes the importance of the BF in promoting cortical arousal and its possible role in the build-up of sleep pressure.

Adenosine in the BF. Adenosine shows a diurnal variation in concentration during the spontaneous sleep-wake cycle (Strecker *et al.*, 2000). Also a diurnal variation in the activity of adenosine metabolic enzymes such as ADK, ecto-5'-nucleotidase and endo-5'-nucleotidase can be observed in the BF and the cortex (except endo-5'-nucleotidase for cortex) (Alanko *et al.*, 2003a; Mackiewicz *et al.*, 2003). Basal adenosine levels in the BF are 60-120 nM in rats and 165-300 nM in cats (Strecker *et al.*, 2000). Compared to waking, its levels decrease during NREM sleep in BF, cortex, ventro-anterior and ventrolateral thalamic nuclei, and POAH as shown by microdialysis (Porkka-Heiskanen *et al.*, 2000). However, during 6-h SD adenosine levels rise selectively in the cat BF and to a lesser extent in the cortex (Porkka-Heiskanen *et al.*, 2000). This difference between the two brain regions might be partially explained by a reduced activity of the ENT1 transporter during SD in the BF but not in the cortex (Alanko *et al.*, 2003b). Other tested regions which are involved in behavior control and comprise ventro-anterior and ventrolateral thalamic nuclei, POAH, PPT and DRN, do not exhibit an adenosine response to SD (Porkka-Heiskanen *et al.*, 2000). Furthermore, inhibition of adenosine transport in the BF but not in the thalamus mimics the sleep-inducing effect of SD (Porkka-

Heiskanen *et al.*, 1997), and in correlation with this data, lesion of the BF cholinergic cells results in nearly complete abolition of the adenosine response to SD as well as of recovery sleep (Kalinchuk *et al.*, 2008). Finally, perfusion of A_1R antisense oligonucleotides to the BF reduces both delta activity in recovery sleep and recovery NREM sleep amount (Thakkar *et al.*, 2003b). Altogether this accentuates the specificity of adenosine effects in the BF for the mediation of sleep homeostasis. However, it should be noted that other brain regions which are active during waking and not tested previously may also show adenosine responses to SD. They include the histaminergic tuberomammillary nucleus (TMN), the noradrenergic LC, the dopaminergic ventral tegmental area (VTA), the cholinergic LDT, and the orexinergic lateral hypothalamic area. Since many of these regions are too small and/or are morphologically scattered, microdialysis used in the aforementioned studies may not be a suitable technique in their case. Also, it would be of interest to explore some of the already tested brain areas with another method, e.g. with microsensors.

Adenosine modulates the discharge rates of BF neurons, thus affecting the state of vigilance. Microdialysis perfusion of adenosine or A_1R agonist into the BF decreases waking and suppresses the discharge activity of BF neurons, while A_1R antagonists have an opposite effect (Rainnie *et al.*, 1994; Portas *et al.*, 1997; Alam *et al.*, 1999; Strecker *et al.*, 2000; Thakkar *et al.*, 2003b). Both the BF cholinergic and non-cholinergic cells are inhibited by adenosine (Arrigoni *et al.*, 2006), the former via an inwardly rectifying K^+ current, the latter via a hyperpolarization-activated cation current (Bjorness and Greene, 2009). However, some BF neurons exhibit excitation in response to adenosine and adenosine transport inhibitor, reduce their discharge rate in response to A_1R antagonist, and therefore are sleep-related (Alam *et al.*, 1999). In another, more recent, study adenosine and A_1R agonist reduced the discharge rate of the BF waking-active neurons but did not affect sleep-active neurons (Thakkar *et al.*, 2003a). Therefore, they may act state-specifically and have their maximum effect in active waking in contrast to NREM sleep (Thakkar *et al.*, 2003a).

3- and 6-h SD induces an increase in A_1R mRNA in the rat BF in contrast to the cortex, while $A_{2A}R$ mRNA is undetectable in the BF (Basheer *et al.*, 2001). 24-h SD raises the A_1R protein amount in the BF and, at the level of tendency, in the cortex (Basheer *et al.*, 2007). A more recent study demonstrated that 24-h SD upregulates A_1R density in the rat parietal cortex, thalamus, and caudate-putamen (Elmenhorst *et al.*, 2009). These data are consistent with human data showing A_1R upregulation in frontal, orbitofrontal, occipital and temporal cortices and striatum following 24-h SD (Elmenhorst *et al.*, 2007). To sum up, it could be speculated that longer sleep loss recruits the homeostatic control via A_1R which is characteristic for many brain regions besides the BF.

Activation of NF-κB via recruiting the A_1R signaling pathway is the most probable cause for the increased levels of A_1R mRNA (Ramesh *et al.*, 2007). When acting through A_1R, adenosine induces intracellular calcium release in the BF exclusively in the cholinergic neurons (Ramesh *et al.*, 2007). This results from phospholipase C and protein kinase C activation and a consequent production of inositol trisphosphate (IP3), which binds to IP3 receptors and mobilizes calcium stores from the endoplasmic reticulum. Calcium release occurs at higher concentrations of adenosine (Basheer *et al.*, 2002); this agrees with the observation that activation of phospholipase C via G_{i3} protein takes place at higher adenosine levels (Biber *et al.*, 1997). Intracellular calcium release might lead to NF-κB nuclear translocation, where the latter would regulate the transcription of many genes including A_1R, interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), and nitric oxide synthase. NF-κB translocation to the nucleus in the BF happens exclusively in the cholinergic neurons following 3-h SD (Ramesh *et al.*, 2007). Moreover, both 3- and 6-h SD enhance DNA binding activity of NF-κB selectively in HDB/SI/MCPO compared to areas of the more medial BF and the cingulate cortex (Basheer *et al.*, 2001). As a blocking of NF-κB nuclear translocation prior to 6-h SD reduces delta activity in recovery sleep (Ramesh *et al.*, 2007), it indicates that recruiting of the NF-κB pathway provides a positive feedback loop for recovery sleep promotion by adenosine and enhances its sleep-inducing effects. This event is specific for sleep homeostasis, since there are no differences in A_1R density in spontaneous wake and rest (Ramesh *et al.*, 2007). The cause for the site-specificity remains unclear, and it has been speculated that the molecular interactions with accessory proteins or receptor homo/heterodimerization might be involved (Basheer *et al.*, 2002).

1.2. ADENOSINE INVOLVEMENT IN MAJOR DEPRESSIVE DISORDER AND DISTURBED SLEEP

1.2.1. MAJOR DEPRESSIVE DISORDER

Definition and classification of major depressive disorder. Major depressive disorder (MDD) is defined by episodes of depressed mood lasting for two weeks at a minimum and accompanied by four or more of the following symptoms: (1) significant loss of interest or pleasure in almost all activities, (2) significant loss or gain of weight, (3) insomnia or hypersomnia, (4) psychomotor agitation or retardation, (5)

fatigue or loss of energy, (6) feelings of guilt or worthlessness, (7) reduced concentration and ability to think, (8) thoughts of death or suicide (American Psychiatric Association and American Psychiatric Association. Task Force on DSM-IV, 1994). In comparison to normal emotions of sadness and bereavement, depression is disproportionate to the feelings' cause and often lasts even after the external cause of the emotions has vanished (Belmaker and Agam, 2008). Five subtypes of MDD are further recognized: melancholic depression, atypical depression, catatonic depression, postpartum depression and seasonal affective disorder (American Psychiatric Association and American Psychiatric Association. Task Force on DSM-IV, 1994). The diagnosis of MDD is complicated by the fact that depressive symptoms might be generated within an undeclared bipolar disorder, thyroid disease, undiagnosed brain tumour, or the earliest neurodegenerative stages of Parkinson's or Huntigton's disease (Matthews *et al.*, 2005). The MDD lifetime prevalence in Finland is 4.1% for men and 7.7% for women (age 30-64 years old) according to the results of the Finnish population survey Health 2000 performed in 2000-2001 (results are published online on http://www.terveys2000.fi/julkaisut/baseline.pdf). Risk factors include stressful life events and emotional trauma, endocrine abnormalities (hypothyroidism and hypercortisolism), viral infections (e.g. Borna virus), cancer, side effects of drugs, and even stochastic processes during brain development (Nestler *et al.*, 2002; Krishnan and Nestler, 2008). Twin studies estimate 37% heritability of MDD, with severe recurrent and early-onset forms having higher genetic contributions (Sullivan *et al.*, 2000). Women demonstrate greater heritability (42-49%) than men (25-29%) (Kendler *et al.*, 2006; Orstavik *et al.*, 2007). However, no major gene has been identified presumably due to the fact that MDD is a complex and highly heterogeneous disorder comprising multiple subtypes with many genes involved. Thus, the effect of a single gene might be relatively small and difficult to detect.

Despite numerous studies, the pathophysiology of MDD remains unclear owing to many factors. First, compared to other diseases, mental disorders are difficult to study since observing pathological changes in the brain is more complicated than in other organs. For obvious reasons, studies of the human brain are limited to non-invasive imaging techniques which give an idea about differential patterns of activity in various brain regions of depressed individuals, but cannot explain all the symptoms and mechanisms of MDD. To explore the molecular mechanisms, gene expression and protein levels can be measured in post-mortem brain tissues. This type of studies comprises numerous limitations reviewed in *Section 1.3.1*. Therefore, animal models are used to mimic symptoms of depression, and their advantages and limitations are described in the same section.

Brain metabolism in MDD. MDD is accompanied by regional and global brain hypometabolism (Iosifescu *et al.*, 2008). Metabolism decreases in the dorsome-

dial and dorso-anterolateral prefrontal cortex and the subgenual cingulate cortex (subregion of the prefrontal cortex) and increases in the amygdala and the ventrolateral prefrontal cortex (Drevets *et al.*, 1997; Drevets *et al.*, 2002; Engels *et al.*, 2010). These metabolic abnormalities are reversed by selective serotonin reuptake inhibitor (SSRI) treatment (Drevets *et al.*, 2002). Also, the antidepressant role of thyroid hormones might be related to their action on brain metabolism (Iosifescu *et al.*, 2008). Other studies have demonstrated increased activity in the amygdala and the subgenual cingulate cortex in depressed individuals, which correlates with negative emotions (Mayberg *et al.*, 1999; Engels *et al.*, 2010).

MDD theories. At present there are several theories attempting to explain all the diversity of the MDD symptoms and the pathophysiological mechanisms. They are described below.

a) Monoamine deficiency theory. Clinical observations which indicated that drugs enhancing serotonin and noradrenaline transmission have antidepressant effects laid the foundation for the monoamine deficiency hypothesis of MDD (Krishnan and Nestler, 2008). Further evidence exists in support of this hypothesis: (1) depressed patients display a 30% increase of the enzyme monoamine oxidase A which metabolizes serotonin and noradrenaline (Meyer *et al.*, 2006); (2) depressed patients show a reduced sensitivity of serotonin 5-HT_{1A} receptors and an increased sensitivity of the α2-noradrenergic receptor (Ordway *et al.*, 2003; Pitchot *et al.*, 2005); (3) a functional single nucleotide polymorphism (SNP) in the 5-HT_{1A} receptor associates with depression (Strobel *et al.*, 2003), as well as a decrease in ligand binding in the forebrain and the raphe (Lesch, 2004); (4) mice with a deletion of the *Slc6a4* gene encoding the serotonin transporter are excessively anxious (Ansorge *et al.*, 2004); (5) humans carrying a short allele in the promoter of the *SLC6A4* gene associated with reduced transporter activity are at slightly higher risk for MDD (Lotrich and Pollock, 2004). The latter fact is especially interesting in terms of gene-environment interactions. When coupled with stressful life events, the short allele is able to moderate their effect on depression (Caspi *et al.*, 2003). Therefore, some researchers suggest that *SLC6A4* SNPs may not directly associate with MDD but modulate the serotonergic response to stress (Uher and McGuffin, 2008). In addition, these short allele carriers demonstrate hyperactivity in the amygdala in response to aversive signals (El Hage *et al.*, 2009), which is a characteristic feature observed in depressed patients.

However, there are findings which demonstrate the insufficiency of the monoamine theory. First, experimental depletion of monoamines produces mild mood reduction in unmedicated depressed patients, but does not alter mood in healthy individuals (Ruhe *et al.*, 2007). Oral tryptophan depletion does not induce depression in healthy subjects, but causes a relapse in patients successfully treated with SSRIs (Ruhe *et al.*, 2007). Second, it takes several weeks before antidepressants

start to act (Covington *et al*., 2010), which could mean that enhanced serotonergic and noradrenergic neurotransmission per se are not responsible for the clinical actions of these drugs. Rather, some gradually developing adaptations might be important, such as neuroplastic changes involving transcriptional and translational alterations. However a recent meta-analysis demonstrated that SSRIs effect is more rapid (Taylor *et al*., 2006). Third, recent meta-analyses and comparison with placebo showed that only patients with severe depression respond to antidepressant treatment, while patients with milder depressive symptoms tend to respond more readily to placebo (Kirsch *et al*., 2008; Turner *et al*., 2008; Fournier *et al*., 2010). It is also possible that severely depressed individuals have a reduced response to placebo effect rather than an increased response to medication. Finally, regardless of the antidepressant used, only half of the patients improve with the first medication, only one-third remit, while even after multiple trials one-third of the patients do not achieve remission (Esposito *et al*., 2009). To conclude, monoamine deficiency may represent a vulnerability trait but might not be the real cause for depression. It can be speculated that the dysfunction at the level of second messenger systems is a primary mechanism in this paradigm, and that antidepressants relieve depression by affecting the second messenger signaling as well as reducing secondary stress caused by dispirited mood rather than by directly elevating mood.

b) Neurotrophin deficiency theory. Support for this hypothesis came from studies which demonstrated that several forms of stress reduce brain-derived neurotrophic factor (BDNF) signaling in the hippocampus, whereas chronic treatment with antidepressants enhances this signaling (Duman and Monteggia, 2006). Furthermore, post-mortem and neuroimaging studies indicate reductions in grey-matter volume and glial density in the prefrontal cortex and the hippocampus, cell loss in the subgenual and the dorsolateral prefrontal cortex as well as the orbitofrontal cortex, and increased numbers of cells in the hypothalamus and the DRN (Rajkowska, 2000; Krishnan and Nestler, 2008). Volumetric changes are also evident in the amygdala and the basal ganglia (Campbell and MacQueen, 2006). Finally, several antidepressants promote hippocampal neurogenesis and elevate the amounts of BDNF and vascular endothelial growth factor (Covington *et al*., 2010). Altogether these data suggest that deficiency in neurotrophic factors may result in the observed neuronal and glial alterations. In accordance with this hypothesis, BDNF polymorphisms might be linked to depression-related vulnerabilities in humans (Duncan *et al*., 2009; Gatt *et al*., 2009). However, a number of studies contradict this theory. First, mice with conditional forebrain deletion of BDNF or its receptor do not display depressive-like behavior, and modulating BDNF or BDNF receptor TrkB activity is clinically ineffective (Tanis *et al*., 2007; Sen and Sanacora, 2008). Interestingly, only female BDNF knockout mice exhibit depressive-like behavior (Monteggia *et al*., 2007). Second, a polymorphism in the BDNF gene which results

in impaired BDNF release and reduced hippocampal volume does not alter vulnerability to depression (Gratacos *et al.*, 2008). Third, in the VTA and the nucleus accumbens BDNF has pro-depressive effects (Eisch *et al.*, 2003). Fourthly, reduced neurogenesis does not produce depression by itself (Sahay and Hen, 2007). Finally, the BDNF precursor pro-BDNF has an opposing influence and can, upon binding to $p75^{NTR}$ receptors, elicit long-term depression, reduce spine density in hippocampal neurons and induce apoptosis in the BF neurons (Covington *et al.*, 2009). To conclude, BDNF seems to have brain region specific effects with dichotomical actions. Reduced neurogenesis might be a result of stress but is not necessarily behaviorally relevant (Henn and Vollmayr, 2004; Castren and Rantamaki, 2010).

c) Hypothalamo-pituitary-adrenal (HPA) axis dysfunction theory. Activation of the HPA system in response to stress involves secretion of the corticotropin-releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus, which induces production and release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH in its turn stimulates glucocorticoid (GC) (cortisol in humans, corticosterone in rodents) production and release from the adrenal cortex. GCs suppress the CRH and ACTH synthesis in a negative feedback loop and exert multiple metabolic and behavioral effects (Nestler *et al.*, 2002).

Patients with depression show elevated levels of cortisol in the plasma (Burke *et al.*, 2005), of CRH in the cerebrospinal fluid (Nestler *et al.*, 2002), and of CRH mRNA and protein in the limbic regions (Merali *et al.*, 2004). CRH has anxiogenic and anorexinegic properties (Nestler *et al.*, 2002). As it affects activity, appetite, and sleep, it has been assumed that CRH hypersecretion may partially contribute to the emotional and behavioral aspects of MDD (Sato *et al.*, 2008). CRH receptor antagonists have antidepressant effects in animal models of depression (Louis *et al.*, 2006). Polymorphism in the *CRHR1* gene associates with such markers of MDD severity as early age onset of the first depressive episode and a seasonal pattern of depression implying a recurrent character (Papiol *et al.*, 2007). Moreover, polymorphisms in genes encoding GC and mineralocorticoid receptors associate with MDD (El Hage *et al.*, 2009). The hyperactivity of the HPA axis in MDD might result from GC resistance as a consequence of decreased GC receptor function or expression in the brain (Pariante, 2006): hypercortisolaemia in depression is manifested as impaired GC-mediated negative feedback, adrenal hyper-responsiveness to ACTH, and hypersecretion of CRH. GC receptor deficient mice represent CRH overexpression and oversecretion in the PVN (Sato *et al.*, 2008). Chronic administration of tricyclic antidepressants and SSRIs reduces CRH mRNA levels in the PVN (Wong *et al.*, 1996), the effect being associated with a suppression of mRNA levels of type I or II GC receptors in the hippocampus.

However, recent studies show that hypercortisolaemia exists almost only during very severe depressive episodes (Brouwer *et al.*, 2005; Schatzberg and Lindley,

2008), and compounds blocking GC receptors are efficacious only in case of severe depression (Flores *et al.*, 2006). Only 35% of the MDD patients might have the HPA axis abnormalities (Stokes *et al.*, 1984). Moreover, the manic phase of bipolar disorder associates to a very similar neuroendocrine dysfunction (Matthews *et al.*, 2005). Interestingly, atypical depression characterised by hyperphagia and hypersomnia associates with hypocortisolaemia and therefore has presumably a different pathogenetic cascade (Gold and Chrousos, 2002; Brouwer *et al.*, 2005). Also, since the HPA axis plays a major role in stress, it is difficult to define the order of events: stress may be causative in some cases and secondary to depressed mood in others. The pre-existing HPA axis abnormalities might contribute to the genesis of severe forms of depression (Matthews *et al.*, 2005). In contrast, there is an interesting speculation that a subgroup of depressed patients without HPA dysfunction during acute phases may develop a chronic form of the disorder (Watson *et al.*, 2002).

d) Cytokine theory. Multiple data indicate that inflammatory processes may underlie depression. Depressed patients display increased levels of proinflammatory cytokines (IL-1β,2,6,8,12, interferon γ, TNF-α) and a relative shortage in the anti-inflammatory cytokine IL-10 (Maes *et al.*, 2009). Around 30% of the individuals treated with interferons develop depression as a side effect (Loftis and Hauser, 2004). In rodents, administration of cytokines induces depressive-like symptoms such as anhedonia and anxiety (Anisman *et al.*, 2002). Also psychological stressors increase IL-1β and IL-6 levels in the blood and various brain regions of experimental animals (Maes *et al.*, 2009). IL-6 and TNF-α receptor knockout mice display antidepressive-like phenotypes (Chourbaji *et al.*, 2006; Simen *et al.*, 2006). In animal models of depression, such as chronic mild stress and olfactory bulbectomy models, a detected inflammation is reversed by antidepressant treatment (Maes *et al.*, 2009). Similarly, blocking proinflammatory cytokine-mediated signaling can produce antidepressant effects (Maes *et al.*, 2009).

However, clinical studies concerning depression-associated cytokine concentrations are inconsistent (Dunn *et al.*, 2005), and cytokine-induced depressive symptoms are temporary and not replicated in all studies (Spalletta *et al.*, 2006). Some data indicate that immune and cytokine alterations are more characteristic for melancholic depression (Maes, 1999). Hence it is possible that immune activation may be a signature of a small subset of depression cases.

e) Gene-environment interactions. Low MDD concordance between monozygotic twins (31% for men and 48% for women) and individual differences in response to stress among inbred rodents suggest that other factors than genes contribute to MDD heritability (Mill and Petronis, 2007). It might be an environmental effect as well as an interaction between genes and environment which is leading to the expression of environmental effects only in the presence of a permissive genetic background. For example, in their pioneer study Caspi et al. demonstrated that po-

lymorphism in the serotonin transporter gene regulates the effect of stressful life events on the susceptibility to depression and is stronger in individuals carrying at least one short allele (Caspi *et al.*, 2003). Next, individual exposure to environmental stressors is itself strongly influenced by genetic factors, and predisposed individuals may choose more risky environments (Plomin *et al.*, 1990; Kendler *et al.*, 1993). These inherited factors strongly correlate with those affecting MDD susceptibility (Kendler and Karkowski-Shuman, 1997). Finally, an individual epigenetic profile may increase susceptibility to depression. Epigenetics refers to the heritable but reversible regulation of various genomic functions which is mediated through changes in DNA methylation and chromatin structure via post-translational modifications of histone N-terminal tails (acetylation, methylation and phosphorylation). For example, poor maternal care expressed as low rates of maternal licking and grooming results in increased methylation of GC receptor gene promoter and low GC receptor expression in the hippocampus and in elevated anxiety in adult offspring (Weaver *et al.*, 2004). Epigenetic mechanisms control quantity, location and timing of gene expression. The investigation of depression focused on two main modifying processes (Mill and Petronis, 2007):

- DNA methylation which occurs at position 5 of cytosine's pyrimidine ring in CpG dinucleotides in promoter regulatory regions of many genes might reflect the effect of maternal care on emotional behavior. Methylation disrupts the binding of transcription factors and attracts methyl-binding proteins associated with gene silencing and chromatin compaction (Mill and Petronis, 2007).
- Histone acetylation associates with transcriptional activation due to chromatin decondensing and seems to be a key substrate for antidepressant action (Tsankova *et al.*, 2004). Antidepressant action of imipramine requires an increased histone acetylation at the *Bdnf* promoter in the hippocampus (Tsankova *et al.*, 2006), and histone deacetylase (HDAC) inhibitors are therapeutically advantageous (Schroeder *et al.*, 2007).

DNA methylation and changes in chromatin structure can interact in a number of ways. For example, methyl CpG-binding protein 2 binds to methylated cytosines and attracts HDACs (Mill and Petronis, 2007). The epigenetic profile of somatic cells is inherited from maternal to daughter chromatids during mitosis (Mill and Petronis, 2007). The pattern is not completely erased during meiosis and thus can be transmitted from generation to generation (Mill and Petronis, 2007). Profound epigenetic differences arise during the lives of monozygotic twins (Fraga *et al.*, 2005). They may result from environmental, hormonal and random stochastic factors including dietary intake (Waterland and Jirtle, 2004), certain drugs (Numachi *et al.*, 2004), and psychosocial effects (Weaver *et al.*, 2004).

To conclude, epi-alleles and epi-haplotypes combining both DNA sequence and epigenetic information may be better predictors for MDD. Some alleles and haplo-

types tend to be associated with specific epigenetic profiles (Dempster *et al.*, 2006; Polesskaya *et al.*, 2006). It should be noted, however, that those epigenetic changes might be also induced by MDD.

f) Circadian disruptions. Multiple rhythms are disrupted in MDD, including profiles of cortisol, growth hormone, melatonin, prolactin, and body temperature, as well as sleep timing and structure (Turek, 2007; Lavebratt *et al.*, 2010). One of the most consistent circadian abnormalities is a phase advance of the circadian rhythm expressed as a short latency to the first REM sleep episode, plus early morning awakening, early morning rise of ACTH and nocturnal elevation of prolactin and growth hormone (Turek, 2007). Most, if not all tissues, might contain molecular circadian clocks (Panda *et al.*, 2002). However, the diversity of dysfunctional rhythms in MDD indicates that the origin of these disruptions is central and locates within the suprachiasmatic nuclei of the anterior hypothalamus. Also, the possibility that the disruption of one rhythm may lead to the other rhythms' abnormalities should not be excluded. For example, early onset of REM sleep may result in advanced phase positions of other circadian rhythms.

Disorganization of the circadian system could trigger neurobiological dysfunctions and a disturbed mental state. A circadian disturbance of the normal sleep-wake cycle could lead to insomnia and other sleep abnormalities, since the circadian clock regulates not only the timing of sleep, but other sleep parameters such as sleep duration, intensity and pattern (see *Section 1.2.1*). For instance, *Clock* mutant mice have their NREM sleep reduced by 1-2 h per day, and an attenuated REM sleep rebound (Naylor *et al.*, 2000). In supportnof this hypothesis, MDD patients with *CLOCK* C/C variant polymorphism are more likely to experience insomnia (Serretti *et al.*, 2003).

Further evidence comes from genome association studies. In humans, *CRY2* and *NPAS2* polymorphisms associate with MDD (Soria *et al.*, 2010). *TIMELESS* variants associate with depression accompanied by symptoms of disturbed sleep both in men and women (Utge *et al.*, 2010b). Consistent with the circadian theory, phase advance therapy in which depressed patients are instructed to go to bed and to wake up several hours earlier than according to their common schedule, has an antidepressant effect when combined with SD (Benca *et al.*, 1997).

Additional MDD theories include altered glutamatergic and reduced GABAergic transmission, impaired endogenous opioid function, monoamine-acetylcholine imbalance (Belmaker and Agam, 2008), glial dysfunction (Lesch, 2004) and abnormalities in feeding behavior peptides (Krishnan and Nestler, 2008).

Integration of the current models. Since brain areas are highly interconnected, an integrative framework between these numerous theories for depression may better explain the diversity of the depressive signs. Reduced serotonergic tone may disinhibit activity within the amygdala and the HPA axis (El Hage *et al.*, 2009),

and an overactive amygdala could mediate fear- and anxiety-like responses, as well as further boost cortisol and CRH production (Nestler *et al.*, 2002). Hypercortisolaemia and GR downregulation might also be inflammation epiphenomena, since proinflammatory cytokines (TNF-α, IL-1β, IL-6) activate the HPA axis (Dunn *et al.*, 2005). In mice, elevated corticosterone levels mediate the critical role of IL-1β in chronic-stress-induced depression (Goshen *et al.*, 2008). Some of the stress-induced effects can be prevented by antagonists of cytokine activity, which suggests that cytokines are HPA stress pathway mediators (Anisman and Matheson, 2005). Furthermore, inflammation reduces tryptophan availability, and consequently may promote serotonin depletion (Maes *et al.*, 2009).

TNF-α and IL-1β decrease neurogenesis in selected brain areas, possibly by affecting *BDNF* expression in neurons (Maes *et al.*, 2009). In addition to cytokines, GCs may also interfere with transcriptional mechanisms which control BDNF expression (e.g. CREB), and this way they may reduce the hippocampal neurogenesis (Krishnan and Nestler, 2008). Since the hippocampus has an inhibitory influence on CRH-containing hypothalamic neurons (Nestler *et al.*, 2002), the hippocampal loss observed in MDD might result in their disinhibition and further hyperactivation of the HPA system.

It is likely that each brain region mediates a particular MDD symptom. For example, neocortex and hippocampus could mediate the cognitive aspects (memory impairments, feelings of worthlessness, hopelessness, guilt, and suicidality). The striatum (particularly the ventral striatum and the nucleus accumbens) and the amygdala account for emotional memory and thus could be responsible for anhedonia, anxiety and reduced motivation. The hypothalamus might mediate neurovegetative symptoms, such as sleep disturbances, reduced appetite and energy, loss of interest in sex and other pleasurable activity (Nestler *et al.*, 2002).

To better understand the MDD pathogenesis and to develop more efficient antidepressants, it would be advantageous to study the mechanisms of resilience defined as an ability to prevent, minimize or overcome the adverse effects of stress (Feder *et al.*, 2009). For this purpose animal models are invaluable. For example, in the learned helplessness paradigm, an upregulation of ΔFOSB (product of the *Fosb* gene) in the midbrain periaqueductal grey nucleus promotes a resilient phenotype (Berton *et al.*, 2007). In the social defeat paradigm, resilient mice upregulate potassium channels in the VTA, thus reducing the excitability which increases in depression, and maintain low BDNF signaling in the nucleus accumbens (Berton *et al.*, 2007). Epigenetic mechanisms might play an important role in creating a resilient phenotype, since they can explain the interindividual differences in stress responses among genetically inbred mice housed under identical environmental conditions.

To conclude, from multiple theories attempting to explain the MDD pathogene-

sis and from the heterogeneity in terms of clinical presentation, age of onset, course of illness, treatment response, it is obvious that MDD does not reflect a single process. Most probably it comprises several subgroups which have distinct causes and pathophysiology and should be treated differently. Antidepressants should not only prevent deleterious effects of stress but also promote resilience. To overcome dependence and tolerance and overdoses of antidepressants, it would be advantageous to use receptor allosteric modulators, since binding to allosteric sites permits to change the efficacy of endogenously available receptor ligands.

1.2.2. SLEEP IN MAJOR DEPRESSIVE DISORDER

The MDD sleep pattern comprises changes in sleep continuity and in NREM and REM sleep (Steiger and Kimura, 2010). Sleep continuity abnormalities include prolonged sleep latency and frequent nocturnal and early morning awakenings (Benca *et al.*, 1997). Changes in NREM sleep are characterized by reduced amounts of deep stages of NREM sleep and delta activity, and by increased duration of light stage 1 (Meerlo *et al.*, 2008). Disinhibition of REM sleep in depressed patients is expressed as reduced latency to the first REM sleep period, sleep onset REM periods, prolonged first REM sleep periods, increased REM sleep amounts in the first half of the night and more rapid eye movements during REM sleep (Benca *et al.*, 1997). Of these features early morning awakenings are more characteristic for the severe, melancholic type of depression (Kiloh and Garside, 1963). Reduced sleep quality resulting from shallow sleep might lead to such a core symptom of depression as fatigue, and indeed the severity of fatigue correlates with compromised sleep quality (Ferentinos *et al.*, 2009). Disturbed sleep may also inhibit neurogenesis in the hippocampus (Meerlo *et al.*, 2009) and increase the levels of proinflammatory cytokines (Vgontzas *et al.*, 2004), which could add to the development of depression or exacerbate its symptoms. EEG changes in depressed patients include reduced delta power throughout the night (Steiger and Kimura, 2010) and amplified alpha and beta activity overall (Meerlo *et al.*, 2008).

80% of depressed patients complain about insomnia, whereas hypersomnia is rarer (15-35%) (Steiger and Kimura, 2010) and rather present in atypical depression (American Psychiatric Association and American Psychiatric Association. Task Force on DSM-IV, 1994). Chronic insomnia is more strongly associated with depression than with any other medical or psychiatric illness (Benca *et al.*, 1997). People with insomnia are at 9.82 times higher risk of MDD than non-insomniacs, as shown by cross-sectional study (Taylor *et al.*, 2005). Longitudinal twin study has demonstrated that poor sleep predicts life dissatisfaction, which is a risk factor

for MDD, whereas the reverse order of events is not evident (Paunio *et al.*, 2009). Also, insomnia can precede depressive episodes (Szklo-Coxe *et al.*, 2010). A variety of symptoms associates with insomnia, including fatigue, difficulty in concentrating, irritability, loss of interest in social activities, which could all eventually lead to depression (Turek, 2005). Although insomnia and depression have a bidirectional relationship, it is clear that not all insomniacs become depressed and not all depressed individuals are insomniacs (Turek, 2005).

Similarly to MDD, insomnia is accompanied by hyperarousal, indicated by increased body temperature, energy metabolism, ACTH and cortisol secretion and sympathetic tone (Staner *et al.*, 2003). The homeostatic process deficiency hypothesis suggests an attenuated build-up of sleep pressure during waking as a cause for reduced delta activity and early emergence of REM sleep (Borbely and Wirz-Justice, 1982). Both reduced sleep propensity and hyperarousal could contribute to sleep initiation and maintenance problems in MDD. However, when comparing depressive insomniacs and patients with primary insomnia, only primary insomniacs display hyperarousal expressed as a lack of gradual decrease of alpha (8.0-12.5 Hz) and beta 1 (13.0-21.5 Hz) power during the sleep onset period (Staner *et al.*, 2003). Depressive insomniacs exhibit less dynamic changes in delta activity during the first NREM period with an earlier peak value, which is consistent with the homeostatic process deficiency hypothesis (Staner *et al.*, 2003). However, this result can not be generalized to all forms of depression with insomnia, since the most severe subtypes, such as melancholic and psychotic depression, are accompanied by physiological hyperarousal (Staner *et al.*, 2003).

The existence of common substrates for sleep and mood regulation could explain the close correlation between poor sleep and MDD. For example, reduced monoaminergic activity and elevated cholinergic sensitivity and neurotransmission might disinhibit REM sleep, resulting in short REM sleep latency and increased REM sleep amount and REM density (Benca and Peterson, 2008; Meerlo *et al.*, 2008). Increased levels of CRH due to the HPA axis hyperactivity would suppress NREM sleep and promote REM sleep (Benca and Peterson, 2008). CRH-1 receptor antagonism increases NREM sleep and reduces the number of awakenings as well as REM density in depressed patients (Steiger and Kimura, 2010). Also elevated cortisol is antagonistic to sleep deepening (Steiger, 2002). Finally, BDNF deficiency might reduce EEG delta activity during NREM sleep (Faraguna *et al.*, 2008), thus leading to shallow sleep.

The antidepressant effect of SD. Intriguingly, although insomnia is one of the most troublesome symptoms of MDD, SD alleviates depression (Giedke and Schwarzler, 2002). In contrast to healthy individuals, total SD leads to increase in depressed mood in morning types (Selvi *et al.*, 2007). SD, whether total or partial, profoundly and rapidly affects depressed mood, while a subsequent sleep reverses

this improvement (Wirz-Justice and Van den Hoofdakker, 1999). Even short bouts of microsleep during SD may prevent its antidepressant effect (Hemmeter *et al.*, 1998). There are several possible mutually non-exclusive mechanisms to explain the alleviating effect of SD: (1) According to the homeostatic process deficiency model, SD restores the level of the homeostatic factor back to normal. Consistent with it, REM SD requires weeks to elicit an antidepressant effect (Vogel *et al.*, 1980). (2) Mood swings might be grounded in the interaction of circadian rhythm and sleep-wake cycle (Wirz-Justice and Van den Hoofdakker, 1999), and SD could change the phase-angle between sleep need and advanced circadian pacemaker, thus exerting its therapeutic effect. A large variability of diurnal mood fluctuations predicts a more favourable response to SD (Gordijn *et al.*, 1994). The fact that relapse can be prevented when a total SD is followed by an acute 6-h phase-advance of sleep and then a gradual 1-h per day return to the normal sleep schedule, supports this hypothesis (Wirz-Justice and Van den Hoofdakker, 1999). However, the possibility that the duration of sleep during a shifting timetable could also change should not be excluded. (3) Positive expectations for patients who perceive sleep loss as a central problem may per se improve their mood. However, it is then not clear why recovery sleep would reverse the effect. (4) In contrast to sleep, SD provokes different patterns of neurotransmitter levels. Down-regulation of the presynaptic 5-HT_{1A} receptors during SD might increase circulating serotonin levels (Prevot *et al.*, 1996). The finding that dopamine reuptake inhibitor prevents the antidepressant effects of repeated SD suggests a dopaminergic mechanism for the therapeutic effect of SD (Wirz-Justice and Van den Hoofdakker, 1999). Consistent with this hypothesis, an antidepressant may potentiate SD's therapeutic effect and prevent relapse (Wirz-Justice and Van den Hoofdakker, 1999). (5) Specific gene expression patterns during SD might have an antidepressant effect. (6) SD-induced cerebral fatigue might break the distressing state of hyperarousal and normalize the abnormally elevated glucose metabolism in the cingulate cortex (Wirz-Justice and Van den Hoofdakker, 1999). In accordance with this point of view, behavioral activation is related to favourable SD response (Wirz-Justice and Van den Hoofdakker, 1999).

1.2.3. ADENOSINE AND MAJOR DEPRESSIVE DISORDER

Indirect evidence for the possible role of adenosine in MDD is currently available. First, consumption of the adenosine receptor antagonist caffeine modifies the mood of healthy volunteers and psychiatric patients, and caffeine withdrawal induces anxiety-like symptoms such as irritability, sleepiness, dysphoria, nervousness or restlessness (Cunha *et al.*, 2008). However, depressed patients tend to have

stronger caffeine-induced anxiety effects (Cunha *et al.*, 2008) and are the least caffeine consumers compared to other psychiatric patients (Rihs *et al.*, 1996). Second, such MDD therapeutic strategies as SD and electroconvulsive therapy cause short- and long-term adaptations of the adenosine modulation system (Cunha *et al.*, 2008). Short-term adaptations are carried out through A_1R affecting delta activity and cerebral metabolic activity, while long-term – through upregulation of A_1R and possibly $A_{2A}R$ (Cunha *et al.*, 2008). Finally, both A_1R and $A_{2A}R$ knockout mice display anxiety and reduced exploratory behavior (El Yacoubi *et al.*, 2003; Fredholm *et al.*, 2005b).

There are several possible mechanisms which could explain these effects. Adenosine is able to modulate the release of acetylcholine, glutamate, GABA, dopamine and serotonin via A_1 and $A_{2A}R$ (Dunwiddie and Masino, 2001), and thus modify mood. Recent evidence indicates that signaling through $A_{2A}R$ might be especially important in affecting mood. For example, extrastriatal $A_{2A}R$ play a prominent role in excitatory effects on psychomotor activity (Shen *et al.*, 2008), and in astrocytes $A_{2A}R$ control the uptake of excitatory aminoacids (Dare *et al.*, 2007). Next, $A_{2A}R$ antagonists prevent restraint-stress-induced synaptic damage in the hippocampus (Cunha *et al.*, 2006). Antidepressant effects of adenosine antagonism might also involve nitric oxide and cyclic GMP effects on capillary vasodilation and actions on opioid systems (Cunha *et al.*, 2008). However, the antidepressant effects of $A_{2A}R$ antagonists as evaluated by forced swim test and tail suspension test (Hodgson *et al.*, 2009) are difficult to interpret correctly, since they might be mediated by adenosine control of motor functions via $A_{2A}R$ in striatopallidal GABAergic neurons (Hodgson *et al.*, 2009). Hence, it is necessary to perform other unbiased tests.

Heterodimerization is another mechanism that could explain $A_{2A}R$ signaling effects. $A_{2A}R$ form heterodimers with dopamine D_2 receptors, which are the targets of psychoactive medications (Fuxe *et al.*, 2005). Also, there is a tight interaction between $A_{2A}R$ and TrkB receptors (Jeanneteau and Chao, 2006). $A_{2A}R$ control BDNF actions, either through acute transactivation, or by chronic normalisation of its signaling (Cunha *et al.*, 2008). Furthermore, costimulation of $A_{2A}R$ and fibroblast growth factor receptors, which presumably have physical interaction, profoundly increases neurite formation and spine density (Flajolet *et al.*, 2008).

Adenosine has neuroprotective properties which may be recruited during inflammatory processes in MDD. Neuroprotection can be mediated by A1R (Aden *et al.*, 2001) or by A2AR antagonism (Aden *et al.*, 2003). The latter may normalize glutamatergic synapses and suppress mitochondria-induced apoptosis and neuroinflammation (Cunha *et al.*, 2008). In addition, adenosine reduces the release of corticotropin and cortisol (Cunha *et al.*, 2008), thus weakening the HPA hyperactivity.

Interestingly, the serum levels of the adenosine-metabolizing enzyme ADA are elevated in patients with MDD (Herken *et al.*, 2007), and this increase persists af-

ter antidepressant treatment. Negative correlation between ADA levels and duration of illness may suggest that ADA is an indicator of acute phases of the disease (Herken *et al.*, 2007). It is difficult to interpret these data in terms of their effects on adenosine levels since another study demonstrated decreased ADA activity in MDD (Elgun *et al.*, 1999).

Apart from the neurochemical mechanisms, adenosine effects on depression may be mediated through its effects on sleep. Depression is characterized by decreased delta power in NREM sleep (Kupfer *et al.*, 1984; Berger *et al.*, 2003), which reflects decreased sleep intensity (Borbely, 1982). One of the hypotheses to explain the abnormal sleep pattern in depressive patients proposes a deficiency in a homeostatic factor formed during waking and determining the strength of sleep drive as measured by delta power in NREM sleep (Borbely and Wirz-Justice, 1982). Reduction of delta power and of NREM sleep stages III and IV could result in a shortened latency for REM sleep, shorter sleep cycles at night which could eventually lead to early morning awakenings, and the fatigue observed in depression. One of the most plausible candidates for a homeostatic factor is adenosine (Porkka-Heiskanen *et al.*, 1997). A reduction of NREM sleep slow oscillations (<1 Hz) has been observed in mice with reduced extracellular adenosine levels due to inhibited gliotransmission (Fellin *et al.*, 2009). Interestingly, quantitative trait loci analysis in mice suggests that genes encoding ADA and SAHH at chromosome 2 might contribute to the rate of sleep pressure accumulation (Franken *et al.*, 2001). Moreover, humans with excessive levels of adenosine due to a functional polymorphism in *ADA*, show increased amounts of slow wave sleep and reduced amounts of nocturnal awakenings (Retey *et al.*, 2005). Altogether it might be hypothesized that adenosine deficiency in depression could result in shallow sleep which may aggravate the depressive state or even have a causal effect.

Finally, adenosine levels reflect a cellular energy status since adenosine serves as a building block for adenosine triphosphate and is a by-product of ATP metabolism. Under increased energy demand when ATP is extensively utilized, the levels of adenosine increase as a result of ATP metabolism (Kalinchuk *et al.*, 2003). It has been suggested that metabolic depression defined as a decrease in standard metabolic rates may partially explain fatigue and the loss of energy characteristic for MDD (Tsiouris, 2005). This downregulation of energy production might partially result from impaired adenosine synthesis which, on the other hand, may lead to disturbed sleep and exacerbate the mentioned symptoms. On the other hand, metabolic depression may simply reflect the reduced utilization of energy stores due to reduced motivation and activity.

1.3. STUDYING DEPRESSION WITH DISTURBED SLEEP BY USING AN ANIMAL MODEL

1.3.1. CLOMIPRAMINE MODEL OF DEPRESSION

Animal models are important tools for the investigation of depression, allowing an evaluation of disturbances at the molecular level with invasive methods which for obvious reasons are impossible to use in humans. By making use of more controllable and homogenous conditions, animal models also avoid the numerous shortfalls of human postmortem brain studies, such as clinical heterogeneity due to amount and frequency of therapeutic treatments, concomitant medical conditions, cause of death, agonal factors, and postmortem interval. The models mimic the behavioral features of depression and should fulfil several criteria. One of them is construct validity, which is defined as the accuracy with which the model replicates the key abnormalities and involves similar neurochemical processes (Anisman and Matheson, 2005). Face validity defined as the similarity in the symptom profile is relatively unrealistic in certain respects, since such symptoms as guilt and suicidality are impossible to reproduce in animal models. Nor does an animal model of depression capture the periodic changes of behavior into and out of depression which are seen in depressed individuals. Predictive validity, defined as an ability to correctly identify effective antidepressant treatments, might be helpful, but better information on differential responses to treatment in humans is required (Matthews *et al.*, 2005). Finally, etiological validity defined as an induction by events which are similar to these triggering the human disorder is also a controversial demand, as a better understanding of MDD causes is needed and MDD is not necessarily always triggered by external events. At the moment there is no animal model that fully fulfils all of these criteria. Instead, most of the models focus on particular aspects of depressive behavior. This fact might reflect the lack of an adequate classification of MDD in humans rather than a failure to develop a valid model (Lesch, 2004). Also, it must be noted that the optimal animal model should not necessarily always lead to behavioral disturbances, since not all humans having some vulnerability trait (genetic trait, age, sex, and previous stressful experience) or encountering adverse events develop MDD (Anisman and Matheson, 2005).

The rat clomipramine (CLI) model is widely applied for investigation of depression. In this model rat pups are treated at neonatal age with CLI, a tricyclic antidepressant inhibiting serotonin and norepinephrine reuptake. In adulthood the treated animals possess a depressive-like phenotype (Vogel *et al.*, 1990a). Adult rats have profound sleep changes including an elevated amount of REM sleep and

REM sleep fragmentation, and reduced REM sleep latency (Vogel *et al.*, 1990c). They also have a decline in pleasure-seeking behavior (Vogel *et al.*, 1990b) and sexual activity (Neill *et al.*, 1990), and an enhanced alcohol consumption (Dwyer and Rosenwasser, 1998). The molecular changes in this model include dysregulation of the HPA system (Prathiba *et al.*, 1998), reduction of serotonin and noradrenaline levels in several brain regions (Vijayakumar and Meti, 1999), decreased expression of serotonin transporter mRNA in the DRN (Hansen and Mikkelsen, 1998), increase of acetylcholine esterase activity in the hippocampus as well as its augmentation in the frontal cortex (Mavanji and Datta, 2002). CLI treatment substantially diminishes orexin A and orexin B levels in neonatal rats, and results in their enhancement in adulthood (Feng *et al.*, 2008).

The mechanism of promoting depressive-like behavior in this model remains incompletely understood. CLI treatment cuts REM sleep in postnatal rats by 44-68% (Feng and Ma, 2002), and neonatal instrumental REM SD produces depressive-like signs (decrease in sexual activity and aggressive behavior, increased REM sleep) in adult rats similarly to the CLI model (Feng and Ma, 2003). These findings led to the speculation that REM sleep suppression might promote depressive-like symptoms. However, another study compared the effects of three drugs which have similar REM sleep suppressing effects but inhibit the uptake of different monoamines. Interestingly, depressive-like sleep deficits in adult rats following neonatal drug treatment were observed only for those drugs that elevated serotonin levels (Frank and Heller, 1997). Thus it is likely that at least the sleep-related signs of depression in the CLI model do not depend on REM sleep-suppressive effects of CLI.

1.3.2. ROLE OF THE HYPOTHALAMUS IN SLEEP AND DEPRESSION

The hypothalamus is a complex structure containing multiple micronuclei with distinct cellular morphologies and functions (Nestler *et al.*, 2002). They regulate such vitally important functions as energy homeostasis, circadian rhythms, sleep and arousal, feeding behavior, sex behavior, and motivation (Nishijo *et al.*, 2000; Nestler *et al.*, 2002). Many of these activities are deteriorated in MDD, and abnormal function of the hypothalamus might well be associated with MDD pathogenesis. Since poor sleep and depression have a bidirectional relationship, these two aspects of the hypothalamic actions will be in focus below.

The hypothalamus and sleep. Four main subdivisions within the hypothalamus are marked out for their major regulatory role in sleep and wakefulness. They are the ventrolateral preoptic area (VLPO), the median preoptic nucleus (MPN), the perifornical lateral hypothalamic area (PFLH) and the TMN.

Lesion of the preoptic area yields profound and persistent sleep loss (Szymusiak and McGinty, 2008). Neuronal discharge within the two clusters of this area, the VLPO and the MPN, anticipates sleep onset by several seconds. Neuronal recording studies imply that the MPN presumably initiates the transition from waking to NREM sleep, since it discharges at its maximum early in the beginning of NREM sleep episodes following waking, and declines its firing across NREM sleep. The VLPO may predominantly maintain sleep stability, since the neuronal activity of the VLPO neurons increases across NREM sleep (Szymusiak and McGinty, 2008). In addition, the preoptic area is one of the key thermosensitive regions in the mammalian brain, and comprises warm-sensitive neurons which express sleep-active discharge profiles in association with the heat loss and decrease in metabolism characteristic for sleep onset. Local warming of the preoptic area induces sleep and increases EEG delta activity (Szymusiak and McGinty, 2008).

In regard to sleep homeostasis, the VLPO neurons are mostly active during recovery sleep and only moderately during SD. In contrast, the MPN neurons are active during NREM and REM SD. Thus the VLPO presumably mediates sleep consolidation in response to sustained waking, whereas the MPN promotes sleep following sustained waking (Szymusiak and McGinty, 2008). Through their GABAergic projections to the DRN, the LC and the PFLH, the VLPO and the MPN provide inhibition of these arousal-maintaining areas. The VLPO additionally projects to the waking-active TMN. Further, the preoptic region is activated by such endogenous somnogens as adenosine, IL-1β, prostaglandin D_2, and growth hormone releasing hormone (GHRH), which produce increased sleep propensity and sleep amounts (Szymusiak and McGinty, 2008).

Orexinergic neurons of the PFLH and histaminergic TMN neurons maintain waking and provide activating inputs to the thalamus, the BF, the neocortex, the limbic telencephalon, the monoamianergic areas (the LC, the DRN, and the VTA) and the brainstem cholinergic nuclei (Szymusiak and McGinty, 2008).

Several hypothalamic hormones and neurotransmitters affect sleep. These include GHRH promoting NREM sleep, and neuropeptide Y which might be a physiological antagonist of CRH acting via the $GABA_A$ receptor. The hypothalamic effects on sleep in terms of the HPA axis functioning are discussed in more detail in *Section 1.2.2*.

The hypothalamus and depression. In the context of MDD the hypothalamus has been mostly studied as a part of the HPA axis (see *Section 1.2.1*). Aside of these hypothalamic effects which are operated through the CRH signaling pathways, several other hormones are implicated in MDD pathophysiology. One of them is vasopressin (VP), which is released by the hypothalamic PVN and the supraoptic nucleus (SON) into the posterior pituitary (Meynen *et al.*, 2006). It can enter the blood stream and then exert its physiological (blood pressure regulation and body's

water retention) and behavioral effects (aggression and pair-bonding). VP potentiates ACTH release by CRH, and in chronic stress VP may become a more preferable pathway for ACTH release stimulation (Meynen *et al.*, 2006). Data showing that plasma VP levels positively correlate with cortisol levels in depression support this assumption (Meynen *et al.*, 2006). Depressed subjects have increased number of VP-immunoreactive neurons in the PVN and elevated VP plasma concentrations, the latter observed particularly in the melancholic subgroup. Melancholic patients demonstrate increased VP mRNA expression both in the SON and the PVN (Meynen *et al.*, 2006). Therefore, in addition to the PVN, the SON might also contribute to the HPA axis hyperdrive.

Another hypothalamic hormone involved in MDD is thyrotropin-releasing hormone (TRH). It induces the release of thyroid-stimulating hormone (TSH) and prolactin by the anterior pituitary. MDD patients often show an impaired TSH response to TRH, which may be partially explained by reduced TRH gene expression in the PVN (Alkemade *et al.*, 2003). Also a mild hypercortisolaemia may contribute, since GCs induce a downregulation of TRH mRNA in the PVN (Alkemade *et al.*, 2003).

Orexins released by the PFLH regulate not only sleep and alertness but also feeding behavior, and might be involved in an antidepressant-like effect of calorie restriction (Lutter *et al.*, 2008). Orexins excite monoaminergic neurons and therefore potentiate a release of monoamines (Liu *et al.*, 2002; Saper *et al.*, 2005), which in MDD is presumably suppressed. Weakened diurnal variation of orexins is observed in depressed subjects (Salomon *et al.*, 2003), and it can be assumed that due to their influence on sleep, feeding and energy homeostasis they may mediate depressive behavior. Melanin-concentrating hormone (MCH) is another potent orexinergic peptide that enhances sexual and anxiety-like behaviors (Nestler *et al.*, 2002).

1.3.3. FEMALES AND DEPRESSION

Women are at a double risk to be affected by MDD compared to men (Weissman *et al.*, 1996). This heightened risk begins at puberty and lasts until menopause (Meerlo *et al.*, 2008), which implies that gonadal hormones are involved in the etiology of MDD. Via binding to their nuclear receptors, sex hormones may modify epigenetic status and gene expression (Mill and Petronis, 2007).

Estrogen treatment may reduce stressful effects of chronic environmental and social stressors (Borbely, 1982; Young *et al.*, 2000). Depressive-like behavioral responses are accompanied by insufficient estrogen receptor beta (ERβ) activation in the brain (Covington *et al.*, 2010). Moreover, genetic deletion of ERβ increases

depressive- and anxiety-like behaviors (Krezel *et al.*, 2001; Imwalle *et al.*, 2005). ERβ are highly expressed in the DRN serotonergic neurons, where estrogen induces gene expression of tryptophan hydroxylase, a rate-limiting enzyme in serotonin biosynthesis, and may consequently increase serotonin levels (Hiroi *et al.*, 2006). ERβ are also expressed in the hippocampus and the amygdala, and the hippocampal activation of estrogen receptors has antidepressant effects (Walf and Frye, 2007). Collectively, these data indicate that a dysregulation of estrogen signaling might promote depressive behavior in women.

Gender-related differences are observed in the functioning of the serotonergic system. Women have lower plasma tryptophan availability and hence a lower rate of serotonin synthesis, making them more susceptible during stressful events (Nishizawa *et al.*, 1997). Also, sex hormones in female mice amplify expression of the serotonin transporter gene (Gubbels Bupp *et al.*, 2008). Interestingly, a polymorphism in the *TPH2* gene encoding tryptophan hydroxylase associates to depression with fatigue in women (Utge *et al.*, 2010a).

Another factor contributing to the overrepresentation of MDD in women might be a risk gene on the X chromosome. Recessive X-linked genes may associate with depression (Vaillant *et al.*, 2005). Interestingly, a compensatory inactivation of the X chromosome in women can be skewed, which means that in some cases the maternal or paternal X-chromosome is preferentially inactivated (Plenge *et al.*, 2002). Also, 15% still show the evidence of expression after the X chromosome inactivation (Craig *et al.*, 2004; Carrel and Willard, 2005). These factors could together add to the MDD sexual dimorphism.

The MDD pathophysiological cascade might differ between the genders. For example, depressed women are more likely to display such atypical symptoms as weight gain, rejection sensitivity, irritability and hypersomnia (Meerlo *et al.*, 2008). Some of the strongest evidence to support this hypothesis comes from EEG data. MDD women display a more dramatic elevation of the fast-frequency alpha and beta activities and a more clearly disturbed sleep microarchitecture (Meerlo *et al.*, 2008). Both healthy and affected women have higher delta activity in response to SD than respective groups of men (Meerlo *et al.*, 2008). These features may signify a stronger response to challenge in women, which might reflect a greater functional plasticity. The hyper-responsivity is more likely to contribute to an increased risk and also the genesis of MDD. Finally, sexual dimorphism in brain morphology or in any of the depression-related brain areas, distribution of neurotransmitter receptors, glucose metabolism in brain and overall metabolic rates, as well as asymmetries during task performance and in cerebral evoked potentials might all contribute to a differential MDD pathogenetic cascade and higher vulnerability in women.

2. AIMS AND HYPOTHESES OF THE STUDY

The study outline and the specific aims are presented in Figure 1.

The general aims of the study were to explore the role of adenosine in the regulation of sleep and depression and to find the common molecular substrates and pathways underlying sleep maintenance and depression. Our hypothesis was that regulatory mechanisms of sleep and depressive behavior may have common pathways, one of them through adenosine.

Specific hypotheses of the study:

1. Adenosine induces recovery NREM sleep acting via inhibitory A_1 receptors in the BF.
2. A dysfunction in the adenosine signaling predisposes to major depression accompanied by signs of disturbed sleep.
3. The analysis of gene expression in the BF of clomipramine-treated rats, which develop depressive-like symptoms in adulthood, will reveal molecular changes at early stages which could predispose to the development of the depressive-like phenotype which also shows disturbed sleep.
4. As the hypothalamus is one of the key brain areas involved in sleep regulation and also the mediation of the neurovegetative symptoms of depression, the investigation of the hypothalamic gene expression in adult clomipramine-treated rats could reveal the common substrates of depressive-like behavior and abnormal sleep.

Specific aims of the study:

I. To study whether adenosine promotes recovery NREM sleep through A_1 or/and A_{2A} receptors in the rat BF by perfusing adenosine A_1R and $A_{2A}R$ antagonists into the BF during SD.

II. To find out whether SNPs in adenosine-related genes would associate to major depression accompanied by signs of disturbed sleep (early morning awakenings and fatigue) in humans.

III. To explore the gene expression patterns in the rat BF immediately after the clomipramine treatment, and in the hypothalamus of adult depressive-like female rats.

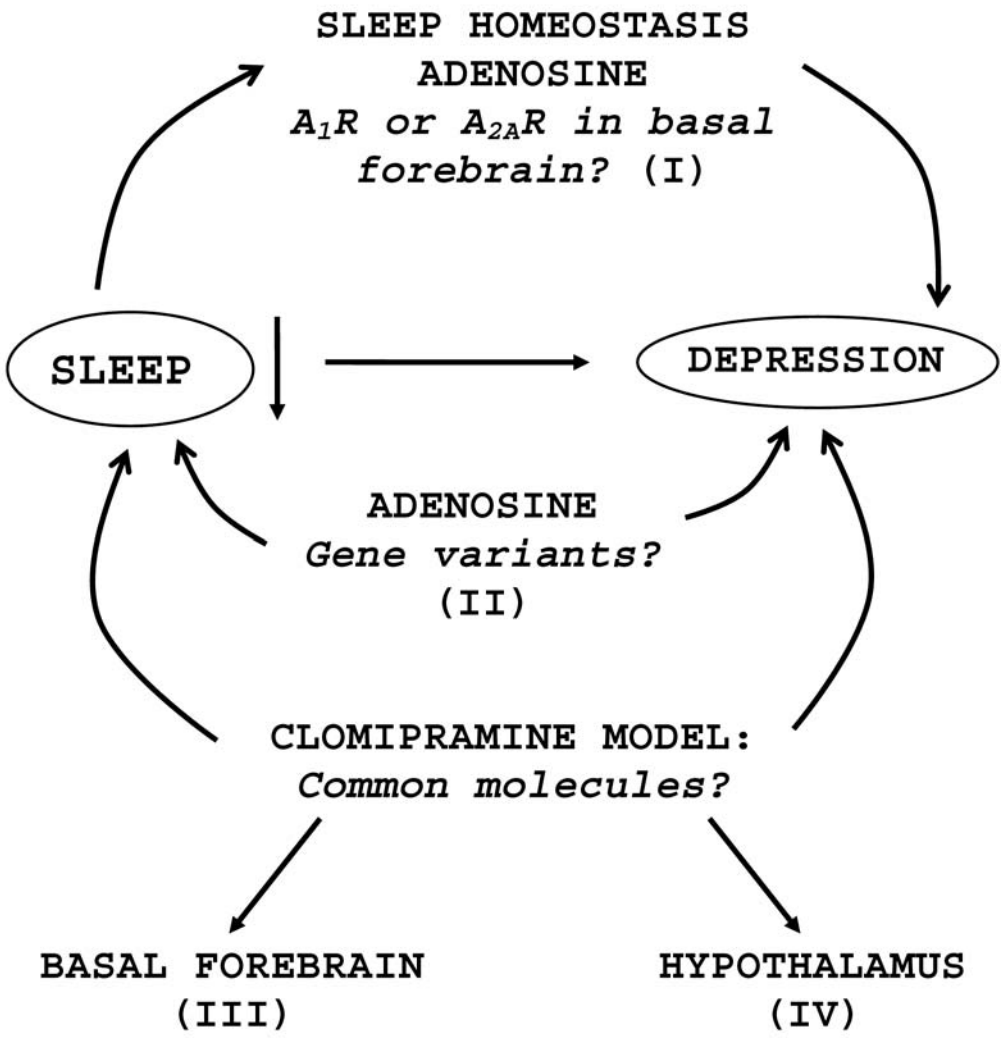

Figure 1. The study outline showing the specific aims *in italics* and original studies by Roman numerals.

3. MATERIALS AND METHODS

The details of materials and methods are given in the original publications.

3.1. EFFECT OF ADENOSINE RECEPTORS ANTAGONISTS ON SLEEP

3.1.1. RATS AND SURGERY

Young male Wistar rats (300-400 g, 11-16 weeks) were housed at constant temperature (23-24°C) in a 12-h light-dark cycle (lights on at 8:30 a.m.) with *ad libitum* access to food and water. Under general anaesthesia rats were implanted with EEG and electromyographic (EMG) electrodes and with a unilateral guide cannula for the microdialysis probe (CMA/11 Guide; CMA/Microdialysis, Stockholm, Sweden), targeting the area at 4 mm above the BF including the HDB, SI and MCPO (anterior = - 0.3; lateral = 2.0; vertical = 5) (Paxinos and Watson, 1998). Two EEG electrodes were put in frontoparietal positions; two silver EMG electrodes were placed into the neck muscles. After one week of recovery the rats were connected to recording leads for 48-h adaptation, upon which the EEG/EMG were recorded for 2 days before the experiments. These recordings were used as a baseline for comparison with the treatments. All animal studies were approved by the University of Helsinki Ethical Committee for Animal Experiments and were carried out according to the European Communities Council Directive of 24 November 1986 (86/609/EEC). Every effort was made to minimize the suffering of the animals and their number.

3.1.2. POLYSOMNOGRAPHIC RECORDINGS AND DATA ANALYSIS

The EEG/EMG signals were recorded (BioAmplifier AC-916, CWE, Inc., Ardmore, PA, USA), amplified (gain 500) and filtered (EEG high-pass filter: 0.1 Hz and EEG low-pass filter: 100 Hz; for EMG – 30Hz and 100 Hz, respectively). Signals were transmitted to a PC via a CED 1401 interface (Cambridge Electronic Devices, Ltd, Cambridge, UK) and sampled with the Spike 2 software (version 5.11; Cambridge Electronic Devices, Ltd, Cambridge, UK). The same program was used to score the EEG data. First, the data were digitally filtered with a 0.75 Hz high-pass filter

to remove low-frequency artefacts, and then 30-s epochs were semi-automatically scored for NREM sleep (described in the original publication). The baseline recordings were used for the normalizing of the experimental data. The effects of the drugs on recovery sleep were evaluated by comparing the experimental data with the 12-h recovery sleep data obtained after SD. The data were analyzed by using Sigma Stat® Statistical Software (SPSS, Chicago, IL, USA). One-way ANOVA followed by Student-Newman-Keuls post hoc test was applied to normally distributed data, and one-way ANOVA on ranks to non-normally distributed data. To compare two groups, parametrical and non-parametrical paired t-tests were used with the significance set at 0.05.

3.1.3. *IN VIVO* MICRODIALYSIS IN RATS

In vivo microdialysis was introduced in its present form in 1984 by Ungerstedt (Marsden, 1984), and is nowadays one of the major methods to continuously monitor the chemical composition of the extracellular fluid in any tissue and organ. Also, pharmacological agents can be administered continuously through the probe in order to study e.g. the correlation between brain chemistry and behavior. The principle of the method is based on passive diffusion of substances across the semipermeable membrane of a microdialysis probe. The membrane permits the diffusion of molecules up to a particular size defined by the membrane's cut-off value, and protects the surrounding tissue from damage by perfusion pressure. The membrane is connected to rigid inlet and outlet tubes. Analysis of the collected fluid provides information about the chemical composition in the site of sampling. When using microdialysis in the brain, artificial cerebrospinal fluid (aCSF) is perfused through the probe to minimize the dialysis effect on the site's ionic composition.

Before starting the experiments several factors should be taken into consideration, including probe recovery, the rate of perfusion, and the probe properties. Probe recovery, defined as the ratio of the concentration in samples collected from the probe to the tissue concentration, depends on the properties of the molecule (molecular mass and lipophilicity), probe membrane area, flow rate of perfusion, temperature and the viscosity of the surrounding fluid (Westerink, 2000; Watson *et al.*, 2006). A higher recovery can be achived by a smaller flow rate (0.1-0.5 μl/min), enlargement of the probe's active area and higher temperature (Westerink, 2000; Watson *et al.*, 2006). However, a lower recovery is advantageous in terms of less interference with the brain's homeostasis. The rate of perfusion is usually 0.1-3 μl/min (Watson *et al.*, 2006), but can vary from 0.1 to 10 μl/min . Since the rate of perfusion affects the probe recovery, it should not be too high. Probe membrane length and diameter are chosen depending on the size of the brain area of interest.

As a technique microdialysis has several important advantages. It can be used in freely moving animals to study the neurochemistry of behavior and drug effects in specific brain regions. Several compounds can be obtained simultaneously in one sample, and since it can be coupled to various analytical techniques, virtually any compound can be measured. Also, fewer animals are needed for study, since they serve as their own controls for each time point. Finally, microdialysis is relatively inexpensive and easy to use.

The disadvantages include the low temporal resolution (5-30 min), removal of endogenous molecules from the site, and tissue damage induced by the probe insertion. The scar tissue is usually formed after 3 days, therefore microdialysis experiments are limited in time (Westerink, 2000). One way to overcome this limitation is to use guide cannula and insert probes several times. Further, the microdialysis probe size is bigger than currently used microelectrodes and causes accordingly more tissue damage, but as microdialysis measurements are taken from bigger areas compared to microelectrodes, they are less prone to artefacts (Watson *et al.*, 2006).

In our study, the day before the experiment, microdialysis probes (CMA/11, membrane length 2 mm, membrane diameter 0.24 mm; CMA/Microdialysis, Stockholm, Sweden) were inserted through the guide cannula. During experiments, the rats were kept connected to the microdialysis leads for 6 h (10:00-16:00), and aCSF (147 mM NaCl, 3 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$) or drug solutions in aCSF were perfused through the microdialysis probe at 1 μl/min. After 16:00 the rats were reconnected to ordinary recording leads. A_1R antagonist 8-cyclopentyl-1,3-dimethylxanthine (CPT) (Sigma-Aldrich, St Louis, MO, USA), and $A_{2A}R$ antagonist 3,7-dimethyl-propargylxanthine (DMPX) (Sigma-Aldrich, St Louis, MO, USA) were used in the study.

Types of experiments:

1. 6-h (10:00-16:00) microdialysis perfusion of aCSF during spontaneous sleep-wake cycle to verify that the probe insertion and perfusion itself did not affect the sleep pattern.
2. 3-h SD (12:00-15:00) accompanied by aCSF microdialysis perfusion (10:00-16:00) (n=7). The gentle handling method was used for the SD; the rats were kept awake by introducing novel objects into the cage, and objects were changed when the animals showed signs of sleepiness.
3. Microdialysis perfusion of antagonists for 4 h (11:00-15:00), starting 1 h prior to the SD (12:00-15:00). In the first hour of recovery sleep (15:00-16:00) drugs were changed for aCSF. The antagonists were perfused at the following concentrations: 1 μM CPT (n=5), 5 μM CPT (n=5), 5 μM DMPX (n=5) and 10 μM DMPX (n=5).

4. Microdialysis perfusion of antagonists for 4 h (11:00-15:00) during the spontaneous sleep-wake cycle. In the last hour of the experiment (15:00-16:00) drugs were changed for aCSF. To exclude circadian differences, drugs were perfused in the same time period as in experiments with SD. The following concentrations were used: 5 μM CPT (n=4) and 10 μM DMPX (n=4).

After the experiments the rats were sacrificed with an overdose of pentobarbital (60 mg/kg i.p.), and the brains were removed and stored at -80°C. Brain sections (20 μm) were cut on a freezing microtome, stained with Toluidine Blue, dehydrated with ethanol, and dried. The probe locations were controlled under the light microscope.

3.2. ADENOSINE-RELATED GENES AND MAJOR DEPRESSIVE DISORDER WITH SIGNS OF DISTURBED SLEEP

3.2.1. HUMAN SUBJECTS

The case-control study sample for depression was selected from the population-based Health 2000 cohort, a Finnish population survey performed in 2000-2001 in order to inspect general health. Details of the survey can be found on http://www.terveys2000.fi/indexe.html. The sample consisted of 1423 unrelated individuals 30-88 years of age from 80 Finnish regions. The health status of all subjects was estimated by interviews conducted at home and health examination at the local health care centre. Depression was diagnosed according to the DSM-IV criteria by using the research version of the Composite International Diagnostic Interview (Pirkola *et al.*, 2005). The presence of early morning awakenings and fatigue was defined by the questionnaire (see Supplementary 1 of the corresponding original publication).

The depression sample comprised 258 women (mean age 49 years) and 125 men (mean age 48 years). In the depression group 109 women and 61 men had also early morning awakenings. The respective numbers for fatigue were 194 and 103. The control group comprised 557 women (mean age 46 years) and 483 men (mean age 45 years) with no depression according to the Composite International Diagnostic Interview. Of these, 554 women and 483 men had no signs of early morning awakenings, and 449 women and 409 men no signs of fatigue. The study protocol was approved by the Ethics Committee of the Helsinki and Uusimaa Hospital District, and all participants gave their signed informed consent.

3.2.2. SELECTION OF GENES AND SINGLE NUCLEOTIDE POLYMORPHISMS

Thirteen genes related to adenosine metabolism, transport and signaling were selected for the study. These comprised 5 genes encoding metabolic enzymes, 6 for adenosine transporters, and 2 for receptors which showed their involvement in sleep regulation: adenosine deaminase (*ADA*), adenosine kinase (*ADK*), cytosolic 5'-nucleotidase (*NT5C1B*), 5'-ectonucleotidase (*NT5E*), S-adenosylhomocysteine hydrolase (*AHCY*); solute carrier family 29 (nucleoside transporters), member 1, ENT1 (*SLC29A1*), solute carrier family 29, member 2, ENT2 (*SLC29A2*), solute carrier family 29, member 3, ENT3 (*SLC29A3*), solute carrier family 29, member 4, ENT4 (*SLC29A4*), solute carrier family 28 (sodium-coupled nucleoside transporter), member 1, CNT1 (*SLC28A1*), solute carrier family 28, member 2, CNT2 (*SLC28A2*); adenosine receptor type A1 (*ADORA1*), adenosine receptor type 2A (*ADORA2A*). Haplotype-tagging single nucleotide polymorphisms (SNPs) were selected based on the HapMap CEU data set (International HapMap Consortium, 2005), using a cut-off valueof 0.05 for minor allele frequency, and of 0.8 for the multimarker tagging coefficient of determination (r^2).

3.2.3. SEQUENOM MASSARRAY TECHNOLOGY

Sequenom is a manufacturer of DNA massarrays, and iPLEX – their newly developed genotyping assay for the MassARRAY® platform (Gabriel *et al.*, 2009). For the SNP genotyping, first, DNA is isolated, then amplified by PCR and treated with shrimp alkaline phosphatase to dephosphorylate (deactivate) unincorporated dNTPs. The single base primer extension step is performed, resulting in an allele-specific difference in mass between extension products. This mass difference allows the data analysis software to differentiate between SNP alleles. The primer extension products are analyzed using a MALDI TOF mass spectrometer.

In our study genomic DNA was isolated from peripheral blood leukocytes by a standard EDTA extraction procedure (Blin and Stafford, 1976). SNPs were genotyped with Sequenom's MassARRAY technology (Sequenom Inc., San Diego, CA, USA), which is based on single base extension and iPLEX Gold chemistry. Genotype clusters were visualized and genotypes of poor quality removed. We calculated genotyping success rate, minor allele frequencies and Hardy-Weinberg equilibrium p-values by using the PLINK software package, web-based version 1.06 (Purcell *et al.*, 2007). SNPs with genotyping success rate <96%, minor allele frequencies <1% (calculated in control individuals), or Hardy-Weinberg equilibrium p-values <0.0001 (calculated in control individuals) were omitted. In the end, 102 of 117 selected SNPs passed the requirements.

3.2.4. STATISTICAL ANALYSIS

When using PLINK software, allele frequencies between cases of depression and controls were compared by chi-square tests. The following groups were compared: (1) all depressed patients against all controls, (2) depressed patients with early morning awakenings against controls without early morning awakenings, and (3) depressed patients with fatigue against controls without fatigue. The genders were analyzed separately, since women and men have highly varying prevalences of depression and probably different pathogenetic mechanisms. SNPs which gave associations of $p<0.05$ were further analyzed for their interaction with gender, using PLINK software. Associations were adjusted for multiple testing by using the Bonferonni approach as the most stringent test for controlling false-positive results. The haplotype analysis by PLINK software with a two- and three-SNP sliding window approach was performed on the genes that survived multiple testing corrections.

3.3. GENE EXPRESSION SIGNATURE IN THE RAT CLOMIPRAMINE MODEL OF DEPRESSION

3.3.1. RATS AND TREATMENT

The pups (Wistar Hannover rats, Harlan Laboratories, Horst, the Netherlands) were treated with two daily subcutaneous injections (9 am and 7 pm) of 20mg/kg clomipramine (Sigma-Aldrich, St. Louis, MO, USA) during postnatal days 5 to 21 (the day of birth was regarded as day 0). The control group received 0.9 % saline (Baxter Oy, Vantaa, Finland). Six CLI-treated (3 males, 3 females) and seven (3 males, 4 females) control rats were sacrificed at the age of 3 weeks by cervical dislocation, and their BFs were dissected. We aimed at the BF cholinergic region including the horizontal limb of the diagonal band of Broca, substantia innominata and magnocellular preoptic area (anterior = -0.3; lateral = 2.0; vertical = 9.0) (Paxinos and Watson, 1998). The dissected areas were 2 mm thick.

After weaning, animals of the same sex and treatment type were group housed in cages under a constant temperature (23-24°C) in a 12-h light/dark cycle (lights on at 08:30) with *ad libitum* access to food and water. Six CLI-treated and five control female rats were decapitated at the age of 12 weeks and the hypothalami were removed *en block* for further analysis. All animal procedures were approved by the University of Helsinki Ethics Committee for Animal Experiments and performed in accordance with the applicable legislation (European Communities Council Directive of 24 November 1986, 86/609/EC). All efforts were made to minimize the suffering of the animals and their number.

3.3.2 MICROARRAY EXPERIMENTS

DNA microarrays are microscope slides containing arrayed series of DNA sequences. The series are arranged in an ordered fashion, so that the data obtained from the microarray can be tracked back. Each of the series, i.e. each probe, contains a specific DNA sequence of a particular gene which hybridizes with a cDNA or cRNA target. The slides contain a chemical matrix (usually aldehydes or primary amines) that helps to attach DNA onto the slide either by covalent bond or electrostatic interactions. Two types of microarrays are currently available: short oligonucleotide and longer cDNA arrays. In the latter type, long (80-2000 bp) DNA sequences are placed on glass surface and cDNA from two samples is labeled with two different fluorescent dyes and hybridized onto the same array (Sequeira and Turecki, 2006).

Affymetrix is one of the biggest array manufacturers. In Affymetrix GeneChip oligonucleotide arrays each probe contains 11 different target-specific 25-mer oligonucleotide probe pairs representing a transcript. Each probe pair comprises one probe that is perfectly complementary to the transcript (perfect match) and one that has a single base pair difference in the 13th position (mismatch). The mistmatch probe serves as an internal control for nonspecific hybridization in order to tear apart background noise. The redundancy of probes negates any significant impact if a sample may have a sequence variation that causes low specific binding to the Perfect Match, and high specific binding to the Mismatch (Wang *et al.*, 2007a).

To analyze gene expression in samples, first, mRNA from the sample is extracted, and its quality and integrity are evaluated by microcapillary electrophoresis which currently is available from Agilent as the 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA). This method generates an RNA integrity number which is a standard quality measure. Then mRNA is labeled with biotin and transcribed to cRNA by a reverse transcription procedure, whereupon cRNA is fragmented and hybridized to the Affymetrix chip. This is followed by washing and staining with streptavidin-phycoerythrin (SAPE), where streptavidin is binding to biotin and phycoerythrin is a fluorescent tag. Then the signal is amplified, first by washing with a solution containing biotinylated anti-streptavidin (goat) antibody, and afterwards with a final staining with SAPE. Gene expression levels are compared by evaluation of the fluorescence signals from each probe on the array.

In our study, the dissected brain tissues were immediately placed in RLT Plus buffer (Qiagen, Hilden, Germany) with added β-mercaptoethanol and homogenized using a handheld rotor homogenizer and a syringe fitted with a 0.9 mm needle, and stored at -80°C. RNA was extracted using the AllPrep DNA/RNA Mini kit by Qiagen according to the supplied protocol. The RNA fraction was treated with DNaseI using the RNase-Free DNase Set by Qiagen as a precaution against RNA contamination with genomic DNA. RNA was measured for purity and concentration using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies,

Rockland, DE, USA). After extraction, RNA quality was checked with the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). The microarray experiments were performed according to the Affymetrix standard protocol (Affymetrix, Santa Clara, CA, USA). Using the Affymetrix GeneChip One-cycle target labeling kit, biotin-labeled cRNA was prepared from 2 μg of RNA. Then 15 μg of the cRNA was fragmentated and hybridized to the Affymetrix Rat 230.2 chip. Hybridization, staining and washing were carried out using the Affymetrix Fluidics Station 450 Hybridization and Hybridization Oven 640 under standard conditions and according to the Affymetrix protocol.

3.3.3. MICROARRAY DATA ANALYSIS

The distribution of the transcripts in rats treated with CLI or saline was compared using two alternative approaches. The first method focused on testing the levels of single transcripts, whereas the second concentrated on analysis of pathways to explore which biological processes were affected. Finally, to reveal common regulatory mechanisms behind the gene expression patterns we searched for enriched binding sites for transcription factors among the most differentially expressed individual genes.

Analysis of individual genes

The expression levels of individual genes of CLI-treated animals and controls were compared using GeneSpring GX (version 7 for the BF; version 9.0.5 for the hypothalamus) software (Agilent Technologies, Santa Clara, CA, USA). The data analysis was executed as follows: array raw intensity values were normalized and converted to expression summaries by the Gene Chip Robust Multichip Averaging adjustment, the signals then normalized to the median per gene and the Affymetrix control probe sets (57) removed. To ensure that the analysis would pick up both differentially expressed genes and ON/OFF genes, we performed a filtering on flags using the CHP files so that flags had to be either present or marginal in at least 4 of 6 of the CLI samples and/or 5 of 7 of the control samples for the BF dataset, and in 67% of the values in any one of two treatment conditions for the hypothalamic dataset. All genes that had normalized data between 0.666 and 1.5 in 11 of 13 BF samples were filtered out, and a fold change (FC) threshold of 1.3 was applied. In the case of the hypothalamic data the obtained list of genes was filtered by FC so that genes up- or down-regulated with less than 1.5 FC were removed. Differential expression was tested by the Mann-Whitney-Wilcoxon test, and significance was set at $p<0.05$.

Pathway analysis

Analysis of functionally related groups of genes was performed by calculating an aggregate score for genes in specific categories, and the significance was estimated by random sampling, using a custom-made program. We used an input list consisting of the total number of probes on the Affymetrix Rat chip, excluding the control probes (31,042 target sequences). Rattus norvegicus core 50.34t and gene ontology (GO) database 50 by Ensembl were chosen to retrieve the GO information for the probes. The nonparametric iterative cumulative hypergeometric distribution was computed and tested using 100,000 permutations.

Promoter analysis of the differentially expressed genes in the BF

The genes that gained statistical significance from the array analysis were subject to a promoter analysis. A list of the mouse orthologues was created by using Ensmart (Biomart) (Durinck *et al.*, 2005). Sequences of +/- 2000 basepairs from the transcription start site were studied for enrichment of binding sites for vertebrate transcription factors using oPOSSUM (www.cisreg.ca/oPOSSUM).

3.3.4. TAQMAN REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

Using an ABI PRISM 7900 Thermal Cycler and TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA), we performed TaqMan real-time quantitative reverse transcription polymerase chain reaction (PCR). 1 μg of RNA was utilized as a starting material for the reverse transcription by the Advantage-RT for the PCR kit (Clontech Carlsbad, CA, USA). Amplification of the BF cDNA samples was performed using a TaqMan Universal PCR MasterMix and the commercial gene expression assays with primers and probes for: *Dnmt2* (Rn01765153_m1), *Sox6* (Rn01507719_m1), *Pde4d* (Rn00566798_m1), *Acatn* (Rn00573865_m1), *Fgfr2* (Rn01269940_m1) and *Igf2* (Rn00580426_m1) from Applied Biosystems. For the hypothalamic samples the assays included probes and primers for *Ttr* (Rn00562124_m1), *Dlg3* (Rn01419350_m1), *Dnajc5* (Rn00577363_m1) and *Snx10* (Rn01763032_m1) from Applied Biosystems. Expression values were normalized to cyclophilin A (*Ppia* (Rn00690933_m1) used as an endogenous control. We selected this common endogenous control gene after we had verified that its expression was constant in the microarrays for all samples. The program was set at 50°C for 2 min, 95°C for 1 min, and 50 repeating cycles at 95°C for 15 s and at 60°C for 1 min. All assays and samples were run in triplicates. Standard curves were prepared

accordingly in order to allow a calculation of changes in gene expression by the comparative delta method. To estimate the statistical significance of the detected changes in expression between the two groups, a Student's t-test was performed. For the hypothalamic study we included 5 CLI-treated and 3 control animals in the PCR analysis, since the RNA from 3 rats (1 CLI-treated and 2 controls) due to the lower RNA concentration in those samples was entirely used up in microarray experiments, which were performed first.

4. RESULTS AND DISCUSSION

4.1. ADENOSINE INVOLVEMENT IN SLEEP HOMEOSTASIS AND DEPRESSION

In Study I we investigated the mechanism by which adenosine regulates recovery sleep in the BF. As disrupted sleep homeostasis is likely to be connected with depression, and poor sleep is a risk factor for depression (Ford and Kamerow, 1989; Paffenbarger *et al.*, 1994), in Study II we explored whether adenosine-related genes contribute to the prevalence of depression and disturbed sleep in humans.

Adenosine and sleep homeostasis. Our data from Study I demonstrate that adenosine is able to induce recovery sleep when acting via the A_1R subtype in the BF, since only A_1R antagonist CPT at both doses reduced recovery NREM sleep amounts (at 1 μM p=0.034; at 5 μM p=0.011) and delta power (at 1 μM p=0.036; at 5μM p=0.042) during 12 h of recovery sleep. The observed effect of CPT might be a result of increased discharge rates of the BF neurons during wakefulness by antagonizing the inhibitory effects of adenosine (Alam *et al.*, 1999; Arrigoni *et al.*, 2006). Only one previous study explored the influence of A_1R blockade in the BF on recovery sleep parameters, but did not study the effects of $A_{2A}R$ antagonism. In that study, microdialysis perfusion of A_1R antisense into the BF reduced recovery NREM sleep amount and delta power after 6-h SD (Thakkar *et al.*, 2003b). We obtained similar results for the A_1R antagonist, and additionally found that $A_{2A}R$ antagonist had no effect on any parameter of recovery NREM sleep (for NREM sleep amount p=0.806; for delta power p=0.250). $A_{2A}R$ does play an important role in sleep regulation, but predominantly in the lateral preoptic area and the prefrontal cortex (Scammell *et al.*, 2001; Methippara *et al.*, 2005; Van Dort *et al.*, 2009). Our results are consistent with data showing that while SD elevates A_1R mRNA, it does not induce any alterations in $A_{2A}R$ mRNA in the BF (Basheer *et al.*, 2001). Also, they match with data demonstrating the inefficacy of $A_{2A}R$ agonist to change BF neuronal firing (Thakkar *et al.*, 2003a).

In regard to the issue of adenosine effects on spontaneous sleep regulation, our results provide evidence that in the BF adenosine is not a key factor, since neither CPT nor DMPX affected spontaneous NREM sleep amounts (p=0.164 for 5 μM CPT; p=0.672 for 10 μM DMPX) and delta power (p=0.236 for 5 μM CPT; p=0.875 for

10 μM DMPX). This conclusion is partially supported by the fact that A_1R knockout mice have the same amount of spontaneous sleep as wild-type mice (Bjorness *et al.*, 2009). There are two previous studies on cats and rats which explored the local BF effects of A_1R blocking on spontaneous sleep (Strecker *et al.*, 2000; Thakkar *et al.*, 2003b). In cats, only 10 μM CPT diminished NREM sleep, while the lower doses of 0.1 and 1 μM were ineffective (Strecker *et al.*, 2000), whereas we used only a 5 μM dose. In rats, the A_1R antisense reduced spontaneous NREM sleep amounts when administered bilaterally for 8 h (Thakkar *et al.*, 2003b), while our study applied a unilateral 4-h perfusion. Thus the differences in the experimental conditions could account for the dissimilarity in results, but additional studies are necessary to provide a clearer understanding of adenosine contribution to the regulation of spontaneous sleep.

Possible mechanisms of adenosine effect on delta activity. Adenosine could affect delta activity at least through two mechanisms. First, the increase in adenosine levels in the BF during prolonged wakefulness could reduce the cholinergic tone of the wake-active BF cells via A_1R, and consequently reduce the cholinergic tone in the thalamocortical and cortical cells which receive the cholinergic BF projections (Bjorness and Greene, 2009). This would result in the disinhibition of delta activity, which is usually inhibited by the cholinergic tone during waking. The same process might occur in the brainstem arousal centers, although an increase in adenosine levels has so far not been demonstrated in these regions. Second, adenosine could hyperpolarize cortical, thalamocortical and thalamic reticular cells by increasing the inwardly rectifying potassium channel conductance via A_1R and degrading the postsynaptic I_h current (Bjorness and Greene, 2009). The presynaptic A_1R may be especially important for the delta-activity-related effects of adenosine (Bjorness and Greene, 2009).

It is important to note that the adenosine effect on A_1R in the BF is only one of the many pathways which regulate sleep homeostasis. For instance, A_{2A}R knockout mice show an attenuated response to SD (Urade *et al.*, 2003). In the prefrontal cortex A_{2A}R agonist, in contrast to A_1R agonist, reduces delta activity (Van Dort *et al.*, 2009). Sleep-active neurons have been found in VLPO, MPN (Sherin *et al.*, 1996; McGinty *et al.*, 2004) and cortex (Gerashchenko *et al.*, 2008). However, currently it is difficult to analyze the regulation of sleep homeostasis by the cortex and the hypothalamus independently of the BF and to define their separate roles, since these areas are reciprocally connected.

Moreover, the hypnogenic properties of adenosine are not limited only to the BF and the cortex. Adenosine also inhibits histaminergic TMN cells (Oishi *et al.*, 2008), LDT/PPT neurons of the brainstem (Rainnie *et al.*, 1994; Arrigoni *et al.*, 2001), and A_1R agonist inhibits orexinergic neurons of the perifornical-lateral hypothalamic area (PFLH), suppressing arousal (Rai *et al.*, 2010). In the lateral preoptic area, A_1R agonist induces waking, while A_{2A}R stimulation has a sleep-inducing effect

(Methippara *et al.*, 2005). In the pons, A_1R agonist increases REM sleep (Marks and Birabil, 1998), while $A_{2A}R$ activation in the pontine reticular formation might promote NREM sleep via GABAergic inhibition of wake-promoting neurons, and REM sleep by increasing acetylcholine release (Coleman *et al.*, 2006).*Adenosine and depression*. Both in women and men several adenosine-related genes contributed to the risk of depression alone as well as when accompanied by early morning awakening and fatigue (see Table 1 for the overview of results). The strongest finding was the association of *SLC29A3* polymorphism rs12256138 with depression in women. This result stayed significant after adjustment for multiple testing. The same polymorphism also suggestively associated with depression accompanied by early morning awakenings and fatigue. Moreover, this result was replicated in women (from an independent set of Health 2000 data), who had early morning awakenings without depression (269 cases, 550 controls, p=0.0433, OR 0.81) (data not shown). This suggests that *SLC29A3* polymorphism might also contribute to disturbed sleep independently of depression. *SLC29A3* codes the equilibrative nucleoside transporter ENT3 and is expressed in multiple tissues (Baldwin *et al.*, 2005), but the function of its product is still largely unclear. It is known that ENT3 is an intracellular protein, possibly involved in lysosomal nucleoside transport (Baldwin *et al.*, 2005). Our data call for further investigations to define the role of ENT3 in adenosine transport and the effect of the given variant on the actual function of the gene.

In addition to *SLC29A3*, SNP rs6905285 in gene *SLC29A1* which encodes the ENT1 transporter also associated with depression alone and depression with early morning awakenings and fatigue in women. Interestingly, *Slc29a1* knockout mice show increased alcohol consumption (Choi *et al.*, 2004a). In our study the same allele from *SLC29A1* polymorphism (minor allele T from rs6905285) consistently yielded an association with alcohol abuse in women from the Health 2000 project (782 controls, 33 cases; p=0.0304, OR=1.75) (data not shown). Collectively this indicates that malfunctioning adenosine transport might be a common causative event for depression and comorbid alcohol abuse (Fergusson *et al.*, 2009).

Men had the strongest association for *SLC28A1* (rs153372) with depression specifically when occurring with early morning awakenings and fatigue. *SLC28A1* encodes the CNT1 transporter, which executes high-affinity transport of adenosine into the cell (Gray *et al.*, 2004). It is remarkable that both men and women yielded the most significant associations for genes encoding transporters and not for the genes related to adenosine signaling and metabolism. One previous study demonstrated the pronounced effect of 22G>A *ADA* polymorphism on sleep duration and intensity (Retey *et al.*, 2005). Unfortunately we could not obtain data for this particular SNP in our study due to the methodological problems. However our results stress the significance of transporters, which control both intra- and extracellular levels of adenosine.

Most of our results were obtained for the polymorphisms located in the intronic regions of the genes. Introns provide efficient splicing, and some introns are also able to enhance gene expression (Rose, 2008). Moreover, multiple intronic sequences encode microRNAs, which are capable of silencing transcription (Ying *et al.*, 2010). It can be speculated that polymorphisms in intronic regions of adenosine-related genes may affect the expression of these genes as well as their splicing.

Gene symbol	Gene product	SNP	p-value, D	OR	p-value, D+EMA	OR	p-value, D+FAT	OR
			Women					
ADORA1	adenosine receptor type A1	rs17530497	0.3458	1.12	0.3968	1.15	**0.0464**	1.30
		rs12026765	0.1445	0.76	0.1710	0.69	**0.0460**	0.64
SLC29A1	equlibrative nucleoside transporter type 1	rs693955	0.4036	0.86	**0.0167**	0.47	0.1657	0.75
		rs324148	0.3499	0.88	**0.0400**	0.66	0.2848	0.85
		rs6905285	**0.0117**	1.31	**0.0351**	1.36	**0.0172**	1.35
NT5E	5'-ectonucleotidase	rs6942065	0.2564	1.22	**0.0185**	1.67	0.1976	1.30
		rs9450282	0.9431	0.99	**0.0404**	0.72	0.3265	0.88
SLC29A3	equlibrative nucleoside transporter type 3	rs10999776	0.0865	0.82	**0.0340**	0.71	0.3333	0.88
		rs2066210	0.1268	0.76	0.7248	0.92	0.0301	0.62
		rs12256138	**0.0004***	0.68	**0.0033**	0.64	**0.0046**	0.71
		rs780659	**0.0327**	1.25	**0.0492**	1.33	0.0662	1.24
		rs780662	**0.0109**	1.52	0.0812	1.47	0.0983	1.35
		rs12767108	**0.0405**	1.46	**0.0476**	1.60	0.5590	1.15
		rs2487067	**0.0212**	1.29	0.2094	1.21	0.5061	1.09
ADK	adenosine kinase	rs7924176	0.2325	0.88	0.1838	0.81	**0.0402**	0.77
SLC28A1	concentartive nucleoside transporter type 1	rs4980345	0.7535	1.11	**0.0420**	0.25	0.7547	0.91
ADA	adenosine deaminase	rs6031682	0.0398	0.74	0.2493	0.78	**0.0467**	0.71
			Men					
ADK	adenosine kinase	rs946185	0.0296	0.72	0.5701	0.87	0.1163	0.76
SLC29A2	equlibrative nucleoside transporter type 2	rs4244813	0.0293	0.69	0.2955	0.79	**0.0281**	0.66
		rs2279861	0.0503	0.72	0.2982	0.79	**0.0323**	0.66
SLC28A1	concentrative nucleoside transporter type 1	rs7182385	0.4113	1.13	**0.0160**	1.61	0.2684	1.19
		rs11853372	0.0130	1.42	**0.0065**	1.67	**0.0069**	1.53
		rs4980345	0.0908	0.47	**0.0164**	0.00	**0.0468**	0.35
		rs4271567	0.0653	0.74	**0.0441**	0.62	0.0903	0.74
		rs12910991	0.0465	1.34	0.1094	1.38	0.0638	1.35
ADA	adenosine deaminase	rs452159	0.2712	0.83	0.0573	0.63	**0.0165**	0.63

Table 1. Genes and single nucleotide polymorphisms (SNPs) which showed associations with depression (D), depression with early morning awakenings (EMA) and depression with fatigue (FAT) in women and men (OR – odds ratio). Significant associations ($p<0.05$) are marked **bold**.
* - finding which survived a multiple testing adjustment.

Indication for a role of adenosine in depression is gradually accumulating (for details see *Section 1.2.3*). Depressed patients show elevated glutamate levels in the cortex (Sanacora *et al*., 2004), and adenosine affects glutamate uptake and release when acting via A_1 and A_{2A} receptors (Dunwiddie and Masino, 2001; Boison *et al*., 2009). In addition, adenosine modulates serotonin, dopamine, acetylcholine and GABA release, which might affect mood (Dunwiddie and Masino, 2001). At the same time, adenosine is one of the key participants in sleep regulation (Basheer *et al*., 2004; Landolt, 2008), and as poor sleep is a risk factor for depression (Ford and Kamerow, 1989; Paffenbarger *et al*., 1994), our results could illustrate this particular aspect of adenosine action. On the other hand, adenosine is also a basic part of the ATP molecule. Insufficient energy production may be part of depression (Tsiouris, 2005), therefore the outcomes in our study could also be explained by adenosine involvement in energy metabolism.

The main limitation of the human study was the relatively small sample size. Nonetheless, as adenosine is well known for its sleep-maintaining effect, it was rational to hypothesize that adenosine-related genes would add to the risk of depression with disturbed sleep, and our data confirm this assumption. In addition, the data heterogeneity was greatly reduced by analysing cases with sleep disturbances, and also the sexes, independently.

Conclusions and future directions. Taken together, our data demonstrate that adenosine induces recovery NREM sleep and regulates sleep homeostasis when acting through A_1R in the BF. The poor sleep quality characteristic for depression as well as depression alone might be genetically determined by the polymorphisms in genes related to adenosine transport. It remains to be elucidated whether the non-BF neurons expressing A_1R and producing synapses with BF cells contribute to this action. Also, it would be important to investigate which types of the BF neurons (cholinergic, glutamatergic and/or GABAergic) express A_1R. Finally, since adenosine receptors form homo- and heterodimers (Sichardt and Nieber, 2007), their occurrence and possible function in the BF should be examined. Our results in humans call for a replication in unrelated samples as well as in different populations. Also the impact of the polymorphisms on protein function should be investigated.

4.2. MOLECULAR PATTERNS IN THE BASAL FOREBRAIN AND THE HYPOTHALAMUS IN DEPRESSION: FROM EARLY-LIFE CHANGES TO THE FULLY DEVELOPED PHENOTYPE

The basal forebrain patterns. Immediately after the CLI treatment 3 week-old rats displayed differential expression patterns for 72 gene transcripts as compared to

those that received saline. Some of these are under epigenetic regulation or are implicated in this process. They include *Dnmt2* (DNA methyltransferase 2) (FC=1.8, p=0.0223), *Igf2* (insulin-like growth factor 2) (FC=-1.4, p=0.0455), *Fgfr2* (fibroblast growth factor receptor 2) (FC=1.8, p=0.0101), *Dpp4* (dipeptidyl-peptidase 4) (FC=-1.4, p=0.0455), *Col1a2* (collagen type I alpha 2) (FC=-1.6, p=0.0082), *Impact* (FC=1.4, p=0.0455) and *Plk2* (polo-like kinase 2) (FC=1.5, p=0.0455) (Okamura *et al.*, 2004; Tsuji *et al.*, 2004; Smith *et al.*, 2006; Zhu *et al.*, 2007; Anderton *et al.*, 2008; Ollikainen *et al.*, 2010). *Dnmt2* encodes a methyltransferase which, unlike conventional DNA methyltransferases, methylates both DNA and transfer RNA (Jeltsch *et al.*, 2006). The functional outcome of the RNA methylation is still unclear, but the strong conservation of *Dnmt2* points to an evolutionary advantageous role (Jeltsch *et al.*, 2006). Next, in consistence with our findings and as also would be expected after treatment with CLI, a potent REM-sleep-suppressive factor, *Igf2* and *Col1a2* were underexpressed after long-term SD (Cirelli *et al.*, 2006). FGFR2 regulates neurogenesis (Frinchi *et al.*, 2008), which is, as mentioned previously, affected in depression. *Fgfr2* is also differentially expressed in CRH-overexpressing mice, which could mean that its expression is under control of CRH, one of the main molecules of the HPA axis influencing mood and sleep (Peeters *et al.*, 2004). *Dpp4* encodes an ADA-complexing protein which is related to the maintenance of mood as well as sleep. Depressed individuals have reduced DPP4 activity (Elgun *et al.*, 1999), which is in accordance with our result. ADA directly affects the levels of adenosine by metabolizing it to inosine, and thus may modulate sleep. In addition, reduced DPP4 levels increase inflammatory processes due to effect of DPP4 on the production of cytokines (Maes *et al.*, 2009), which are also involved in the neurobiology of depression and sleep regulation. *Impact* is imprinted gene and associates with bipolar disorder (Kosaki *et al.*, 2001). PLK2 regulates synaptic strength during neuronal activity (Seeburg *et al.*, 2005).

Serotonergic (*Htr2c* and *Htr3a*), cholinergic (*Acatn, Gna15*) and glutamatergic (*Grm8*) genes demonstrated enhanced expression in CLI-treated rats (FC=1.7, p=0.0223; FC=1.8, p=0.0455; FC=1.7, p=0.0455; FC=1.8, p=0.0321; FC=1.7, p=0.0383, respectively). Remarkably, *Htr2c* (serotonin receptor 2C) null-mice have less NREM sleep and a greater homeostatic response to SD (Frank *et al.*, 2002), which would be a reason to speculate that the overexpression of this gene in our study might reflect sleep abnormalities already happening during CLI treatment. Overexpression of cholinergic and glutamatergic genes could disturb the functions of the BF neurons, which are in fact essentially glutamatergic and cholinergic (Henny and Jones, 2008). On the other hand, these data might as well reflect the effect of disturbed sleep pattern during CLI treatment on the BF activity.

In addition, *Pde4d* (phosphodiesterase 4D) encoding an enzyme which degrades cyclic AMP and cyclic GMP was upregulated in the CLI-treated rats (FC=1.7,

p=0.0383). Degradation of cyclic AMP elevates extracellular adenosine levels and thus might affect sleep. Interestingly, a *PDE4D* polymorphism associates with sleepiness in humans (Gottlieb *et al.*, 2007). In addition, cyclic AMP is necessary for CREB activation and thus participates in the CREB-regulated gene expression (Carlezon *et al.*, 2005). CREB has numerous target genes which are important in both sleep and mood regulation (Carlezon *et al.*, 2005). Finally, phosphodiesterases interact with protein DISC1, which is a risk factor for many affective illnesses including MDD, and these complexes may serve as a basis for some depressive symptoms (Millar *et al.*, 2007). Noteworthy, PDE4D inhibitors are considered as possible antidepressants (Esposito *et al.*, 2009).

Furthermore, the gene *Ifnar1* encoding interferon receptor 1 exhibited increased expression in the CLI-treated rats (FC=1.5, p=0.0383). *Ifnar1* knockout mice have increased orexin A mRNA in their hypothalamus, so IFNAR1 presumably depresses the transcription of the orexin gene (Bohnet *et al.*, 2004). It is conceivable that the overexpression of *Ifnar1* in the BF could diminish orexin production in the hypothalamus, since the BF has widespread hypothalamic projections. A recent study indeed demonstrated that the juvenile CLI-treated rats have reduced levels of orexin A in the hypothalamus as well as in the frontal cortex and pons (Feng *et al.*, 2008).

The pathway analysis revealed the strongest alterations in the synaptic transmission (permuted p=0.00001), in the cellular component of the synapse (permuted p=0.00001), in extracellular-glycine-gated ion channel activity (permuted p=0.00024), and in GABA and $GABA_A$ receptor activity (permuted p=0.00033, permuted p=0.00079, respectively). It is noteworthy that 72 transcripts obtained from the analysis for individual genes and the 205 transcripts providing the critical input in the pathway analysis have a small percentage of overlap, which demonstrates the capacity of the pathway analysis to spot genes which otherwise are overlooked by a standard approach.

Promoter analysis identified CREB1 as a common transcription factor for the differentially expressed genes in the CLI-treated rats (permuted p=0.000059). 23 genes had the binding sites for CREB1, and these included *Pde4d, Htr2c, Sox6, Col1a2* and *Fgfr2*. Immediately after CLI treatment, *Creb1* was slightly upregulated in the CLI-treated rats (FC=1.35, p=0.0450). CREB phosphorylation, that is necessary for its transcriptional function, is triggered by multiple cellular events including the binding of neurotransmitters and neurotrophins to TrkB, AMPA, NMDA and G-protein-coupled receptors (Carlezon *et al.*, 2005), and thus could reflect the normal functionality of these signaling processes. DNA methylation abolishes the binding of CREB1 to its target sequences and thus its transcriptional activation (Iguchi-Ariga and Schaffner, 1989; Zhang *et al.*, 2005). Moreover, a CREB-binding protein possesses a histone acetyltransferase activity and therefore is itself an epigenetic factor (Korzus *et al.*, 2004).

CREB targets include genes encoding tyrosine hydroxylase, CRH, BDNF, dynorphin and enekaphalin (Carlezon *et al.*, 2005), which are involved in MDD pathogenesis. CREB has brain region-specific effects, with antidepressant activity in the hippocampus correlating with BDNF expression, and depressive-like activity in the nucleus accumbens correlating with increased dynorphin expression and stimulation of κ opioid receptors (Carlezon *et al.*, 2005). In the amygdala CREB mediates both aversive and rewarding behavior (Nestler *et al.*, 2002). CREB1 is also involved in maintaining cortical arousal (Graves *et al.*, 2003). Interestingly, *CREB1* polymorphisms associate to depression alone in men and depression with fatigue in women (Utge *et al.*, 2010a). Also several genetic loci in *CREB1* associate to depression in women (Zubenko *et al.*, 2003). The abundance of CREB1 binding sites in the differentially expressed transcripts in the BF of CLI-treated animals points out CREB1 as one of the factors connecting abnormal sleep and depression. CLI treatment and interference with the normal sleep-wake cycle might promote a depressive-like phenotype in adult rats through genetic and epigenetic modifications exerted by CREB1.

The hypothalamic patterns. Adult female CLI-treated rats exhibited 62 of the 31,042 transcripts (0.2%) with distinct expression: 54 were overexpressed, and 8 underexpressed. We divided them into several functional groups. The first group included two K^+-channel-activity genes probably involved in sleep maintenance. CLI-treated rats had an increased number of *Kcnc2* (potassium voltage gated channel, member 2) transcripts (FC=2.14, p=0.0173). KCNC2 is a subunit of the K.v.3.2 K^+ channel responsible for voltage-dependent K^+ permeability of excitable membranes and activated by high depolarization (Wang *et al.*, 2007b). *Kcnc2* null mice show reduced power in the upper delta range in NREM sleep and low theta in REM sleep (Vyazovskiy *et al.*, 2002), which could reflect KCNC2 involvement in the formation of sleep rhythms. Also, *Kcnc2* closely resembles the sequence of the Drosophila's *Shaker* gene, and mutation in this gene results in greatly reduced sleep duration (Cirelli *et al.*, 2005). The other upregulated gene was *Kcnip2* (Kv channel-interacting protein 2) (FC=1.51, p=0.0303) coding the Ca^{2+}-binding protein in K^+ channels, which regulates A-type K^+ currents in response to Ca^{2+} alterations (Rhodes *et al.*, 2004). Altogether, the upregulation of these genes may disrupt neuronal excitability and cause disruptions in sleep organization.

The second group included three overexpressed glutamatergic genes. One of them was *Gls* (glutaminase) (FC=1.63, p=0.0454) coding an enzyme which catalyzes glutamate production. This result is consistent with human data demonstrating a decreased expression of the glutamine synthetase gene *Glul*, which would cause elevated glutamate concentration by reducing glutamate metabolism (Choudary *et al.*, 2005). Indeed, individuals with major depression exhibit elevated glutamate in the cortex (Sanacora *et al.*, 2004), and antidepressants may normalize glutamater-

gic activity (Klempan *et al.*, 2009). The remaining two genes were *Dlg3* (discs large homolog 3) (FC=2.06, p=0.0173) and *Nmt1* (N-myristoyltransferase 1) (FC=1.59, p=0.0043), both mediating the activity of glutamate NMDA receptors. DLG3 maintains NMDA receptor synaptic trafficking (Sans *et al.*, 2003), while the enzyme NMT1 directs the attachment of fatty acid myristate to Src family protein kinases, which control NMDA receptor activity (Selvakumar *et al.*, 2005). Epileptic patients have upregulated *Nmt1* (Selvakumar *et al.*, 2005), which might be involved in seizure generation and epileptogenesis. Pooling these data with those for *Kcnc2* and *Kcnip2*, we could speculate that in the hypothalamus a possible inequality in neuronal excitation and inhibition processes could cause sleep disruptions. Increased glutamate and NMDA receptor activity may underlie the HPA system hyperactivity characteristic for MDD (Nestler *et al.*, 2002). Finally, the same activity could also result in excitotoxicity, which in its turn increases a synthesis of reactive oxygen species, induces malfunction of mitochondria, and errors in protein folding.

Our impression is substantiated by the observation of increased numbers of transcripts related to unfolded protein binding, such as *Dnajc5* (DnaJ (Hsp40) homolog, subfamily C, member 5) (FC=1.74, p=0.0043), *Cct6a* (chaperonin containing Tcp1, subunit 6A) (FC=2.09, p=0.0130), and *Canx* (calnexin) (FC=1.69, p=0.0303), as well as to mitochondrial function. The latter transcripts include *Dnm1l* (dynamin 1-like) (FC=1.59, p=0.0454) maintaining mitochondrial fission (Li *et al.*, 2010) and *Slc25a30* (solute carrier family 25 member 30) (FC=1.60, p=0.0173) encoding a mitochondrial carrier transporting metabolites over the inner membrane (Haguenauer *et al.*, 2005). Upregulation of *Slc25a30* may disrupt energy homeostasis and synaptic activity. As mitochondrial fission is critical for normal cell activity and vitality, increased transcription of *Dnm1l* by disturbing the fission/fusion cycle might induce cell degeneration. Notably, mitochondria provide more than 95% of the energy in demand (Rezin *et al.*, 2009). The evidence for an underlying role of mitochondrial abnormal functioning in the development of neurodegenerative (Alzheimer's disease, Parkinson's disease, and Huntington's disease) and affective (depression, bipolar disorder, schizophrenia) disorders is constantly increasing (Lin and Beal, 2006; Rezin *et al.*, 2009). Collectively, excitotoxicity, the HPA system hyperactivity, and the hypothalamic cell loss may induce physical and biochemical changes also in the target areas of the hypothalamic projections, such as e.g. the cingulate gyrus and the hippocampus, which show in depression a reduced volume (Konarski *et al.*, 2008).

Furthermore, several genes related to synaptic functioning showed an increased expression. The strongest significance was detected for *Aplp2* (amyloid beta precursor-like protein 2) (FC=2.76, p=0.0087), whose product maintains synaptic maturation (Gralle and Ferreira, 2007). Consistently, depressed subjects have elevated APLP2 concentrations in the cerebrospinal fluid (Post *et al.*, 2006). Densi-

ty of vesicles and synaptic active zone size are lower in *App/Aplp2* deficient mice (Yang *et al.*, 2005). *Aplp2* upregulation in CLI-treated rats may cause synaptic dysfunctions in the hypothalamus.

CLI-treated animals had an increased number of transcripts for genes related to the processing, transport and translation of RNA, such as *Prpf38b* (pre-mRNA processing factor 38 (yeast) domain containing B) (FC=1.76, p=0.0195), *Ddx3x* (DEAD box polypeptide 3 X-linked) (FC=1.64, p=0.0454), and *Caprin1* (cell cycle-associated protein 1) (FC=1.81, p=0.0303). Also *Spin1* (spindlin 1) was upregulated (FC=1.53, p=0.0454), which could promote chromosome instability and apoptosis (Yuan *et al.*, 2008; Zhang *et al.*, 2008). These findings point together at possible transcriptional and translational disruptions in the hypothalamus of CLI-treated rats.

Eight transcripts were underexpressed in the CLI-treated animals. Among the most interesting was *Ttr* (transthyretin) (FC=-1.74, p=0.0303) whose product transports thyroid hormone and vitamin A (Raghu and Sivakumar, 2004; Prapunpoj and Leelawatwattana, 2009). Hypothyroidism associates to depression (Jackson, 1998), while retinoic acid, a vitamin A product, regulates neurogenesis and dopaminergic signaling, thus affecting the susceptibility to depression (Bremner and McCaffery, 2008). Also, thyroid hormones contribute to neurogenesis and plasticity by regulating the transcription of genes coding neurotrophic receptors p75 and TrkB (Tapia-Arancibia *et al.*, 2004). Our result is in agreement with human data on depression which show decreased concentrations of transthyretin in the cerebrospinal fluid (Sullivan *et al.*, 2006).

Another underexpressed transcript was the one predicted for *Foxo1* (forkhead box O1) (FC=-1.53, p=0.0303). FOXO1 promotes food consumption by enhancing the expression of *Npy*, whose product is the orexigenic neuropeptide Y, and by diminishing the expression of anorexigenic proopiomelanocortin (Kim *et al.*, 2006). Inhibition of *Foxo1* in the hypothalamus causes decreases in food consumption and body weight (Kim *et al.*, 2006). *Foxo1* downregulation in CLI-treated rats might be responsible for the reduction in food intake and body weight detected in these animals, which also constitutes characteristic features of MDD. Remarkably, *Foxo1* deficient mice have a depressive-like phenotype (Polter *et al.*, 2009). However, we should not over-interpret the finding for Foxo1-predicted transcript, as it does not absolutely match the gene sequence.

The pathway analysis revealed numerous pathways affected in CLI-treated rats. Several synaptic pathways displayed differential expression, such as those for the regulation of neurotransmitter levels (permuted p=0.0007), Golgi vesicle transport (permuted p=0.0003), synaptic vesicle (permuted p=0.00003), synapse (permuted p=0.0005), and synaptic transmission (permuted p=0.00005). These results suggest profound synaptic disturbances in the hypothalamus of CLI-treated rats. From the molecular function category, $GABA_A$ receptor activity and chloride channel ac-

tivity demonstrated altered expression (permuted p=0.0007, permuted p=0.0005, repectively). Human data indicate a decreased expression of $GABA_A$ receptors in depressed individuals: $GABA_A$ receptors are hypermethylated at their promoter sites in the frontoparietal cortex of suicide victims and mRNA levels of receptor subunits are greatly reduced (Merali *et al.*, 2004; Poulter *et al.*, 2008). Changes in $GABA_A$ receptor activity together with the overexpression of *Kcnc2* and *Kcnip2* in the hypothalamus could disrupt the sleep cycle. On the other hand, these changes might also be a counteractive reaction for the purpose of neutralizing the suggestively higher glutamatergic activity in the CLI-treated rats.

Of the cellular component category, the neuron projection pathway showed distinct expression (permuted p=0.0002) with *Ntrk2* (BDNF/neurotrophin 3 receptor precursor) as one of the top genes. This supports the hypothesis of disturbances in neurogenesis and neuronal survival in the depressive phenotype. Finally, the biological process category was represented by changes in RNA processing pathways (mRNA processing and RNA splicing) (permuted p=0.00001, permuted p=0.00002, respectively), which suggests transcriptional abnormalities and strengthen our aforementioned conclusions. Pathways which were differentially expressed in CLI-treated rats comprised some genes which associate to human depression. These are *Gad1* (glutamate decarboxylase 1) (Hettema *et al.*, 2006), *Gabrg1* ($GABA_A$ receptor, subunit gamma 1) (Klempan *et al.*, 2009), *Htr2a* (serotonin receptor 2A), *Htr2c* (serotonin receptor 2c) (Choi *et al.*, 2004b; Koks *et al.*, 2006), *Gria3* (glutamate ionotrpic receptor, AMPA-3) (Klempan *et al.*, 2009), and *Ntrk2* (Altar *et al.*, 2009).

The main limitation of the study was the lack of the information about the estrous cycle stage of the female rats and the low number of the animals. However, for the genes in the main body of the discussed data there is a consistency in the direction and the values of gene expression within the experimental groups. If the estrous cycle would affect the expression of these genes, we would observe less consistency.

Methodological considerations. In order to rule out technical and methodological errors we validated several genes in both studies by TaqMan RT-PCR. In Study III six genes (*Dnmt2*, *Sox6*, *Pde4d*, *Acatn*, *Fgfr2*, and *Igf2*) were evaluated. All genes except *Igf2* showed the same direction of expression (*Dnmt2*: FC=1.95; *Sox6*: FC=1.94; *Pde4d*: FC=1.90; *Acatn*: FC=1.92; *Fgfr2*: FC=1.94; *Igf2*: FC=-2.0). In Study IV four genes (*Ttr*, *Dnajc5, Snx10* and *Dlg3*) were analyzed, and three of them clearly agreed with array results (Ttr: FC=-1.54; Dnajc5: FC=1.19; Snx10: FC=1.22). The finding for *Dlg3* was less pronounced (FC=1.02), although showing the same tendency as in arrays. Thus, the two approaches of RNA quantification showed an acceptable accordance, but as the fold changes are moderate, the results should not be overinterpreted. Also the assessed hypotheses are not independent, and the expression alterations in this type of paradigm are anticipated to be slight

(Sequeira and Turecki, 2006). Presumably for the same reason the results for the single transcripts did not pass the multiple testing correction procedure and are merely indicative. Therefore, the validity of the obtained data should be evaluated rather on the basis of the results consistency, than on stringent correction adjustments (Klempan *et al.*, 2009). The pathway approach might be more suitable for an expression analysis of mood disorders or of other phenomena with less pronounced changes.

Conclusions. Our data demonstrate that the neonatal CLI treatment initiates early-life molecular alterations in the BF which are mediated through epigenetic mechanisms, as several differentially expressed transcripts and a common transcription factor CREB1 are involved in epigenetic modifications. These changes could induce behavioral changes in sleep and mood. Similar mechanisms might also be functional in humans during the development of depression. It will be important to compare these results with the expression patterns in the BF of adult rats. In the hypothalamus of adult female rats the most pronounced variations were observed for the K^+-channel and glutamatergic genes, mitochondrial genes, as well as for $GABA_A$ receptor and synaptic pathways, pointing at a disruption of synaptic transmission, of neuronal excitation-inhibition processes, and at possible oxidative stress with consequent cell death. Remarkably, $GABA_A$ receptor activity was affected in both brain regions and in both ages. This could signify GABA signaling as one of the crucial mechanisms for depressive pathophysiology. As the hypothalamus regulates a number of physiological processes including food intake, sleep, and reproduction, which are all affected in depression, the detected molecular disruptions might disturb its functioning and underlie the depressive symptoms. Our hypothalamic results should be paralleled to male data in order to understand whether they show similar changes.

It would be valuable to compare our current findings with human data from postmortem brain tissues as well as with other animal models. Our results call for a detailed examination of the individual genes identified in this study, by silencing or overexpressing them in order to arrive at a clear interpretation of their contribution to depression and in order to detect if they could be possible targets for treatment. And of course, also other brain areas maintaining sleep and mood should be explored.

5. SUMMARY AND CONCLUSIONS

The main results of the original publications can be summarized as follows:

I. In contrast to A_{2A} receptor antagonist, A_1 receptor antagonist perfused into the BF during 3 h of SD significantly reduced both recovery NREM sleep amount and delta power. Thus, in the BF adenosine induces recovery NREM sleep when acting through A_1 receptors.

II. Malfunctioning adenosine transport due to variation in the nucleoside transporter gene *SLC29A3* in women and *SLC28A1* in men could predispose to depression accompanied by early morning awakenings and fatigue.

III: Multiple changes in gene expression patterns occur in the BF long before the manifestation of depressive-like symptoms in the rat model of depression. One of the possible causes for these changes might be a differential epigenetic regulation, since several differentially expressed genes (*Dnmt2*, *Igf2*, *Fgfr2*, *Dpp4*, *Col1a2*, *Impact* and *Plk2*) and a common transcription factor CREB1 mediate epigenetic modifications. Also synaptic transmission and $GABA_A$ receptor signaling are effected. Altogether these findings demonstrate how the early-life events may affect the adult-life phenotype through numerous synaptic disruptions and epigenetic mechanisms.

IV. The variations in expression of the K^+-channel, glutamatergic and mitochondrial genes indicate possible hypothalamic disruptions in neuronal excitation-inhibition processes, oxidative stress and excitotoxicity in the adult depressive-like phenotype. $GABA_A$ receptor signaling and synaptic transmission were also affected in this region, similarly to the effects in the BF of postnatal rats. This finding stresses GABA signaling as one of the basic mechanisms malfunctioning in depression.

ACKNOWLEDGEMENTS

This work was carried out during the years 2005-2010 at the University of Helsinki, Institute of Biomedicine, Department of Physiology and at the Finnish National Institute of Health and Welfare, Public Health Genomics Unit. I would like to thank the heads of the departments for providing the research facilities.

I wish to express my gratitude to everyone who guided and encouraged me in my research projects during those years.

First and foremost my excellent supervisor Tarja Stenberg who guided and supported me throughout my dissertation projects while also allowing me to work in my own way. Tarja's professional knowledge and expertise in diverse scientific areas made this work a versatile experience, and this thesis would not have been possible without her patience, persistent help and talent for teaching. I am grateful to Tarja in every possible way and I consider myself extraordinary fortunate to have had her as a supervisor.

I owe my deepest gratitude to Tiina Paunio for her invaluable support and introducing me to the genetic aspects of behavior regulation. Without Tiina this work would never be completed.

Anna Kalinchuk for her support, teaching the methods and data analysis and help in writing. Also for introducing to the international sleep research community and a great pleasure as a company at conferences.

The late Dag Stenberg for teaching me the data analysis and for the intellectual and stimulating discussions.

Pirjo Saransaari and Olli Vuolteenaho for kindly reviewing the thesis and for their constructive comments.

All the co-authors of the papers, in particular Markus Lagus, Hanna Ollila and Siddeshwar Utge for their fundamental effort in experimental setup and data analysis. It was fun to learn genetic research tools with them.

Ernst Mecke for his invaluable help in the experiments, language revision of my papers and thesis, and for nice company. Pirjo Saarelainen for her kind help in the experiments, keeping me stocked with laboratory supplies and for good-humoured company.

All the past and present members of Sleep Team Helsinki for the cheerful atmosphere in the lab, insightful discussions and great company at conferences. In particular I note Andrey Kostin and Sergey Savelyev for their warm friendship and sharing many nice moments in my life which I remember with good laugh. I wish to thank Henna-Kaisa Wigren for teaching me the basics of EEG analysis and discussions which were very fruitful in shaping up my ideas. I thank Kirsi-Marja Rytkönen and

Janneke Zant for the lively atmosphere in the lab, many bright discussions about both science and everything else except science. I will remember our samba performances at conferences with a kind smile. I also note Anna-Sofia Urrila, Leena Laine, Maija Vainikka and Sari Levo-Siitari, as well as Veera Holopainen and Vilja Eskelinen for their help in experiments and sleep scoring.

Marja-Liisa Tiainen, Anja Talvitie and Anne Simola for their excellent secretarial assistance and dealing with travel funds.

My dear family for their presence, unconditional love and support and persistent confidence in me throughout all these years. Without them I would never succeed.

I am indebted to Finland because it provided such good conditions for my work and it gave me opportunities to develop in many ways. It was a very enjoyable period of my life which I will always remember. I would like to thank my dear friends in Finland for their support and presence.

The work was financially supported by the Finnish Graduate School of Neuroscience, the Center for International Mobility, Academy of Finland, the European Union (LSHM-CT-2005-518189), Helsinki University Central Hospital (EVO, TYH6254) and Finska Läkaresällskapet.

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