NATIONAL INSTITUTE FOR HEALTH AND WELFARE

Markus Lagus

Molecular Mechanisms of Sleep and Mood

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MOLECULAR MECHANISMS OF SLEEP AND MOOD

Academic dissertation

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University of Helsinki
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Department of Biosciences, Biotechnology
and
University of Helsinki
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Supervised by

Prof. Tiina Paunio
Public Health Genomics Unit and
Institute for Molecular Medicine FIMM,
National Institute for Health and Welfare, Helsinki, Finland
Department of Psychiatry,
Helsinki University Central Hospital,
Helsinki, Finland

and

Doc. Tarja Stenberg
Institute of Biomedicine, Department of Physiology
University of Helsinki, Finland

Reviewers

Prof. Juha Partanen
Department of Biosciences,
Faculty of Biological and Environmental Sciences,
University of Helsinki, Finland

Doc. Dario Greco
Department of Biosciences and Nutrition,
Karolinska Institutet,
Stockholm, Sweden

Opponent

Prof. Björn Appelberg
Department of Psychiatry,
University of Helsinki, Finland

Custos

Prof. Kari Keinänen
Faculty of Biological and Environmental Sciences
Department of biosciences,
University of Helsinki, Finland

To: Pati, Pekka, Pedro and Lotta

Abstract

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Background Sleep disturbances and mood alterations are highly interrelated. The majority of patients suffering from depression report a reduced sleep quality. Inversely, people with sleep complaints are at elevated risk to develop depression. The complex regulation of these phenomena involves several brain areas and mechanisms. The susceptibility to change in this system is influenced by several factors, such as age and stressful lifestyle that are considered in this study.
Hypothesis The hypothesis of this study was that sleep and mood share common genetic/molecular regulatory networks and that both are also regulated by epigenetic mechanisms and neural plasticity.

Methods The studies were conducted both on humans and using an animal model for depression. In the animal model we measured the genome wide expression of genes in different brain areas of clomipramine-treated pups and adults. Using these data we conducted both individual area and inter-area network analyses of basal forebrain, frontal cortex, hypothalamus and hippocampus. We also measured the amount of BDNF, one of the plasticity-related factors, in sleep restriction and under aging. In the human study we conducted epigenetic analysis of the serotonin transporter gene and related the epigenetic changes to stress in a stressful working environment.

Results In the models investigated changes were observed on the system, protein, transcript and transcriptional regulatory levels. Inter-tissue pathways related to synaptic transmission, regulation of translation and ubiquitinylation were disrupted. The involved pathways are within the cellular components of the axons, growth cones, melanosomes and pigment granules. The disturbed networks are centred around serotonin, Mn(II) and Rhoa. In the basal forebrain the imbalance in gene expression is widely controlled by CREB1. Some of the changes seem to be epigenetically induced by sleep deprivation and stress. Individuals working in a high stress environment have significantly less methylation in the promoter area of serotonin transporter gene SLC6A4, as compared to individuals working in a low stress environment. We also found that the expression of cortical BDNF correlated with the recovery non-REM (NREM) slow wave activity (SWA) response, and that both the cortical BDNF and the SWA response to sleep deprivation were decreased in the aged animals, as were the changes in sleep latency.

Conclusions The disturbances in the models investigated, arise, largely, but not solely, due to disruption in neurological systems previously related to the regulation

of sleep and mood. Novelty value could be ascribed to findings that suggest involvement of inter-tissue networks, and more precisely, imbalance of melanosome related gene expression and gene networks connected to Mn(II). The stress induced demethylation of the SLC6A4 promoter suggests a mechanism for the body to cope with prolonged excessive stress. The downside of this coping mechanism is the possibility that this reprogramming increases the long-term risk for mood disorders. The findings in the sleep deprived aging rats support the hypothesis that the age related decrease in homeostatic NREM SWA is related to a reduced sleep need.

Keywords: sleep, mood, genes, pathways, networks

Tiivistelmä

Markus Lagus. Molecular mechanisms of sleep and mood [Uni- ja mielialahäiriöiden molekyylitausta]. Terveyden ja hyvinvoinnin laitos (THL), Tutkimus 100. 156 sivua. Helsinki 2011.
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Tausta Uni- ja mielialahäiriöt ovat tiiviisti kytköksissä toisiinsa. Valtaosa masennuspotilaista ilmoittavat myös unen laadun heikentyneen. Käänteisesti, univaikeuksista kärsivät henkilöt ovat alttiimpia masennukselle. Näiden ilmiöiden säätely on monimutkainen ja tapahtuu usean mekanismin avulla monessa eri kohdassa aivoja. Useat eri tekijät, kuten rasittava elämäntyyli ja ikä jotka ovat huomioitu tässä tutkimuksessa, vaikuttavat tähän järjestelmään.

Hypoteesi Tämän tutkimuksen hypoteesi oli että unea ja mielialaa säätelevät samat geneettiset ja molekyylitason järjestelmät joita vuorossaan säätelee epigeneettiset mekanismit ja neuronaalinen plastisuus.

Menetelmät Tässä tutkimuksessa käytetään sekä ihmisnäytteitä että eläinmalleja. Masennusta jäljittelevässä eläinmallissa mitataan perimänlaajuisesti usean eri aivoalueen geeniekspressiota. Näihin mittaustuloksiin perustaen, suoritettiin analyysejä sekä yksittäisissä aivoalueissa että geeniverkkoanalyysejä useista aivoalueista. Toisessa eläinmallissa mitattiin plastisuusgeenin BDNF:än ilmentymistasoja ikääntymisessä ja olosuhteissa joissa unta häiritään. Ihmistutkimuksessa tarkkaillaan muutoksia serotoniinikuljettajageenin epigeneettisessä säätelyssä suhteessa rasittavaan työympäristöön.

Tulokset Tutkituissa malleissa todetaan muutoksia niin järjestelmä-, proteiini-, transkripti- että transkriptiota säätelevällä tasolla. Useita aivoalueita kattavia geenipolkuja liittyen synapsien välitykseen, translaation säätelyyn ja ubiquitinylaatioon todetaan muuttuneen. Muuttuneet verkostot keskittyvät Mn(II) ja Rhoa:n ympärille. Basaalisissa etuaivoissa muuttunut geeniekpressiotasapaino on laajalti CREB1 säätelemä. Jotkut muutokset näyttävät johtuvan univajeen ja stressin käynnistämästä epigeneettisestä säätelystä. Yksilöissä jotka työskentelevät rasittavassa työympäristössä todetaan alhaisempi metylaatioaste serotoniinikuljettajageenin (SLC6A4) promootterialueella, verratuna vähemmän rasittavassa työympäristössä työsketeleviin. Todetaan myös että aivokuoressa tapahtuva BDNF ekspressio korreloi palautusunen non-REM (NREM) matalaaaltopituisen aktiviteettivastineen (SWA) kanssa, ja että BDNF ekspressio, SWA vastine sekä muutokset univiiveessä heikentyvät iän myötä. Johtopäätökset Häiriöt näissä tutkituissa malleissa johtuvat pääasiallisesti muutoksista jo ennestään tunnetuissa unta ja mielialaa säätelevissä neurologisissa järjestelmissä. Todettakoon kuitenkin että löydökset jotka viittaavat eri aivoalueita yh-

distävissä järjestelmissä, eritysesti melanosomeihin liittyvässä geenieksпressiossa sekä Mn(II) liittyvien verkkojen olevaan epätasapainoon ovat ennennäkemättömiä ja avaavat uusia alueita uni- ja mielialatutkimuksessa. Stressin aiheuttama demetylaatio SLC6A4 promootterialueella taas osoittaa kehon menetelmää selvitä pitkäaikaisen stressin kanssa. Tämän mekanismin haittapuolena saattaa kuitenkin olla että se pidemmän päälle lisää riskiä sairastua mielialahäiriöön. Ikääntyneissä eläimissä suoritetut unihäirintäkokeet taas tukevat hypoteesiä jonka mukaan ikääntymiseen liittyvä homeostaattisesen NREM SWA vastineen väheneminen johtuisi pienentyneestä unentarpeesta.

Avainsanat: uni, mieliala, geenit, polkuanalyysi, geeniverkko

Contents

List of original papers

I Lagus M, Gass N, Saharinen J, Saarela J, Porkka-Heiskanen T, Paunio T. Gene expression patterns in a rodent model for depression. European Journal of Neuroscience, 06 Apr. 2012.

II Lagus M, Gass N, Saharinen J, Savelyev S, Porkka-Heiskanen T, Paunio T. Intertissue networks between the basal forebrain, hippocampus and prefrontal cortex in a model for depression caused by disturbed sleep. Journal of Neurogenetics, 07 Sept. 2012.

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

IV Alasaari JS, Lagus M, Ollila H, Kivimäki M, Kronholm E, Härmä M, Puttonen S, Paunio T. Environmental stress affects DNA methylation of a CpG rich promoter region of serotonin transporter gene in a nurse cohort. PLOS ONE, 28 Sept. 2012.

V Rytkönen KM, Lagus M, Rantamäki T, Castren E, Paunio T, Porkka-Heiskanen T. Aging and sleep: Insights from multiple-sleep latency test and cortical BDNF. Submitted.

Abbreviations

BF	Basal Forebrain
bp	Base-pairs
CLI	Clomipramine
CpG	Cytosine-Guanine dinucleotide
cRNA	Complementary RNA
EEG	Electroencephalography
EMG	Electromyogram
FC	Fold Change
FCX	Frontal Cortex
GBD	Global Burden of Disease
HIP	Hippocampus
HPA	Hypothalamic-Pituitary-Adrenal
HYP	Hypothalamus
MBI-GS	Maslach Burnout Index General Survey
MDD	Major Depressive Disorder
MSLT	Multiple Sleep Latency Test
PCA	Principal Component Analysis
REM	Rapid Eye Movements
REMS	REM Sleep
SAL	Saline
SD	Sleep Deprivation
SL	Sleep Latency
YLDs	Years Lived with Disability

1 Introduction

Sleep, mood and stress are all phenomena that express fluctuations in both frequency and duration, as well as in intensity and quality. Limits have to be defined for changes to qualify as abnormal. This means that either large sample size or radical models have to be used when studying these phenomena. The absence of definite boarders nevertheless does not mean that individuals suffering from disturbances in any of these phenomena could be left unattended to. The main focus in this study is the effect of changes in sleep and stress, with the outcome of depressed mood. Most of the work represented in this study is presumption free, with the exception of the candidate gene approaches used for the BDNF and SLC6A4 studies in relation to mood, sleep and age, respectively stress. Nevertheless, the findings to a large extent comprised of changes in molecular systems, such as the cholinergic, glutamatergic, serotonergic, GABAergic and components of the hypothalamic-pituitary-adrenal (HPA) axis, previously related to the regulation of both sleep and mood.

1.1 Sleep

Sleep is a reversible state of reduced consciousness and response to outside stimuli. Most animals studied present a state of sleep, whereas in some the term "rest" is preferred to avoid complications with the definition of sleep (Cirelli & Tononi, 2008). Mammalian sleep is divided into two distinctive types of sleep; rapid eye movement (REM) sleep and non-rapid eye movement (NREM) sleep. The characteristics of REM sleep are; a high activity in the high frequency spectra of the electroencephalogram (EEG) and rapid eye movements, as implied by the name of the sleep type. In NREM sleep the eyes remain still and the EEG reveals low frequency waves of high amplitude. The NREM sleep can be further divided into three stages (N1, N2, N3). During the progression from stage N1 to N3 the frequency drops and the amplitude rises. Stage N3 is also referred to as slow wave sleep (SWS). Sleep duration and timing is regulated by two different components: the homeostatic, and the circadian component. The homeostatic sleep pressure rises according to the time spent awake, whereas the sleep pressure arising from the circadian component varies during the daily cycle. The additive effects of the two components amounts to the total sleep pressure (Borbely, 1982).

1.2 Major depressive disorder (MDD)

MDD is a common mental disorder with a worldwide lifetime prevalence estimate of 8-12% (Andrade et al., 2003). Depression is the leading cause of disability as meas-

ured by years lived with disability (YLDs) and in year 2002 MDD was estimated to be the 4:th leading contributor to the global burden of disease (GBD), whereas it is estimated to be ranked 2:nd on the list by year 2030 (Mathers & Loncar, 2006). To some extent the high GBD for MDD is due to the high mortality by suicide. A meta-analysis showed that 87.3% of suicide cases had been diagnosed with a mental disorder, mainly MDD (Kessler, 2007) and another meta-analysis showed that 16-19% of MDD diagnosed patients complete suicide (Wulsin, Vaillant, & Wells, 1999). The healthcare costs for MDD are substantial and it has been suggested that the development of cost-effective treatments for mild disorders might prevent a large proportion of future serious disorders (Kessler, 2007).

1.3 The impact of disturbed sleep on mood

The existence of interplay between sleep and mood is hard to deny. People that report sleep complaints are at 3-4 times the elevated risk of developing a new major depression within one year (Breslau, Roth, Rosenthal, & Andreski, 1996; Ford & Kamerow, 1989). Sleep disturbances are key features of depressive symptomatology and up to 90% of depressed patients complain of poor quality of sleep (Tsuno, Besset, & Ritchie, 2005). The mood of healthy individuals is affected by both acute and cumulative partial sleep deprivation (SD) (Ferrara & De Gennaro, 2001). The instant, but unfortunately temporary, antidepressant effect of SD in depressed patients (Wirz-Justice & Van den Hoofdakker, 1999) is, inversely, another example of the interplay. Yet very little is known about the causal nexus of disturbed sleep and depressed mood, especially on a molecular level. One way to address this relationship is to use controlled SD, in either healthy volunteers or animals. Another way is to observe a large cohort, with data of the parameters of interest, linked to it.

1.4 The impact of stress on mood

An overall trend in the increase of work stress can be observed in Europe (Eurofound, 2012). A high work strain and stressful working environment significantly increases the risk of a depressive episode (Blackmore et al., 2007; Kopp, Stauder, Purebl, Janszky, & Skrabski, 2008; Melchior et al., 2007). That chronic stress has a negative effect on mood is well known, but the molecular mechanisms underlying the connection are under dispute. Recent findings have revealed the vastness of adult neurogenesis and stress has been shown to be one of the most potent environmental parameters that suppress adult neurogenesis (Mirescu & Gould, 2006). This suppression of neurogenesis, especially in the HIP and FCX, is thought to increase vulnerability to depression and (according speculations), also sleep disruption would exert its negative effects on mood via the same mechanism (Lucassen et al., 2010).

On a molecular level the serotonergic system, the serotonin transporter gene (SLC6A4) in particular, has received much attention in stress related studies. The SLC6A4 encodes the serotonin transporter protein 5HTT, responsible for the reuptake of 5-HT to the presynaptic neuron. 5HTT determines the magnitude and duration of serotonergic response and is thus central to the fine-tuning of serotonergic neurotransmission (Mossner et al., 2000). One of the mechanisms regulating 5HTT activity is regulation at the level of transcription by DNA methylation. This methylation of the SLC6A4 promoter is associated with a decrease in SLC6A4 mRNA and thought to add to the vulnerability to psychiatric illnesses (Philibert et al., 2007).

Furthermore, the serotonergic system as a whole is gaining an increasing amount of attention in all the subsystems observed in this study: sleep (Ursin, 2002), depression (Blier & de Montigny, 1994), stress and ageing (Rodriguez, Noristani, & Verkhratsky, 2012).

1.5 Age – sleep

Changes in circadian timing and the regulation of homeostatic sleep pressure, accompanied by changes in sleep quality and architecture, occur with age. The total sleep time is reduced and, accordingly, the total amount of time spent awake, in episodes, after sleep onset is increased with age (Buysse, Monk, Carrier, & Begley, 2005). The sleep efficiency, percentage of slow-wave sleep, percentage of REM sleep and REM latency decrease with age, while overall sleep latency (SL), proportion of stage 1 sleep, proportion of stage 2 sleep, increase with age. However, only sleep efficiency continues to decrease after 60 years of age (Ohayon, Carskadon, Guilleminault, & Vitiello, 2004).

In detail, the decrease in slow-wave activity (SWA) at electroencephalographic (EEG) power between 0.5 and 4 Hz, during NREM sleep is a marker of sleep need and SWA increases according to wake quality (Huber, Tononi, & Cirelli, 2007; Wigren, Schepens, Matto, Stenberg, & Porkka-Heiskanen, 2007; Vyazovskiy & Tobler, 2005) and time spent awake before falling asleep (Achermann, Dijk, Brunner, & Borbely, 1993; Borbely, 1982) and decreases during subsequent recovery sleep. Plastic processes that occur during wakefulness increase sleep pressure and result in an increase in synaptic strength, which is renormalized during sleep and a recent hypothesis suggests that changes in SWA follow changes in cortical synaptic strength (Tononi & Cirelli, 2003, 2006). The expression of genes related to synaptic plasticity, e.g. the brain-derived neurotrophic factor (BDNF), have been shown to increase in the cortex during spontaneous and prolonged waking, as compared to the state of sleep (Cirelli & Tononi, 2000), in accordance with the aforementioned hypothesis.

The possibility exists that BDNF has a central role in the interaction between all the phenomena of interest in this study. BDNF has been linked to regulation of mood (Hashimoto, Shimizu, & Iyo, 2004) (at least partly via the serotonergic system), stress response (Martinowich & Lu, 2008), sleep pressure (Faraguna, Vyazovskiy, Nelson, Tononi, & Cirelli, 2008); and finally, its expression is reduced along with age (Zeng et al., 2011).

1.6 The clomipramine (CLI) model of depression (caused by disturbed sleep)

CLI is a tricyclic antidepressant. It is known to dramatically reduce REM sleep (REMS) in neonatal rats (Mirmiran, van de Poll, Corner, van Oyen, & Bour, 1981). The suppression of REMS, at neonatal age, by CLI treatment, results in an infested depressive-like behaviour at adult age. The signs of depressive-like behaviour include: increased alcohol intake, decreased aggression, motor hyperactivity in a stressful situation, increased immobility in "behavioural despair" test and anhedonia in form of decreased sexual activity, sucrose preference and intracranial self-stimulation (Feng & Ma, 2002; Vogel, Neill, Hagler, Kors, & Hartley, 1990). The animals also show abnormalities in sleep that resemble the disturbed REMS pattern observed in human depression (Vogel, Neill, Kors, & Hagler, 1990). Earlier studies have addressed the effect of CLI on gene expression using a candidate gene approach (Cassano, Hidalgo, Burgos, Adris, & Argibay, 2006; Feng, Guan, Yang, & Fang, 2003; Hansen & Mikkelsen, 1998) but a systematic survey at the whole-genome level has not been previously performed.

1.7 Basal forebrain (BF) – Sleep and Mood

The BF has been implicated in the regulation of both NREM (Porkka-Heiskanen et al., 1997) and REM sleep; or more precisely; the cholinergic BF neurons have been shown to contribute to generation of theta rhythm (Lee, Hassani, Alonso, & Jones, 2005), presumably a result of an increased cholinergic activity in the BF (Vazquez & Baghdoyan, 2001); whereas glutamatergic activity in the BF has been related to sleep pressure (Wigren et al., 2007). Furthermore, the cholinergic cells of BF degenerate in Alzheimer's disease patients (Teipel et al., 2005; Vogels et al., 1990), who also frequently suffer both from problems in sleep and depressive disorder (Catania et al., 2009; Forstl et al., 1992). The anxiolytic effects of benzodiazepines have been ascribed to a mechanism of action where benzodiazepines inhibit the forebrain control system for wakefulness (Kajimura et al., 2004), thereby ascertaining the position of BF in both mood and sleep regulation.

1.8 Frontal cortex (FCX) – Sleep and Mood

The FCX is mediating normal sleep physiology, dreaming and sleep-deprivation phenomena. During non-REM (NREM) sleep, the FCX exerts the highest voltage and the slowest brain waves compared to other cortical regions. The differences between the self-awareness experienced in waking and its diminution in dreaming can be explained by deactivation of the dorsolateral prefrontal cortex during REM sleep. This deactivation has been proposed to result from a direct inhibition of the dorsolateral prefron-

tal cortical neurons by an enhanced release of acetylcholine during REM sleep (Muzur, Pace-Schott, & Hobson, 2002). The FCX has, in several studies, been described as a central area involved in the development of depression. Volumetric studies have revealed a reduction of grey matter in FCX amongst depressed patients (Drevets et al., 1997; Nugent et al., 2006; Rajkowska, 1997). Also within the field of neuroimaging, changes in the activation intensity of FCX have been detected in depressed individuals during different emotion-related tasks (Murray, Wise, & Drevets, 2011).

1.9 Hippocampus (HIP) – Sleep and Mood

The HIP is another brain area actively involved in sleep and in the handling of memories and emotions (Walker, 2009). Changes in hippocampal volume have been observed in major depressive disorder (MDD) (Lorenzetti, Allen, Fornito, & Yucel, 2009). The recent discovery that depressed patients show abnormal hippocampal theta activity (Cornwell et al., 2010), further contributes to the spatial conception of mood disorders. This regulation of hippocampal theta activity is under the influence of GABAergic neurons (Xu, Datta, Wu, & Alreja, 2004).

1.10 Hypothalamus (HYP) – Sleep and Mood

The importance of the hypothalamic region in relation to sleep regulation (Economo, 1930) had already been discovered at the beginning of the 20th century. The HYP consists of a large number of nuclei, many of which are involved in sleep regulation. The HYP partakes in both homeostatic and circadian regulation of sleep, as well as in allosteric alterations of sleep (Saper, Scammell, & Lu, 2005). A decrease in hypothalamic volume can be observed in several affective disorders (Schindler et al., 2012) and as the HYP is part of the hypothalamic-pituitary-adrenal (HPA) axis, it is also thereby an important regulator of sleep, stress response and mood on a system level (Mormede et al., 2011).

1.11 Connections between Basal forebrain-Hippocampus-Frontal cortex (BF-HIP-FCX)

Connections between the BF, FCX and HIP have been identified and some of these connections have been associated with regulation of mood or sleep. According to the hypothesis of this study (II), the mood alterations observed in the CLI model would be caused by gene expressional changes in these tissues and disruptions of the connections between them.

1.11.1 Basal Forebrain-Frontal cortex (BF-FCX)

The cells of the BF project widely to the cortex and the limbic system (Porkka-Heiskanen et al., 1997; Zaborszky, Pang, Somogyi, Nadasdy, & Kallo, 1999). Through widespread projections to the cerebral cortex, including dense projections to the FCX, the BF plays important and diverse roles in the modulation of cortical activity in association with different behavioural states (Everitt & Robbins, 1997; Henny & Jones, 2008; Sarter, Bruno, & Givens, 2003; Weinberger, 2003). These projections are glutamatergic, GABAergic and cholinergic, thus all contributing to the diverse manner in which the BF influences cortical activity (Henny & Jones, 2008).

1.11.2 Hippocampus-Frontal cortex (HIP-FCX)

The HIP presents projections to the FCX and some of the projections seem to be reciprocal (Goldman-Rakic, Selemon, & Schwartz, 1984). These projections play an important role in anxiety disorders, as shown by mice models for anxiety research (Adhikari, Topiwala, & Gordon, 2010).

1.11.3 Basal forebrain-Hippocampus (BF-HIP)

Neuronal projections between the BF and HIP exist, and although less dense and less well studied, compared to the BF and HIP projections to the FCX, they have been shown to be involved in the generation of theta rhythm (Xu et al., 2004).

2 Aims of the study

The interplay between stress, mood and sleep is well known in all cultures and throughout history. Nevertheless, very little is known about the interplay on a molecular level. The ambitious aim of this study was to unravel these mechanisms. When feasible, the research was conducted on a genome-wide scale without a priori designated genes, pathways or cell-types. The candidate gene based studies on SLC6A and BDNF were performed due to financial and methodological restrictions..

Some of the approaches turned out failures, whereas some, discussed here, were successful and contribute to the constantly growing amount of information about this intricate interplay.

Aim 1. Using the rodent clomipramine model of depression we aimed at identifying changes in expression of genes and genetic networks in four brain areas of rat pups (I, II) and adult animals (III) that are targeted by the treatment and could thus mediate the harmful effects of the treatment on the regulation of sleep, emotions and plasticity (II).

Aim 2. In order to clarify the interplay between different brain areas in response to clomipramine treatment we aimed at identifying pathways and networks that span two or more tissues.(II).

Aim 3. In human white blood cells, identify epigenetic changes in the promoter of a serotonin transporter gene, (a gene closely related to regulation of mood/emotions), in relation to stressful environment (workstress)(IV).

Aim 4. Identify age-related changes in neuronal plasticity and slow-wave sleep as response to sleep restriction (V).

3 Material and methods

3.1 Animals and treatments

3.1.1 Ethical issues

All animal procedures were approved by the University of Helsinki Ethical Committee for Animal Experiments and performed according to applicable legislation (European Communities Council Directive of 24 November 1986, 86/609/EC). All efforts were made to minimize the number of animals used and their suffering.

3.1.2 Animals and treatments – the clomipramine (CLI) model (I, II, III)

The animals (Wistar Hannover rats, Harlan Laboratories, Horst, The Netherlands) were treated with bidaily subcutaneous injections (9 am and 7 pm) of 20mg/kg clomipramine (Sigma-Aldrich, St. Louis, MO, USA) from post-natal day 5 to 21. The control group was treated with 0.9 % saline (Baxter Oy, Vantaa, Finland). The animals were sacrificed by cervical dislocation.

Number, age and sex of the animals used in the different studies: (I) For expression analysis: Six CLI treated (3 males, 3 females) and seven (3 males, 4 females) controls were sacrificed at the age of 3 weeks (on the day after the last treatment) and the BF dissected for extraction of RNA and further analysis with the Affymetrix Rat 230.2 chip and TaqMan Realtime RT-PCR. (II) For expression analysis: Six CLI treated (3 males, 3 females) and six (3 males, 3 females) controls were sacrificed at the age of 3 weeks and the BF, FCX and HIP were dissected for RNA extraction. For sleep recordings: 12 male animals underwent the same experimental conditions (6 CLI-treated and 6 controls) and were used for sleep recording at the age of 3 months. For sucrose preference test: an additional 26 animals, all males (12 CLI, 14 SAL), were used (at the age of 3 months) for the sucrose preference test. (III) For expression analysis: Six CLI treated animals and five controls (all females) were sacrificed at the age of 3 months and their HYP dissected for extraction of RNA and further analysis with the Affymetrix Rat 230.2 chip and TaqMan Realtime RT-PCR.

3.1.3 Animals and treatments – the effect of ageing on sleep (V)

Three age groups of male Hannover Wistar rats were used in the experiment: young (Y) (3-4 months; 388 ± 11 g; n = 35), middle-aged (M) (12-14 months; 744 ± 25 g; n =

28), and old (O) (23-25 months; 698 ± 29 g; n = 27). The animals were housed on a 12 h light/dark cycle (lights on at 8:30 A.M.) at constant temperature (+22 ± 1°C). Food and water were provided ad libitum.

3.2 Human subjects (IV)

Clinical data were obtained from a Finnish cohort comprised of female health care professionals, mainly nurses, midwives and nursing assistants. 422 individuals were recruited from a total of 5615 health care professionals that were part of a Finnish Public Sector Study (Kivimaki et al., 2007). This group represents the entire personnel of 21 Finnish public hospitals. These subjects were classified into lowest and highest quartile on work stress as in Karasek's model (Karasek, 1998) in which high work stress is implicated by high demands and low control, while low stress is defined by low demands and high control. Inclusion criteria was age between 30-60, mother tongue Finnish, BMI under 35, at least 3 years of work experience in the same ward and no greater than 6 months of absenteeism from work during the past 3 years. Ninety-nine participants took part in laboratory measurements. All laboratory assessments were performed blind to ward work stress status.

Exclusion criteria for the current study were use of medication affecting cognitive functions, use of hormonal medication, heavy smoking (reported daily smoking for at least 10 consecutive years) and high alcohol intake (4 or more doses of alcohol over 4 times a week). After exclusion criteria, a total of 67 subjects were selected for analysis. Peripheral blood samples were obtained from well-rested individuals. Written consent was obtained from all participants. A total of 49 subjects (73%) were bisulphite sequenced and included in the final analysis for this study. A detailed flow chart for sample selection is provided in Figure S1 of Supporting Information (I).

3.3 Dissections (I, II, III, V)

The tissues were dissected according to The rat brain in stereotaxic coordinates by Paxinos and Watson (Paxinos & Watson, 1986). All the dissections were performed by hand on fresh tissue on ice.

The BF and FCX were dissected bilaterally while the HIP was dissected unilaterally, thereby quickly getting samples of suitable size, making the RNA extraction more precise and saving time to reduce the RNA degradation. 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). The dissected area was 2 mm thick. The frontal cortex was dissected from the part of the brain rostral to bregma -0.3 and included the cortex above corpus callosum and external capsule. The hippocampus and hypothalamus were dissected en block.

3.4 DNA / RNA / Protein - extractions (I, II, III, V)

(I, II, III) The dissected brain tissues were immediately homogenized in cold RLT Plus buffer (Qiagen, Hilden, Germany) with added β-mercaptoethanol 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 following the supplied protocol. DnaseI treatment of the RNA fraction was carried out using the Rnase-Free Dnase Set by Qiagen as a precaution against gDNA contamination of the RNA. Both fractions were measured for purity and concentration using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA).

(V) The dissected brain tissues were stored in the AllProtect Tissue Reagent stabilizer (Qiagen, Hilden, Germany) at -80°C. Half of the tissue was used for measuring BDNF mRNA expression and the other half for measuring BDNF protein expression. Tissue for BDNF mRNA expression was homogenized in Qiazol Lysis Reagent using a bead mill Tissue Lyser (Qiagen). Chloroform was added and the homogenates were centrifuged to enhance separation of the RNA phase. After the homogenization RNA was immediately extracted using RNA Later Lipid Mini Tissue Kit according to the protocol supplied (Qiagen). DNase treatment of the RNA fraction was carried out using the RNAse-Free DNase Set (Qiagen) as a precaution against genomic DNA contamination. The RNA fraction was measured for purity and concentration using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). Due to technical problems RNA was not obtained from the middle-aged rats deprived for 3 hours.

The tissues for the BDNF protein expression analysis, were homogenized in 1:5 ratio buffer (50mM Tris–HCl, pH 7.5, 130mM NaCl, 1mM EDTA, 1mM EGTA, 1% (v/v) TX100, 2.5mM sodium pyrophosphate, 1mM betaglyserol-2-phosphate and 1mM phenylmethylsulphonyl fluoride, including inhibitor cocktails (Sigma–Aldrich Co., St. Louis, MO)) and centrifuged for 30 min at 6 °C. The protein homogenates were stored in -80 °C.

3.5 Gene expression

3.5.1 Gene expression analysis – array (I, II, III)

After extraction RNA quality was further tested with the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). The arrays were performed according to the Affymetrix standard protocol (Affymetrix, Santa Clara, CA, USA). In brief, 2 μg of RNA was used to prepare biotinylated cRNA using the Affymetrix GeneChip One-cycle target labelling kit after which 15μg of the cRNA was fragmentated and hybridized to the Affymetrix Rat 230.2 chip. Hybridization, staining and washing were per-

formed using the Affymetrix Fluidics Station 450 and Hybridization Oven 640 under standard conditions and according to the Affymetrix protocol.

(I) For the array data analysis we used the commercially available software GeneSpring GX 7 (Agilent Technologies, Santa Clara, CA). The analysis was executed as follows: after a GC-RMA preprocessing of the raw data the signals were normalized to the median per gene and the Affymetrix control probes were subtracted. To ensure that the analysis would pick up both differentially expressed genes and ON/OFF genes, a filtering on flags using the CHP files so that flags had to be present or marginal in at least 4 of 6 in the CLI samples and / or 5 of 7 in the control samples was done. All genes that had normalized data between 0.666 and 1.5 in 11 of 13 samples were filtered out and a fold change (FC) limit of 1.3 was set to describe our criteria on differential expression. The p-value (Wilcoxon-Mann-Whitney test) limit was set to 0.05. To take into account the multiple testing performed and the false discovery rate, the q-values (Storey & Tibshirani, 2003) were calculated by bootstrapping, for each gene. For this we used the QVALUE package running in R environment. As input we used all the 956 genes left before any statistical testing.

(II) In the array analysis we used the free CHIPSTER v1.4.0 software provided by CSC (IT Center for Science, Helsinki, Finland). The expression data were analyzed by comparing the expression levels of single genes in the CLI treated animals with the controls, separately in the three different tissues. The data analysis was executed as follows: after a Gene Chip Robust Multichip Averaging (GC-RMA) pre-processing of the raw data the samples were linked to the phenodata and the Affymetrix control probes were subtracted. To ensure that the analysis would pick up both differentially expressed genes and ON/OFF genes, a filtering on flags using the probe set information chip files generated by Affymetrix (CHP files) was done so that genes that presented two or more Absent flags in both groups were filtered out. The average fold change (FC) limit was set at 1.5 to describe our criteria for differential expression. The significance for the differentially expressed genes was calculated by students T-test and a limit was set to 0.05.

To take into account the multiple testing performed and the false discovery rate, the q-values (Storey & Tibshirani, 2003) were calculated by bootstrapping, for each gene. For this we used the QVALUE package running in R environment. As input we used all the genes left before any statistical testing (BF=655, FCX=195 and HIP=611).

(III) The expression levels of individual genes of CLI-treated animals and controls were compared using GeneSpring GX 9.0.5 software (Agilent Technologies, Santa Clara, CA, USA). Array raw intensity values were normalized and converted to expression summaries by GC Robust Multichip.

Average (GC-RMA), the signals were then normalized to the median per gene and the Affymetrix control probe sets (57) were removed. To ensure that the anal-

ysis would pick up both differentially expressed genes and ON/OFF genes, a filtering on flags using the CHP files (so that flags had to be present or marginal in 67% of the samples in any 1 of 2 treatment conditions) was performed. The obtained list of genes was further filtered by fold-change (FC) so that genes up- or down-regulated with less than 1.5 FC were removed. Differential expression was tested by Mann-Whitney unpaired test, and significance was set at $p<0.05$.

3.5.2 Gene expression analysis – Realtime RT-PCR (I,III,V)

TaqMan real-time quantitative reverse transcriptase PCR was performed using ABI PRISM 7900 Thermal Cycler and TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA). 1 µg of RNA was used as starting material for the reverse transcription using the Advantage-RT for PCR kit (Clontech Carlsbad, CA, USA). cDNA samples were amplified using a TaqMan Universal PCR MasterMix and the commercial gene expression assays with primers and probes. The program was set on 50°C 2min - 95°C 1min and 50 cycles of repeating 95°C for 15 seconds and 60°C for 1min. All samples and assays were run in triplicates. Expression values were normalized to Cyclophilin A (PPIA(Rn00690933_m1), which was used as endogenous control. The endogenous control was chosen based on its steady expression throughout the array experiment. Standard curves were prepared accordingly to be able to calculate the changes in gene expression using the comparative delta (CT) method. A Students T-test was performed to estimate the statistical significance of the detected changes in expression between the two groups.

(I) TaqMan probes (Applied Biosystems) for: DNMT2(Rn01765153_m1), SOX6(Rn01507719_m1), PDE4D(Rn00566798_m1), ACATN(Rn00573865_m1), FGFR2(Rn01269940_m1), IGF2(Rn00580426_m1) and PPIA(Rn00690933_m1).

The endogenous control was chosen by examining the expression profiles and intensities (on the arrays) of 30 genes commonly used as endogenous controls in order to find the best endogenous control for this specific experiment. Cyclophilin A was present at a suitable intensity and showed very little variation between samples.

(III) TaqMan probes (Applied Biosystems) for: TTR (Rn00562124_m1), DLG3 (Rn01419350_m1), DNAJC5 (Rn00577363_m1), SNX10 (Rn01763032_m1) and PPIA (Rn00690933_m1) as endogenous control.

(V) TaqMan probes (Applied Biosystems) for: BDNF(Rn02531967_s1) and PPIA(Rn00690933_m1) as endogenous control.

3.5.3 Gene expression analysis – protein (V)

Acidified brain homogenates were analyzed for BDNF expression using sandwich ELISA, as described in (Karpova et al., 2010). The protein expression was calculated using standard curves and normalized to total protein. The total protein concentration was measured from the protein homogenates, using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and used for data normalization.

3.6 Quantitative analysis of site-specific DNA methylation (IV,N/A)

(IV) We isolated DNA from peripheral blood leucocytes using QIAGEN Autopure (Qiagen). CpG methylation status of the CpG-rich region in the SLC6A4 gene promoter was investigated by bisulphite sequencing (Frommer et al., 1992). The region of SLC6A4 that was included in the analysis has been previously shown to be differentially methylated in infants exposed to prenatal maternal depressed mood (Devlin, Brain, Austin, & Oberlander, 2010).

500ng of genomic DNA from leucocytes was treated with sodium bisulphite using the EZ DNA Methylation-Gold Kit (Zymoresearch) following the manufacturer's protocol. The region of interest was amplified by standard PCR from bisulfite-treated DNA using AmpliTaq Gold Hot Start Polymerase (Applied Biosystems) based on previously published primers (Devlin et al., 2010) with slight modifications in region coverage as a necessity to produce good quality sequence with direct sequencing. SLC6A4F: gtattgttaggttttaggaagaaagagaga and SLC6A4R: aaaaatcctaactttcctrctctttaactt.

Overhanging tails in the 5' end were designed for both forward and reverse primers that match with common T7 (taatacgactcactataggg) and T3 (attaaccctcactaaaggga) sequencing primers respectively. Cycling conditions were 95°C for 11 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 40 seconds with a final extension of 10 minutes at 72°C. PCR products were verified randomly on a 2% agarose gel (GellyPhor, Euroclone). The remaining PCR product was purified using the Quickstep 2 PCR Purification Kit (Edge Bio).

Cycle sequencing was performed with the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) using T7 and T3 sequencing primers. Sequencing was performed by capillary sequencing (Finnish Institute for Molecular Medicine). A similar method has been previously described (Lewin, Schmitt, Adorjan, Hildmann, & Piepenbrock, 2004).

Methylation percentage at each CpG site was quantified manually using the Sequence Scanner version 1.0 (Applied Biosystems) by two independent examiners blind to the sample identification codes. The peak heights of C versus the combined

heights of C + T peaks (C/C+T) at each CpG locus were calculated as a percentage (Lewin et al., 2004). The five CpG locations were 28 563 120 (CpG5), 28 563 109 (CpG4), 28 563 107 (CpG3), 28 563 102 (CpG2), and 28 563 090 (CpG1) as per hg19 assembly of the UCSC Genome Browser. The success rate for DNA amplification and bisulfite sequencing was 73% (49/67).

Correlations between methylation levels in all five CpG residues were calculated using the Pearson's Correlation. Structural equation measurement model was analyzed in order to test the hypothesis that these correlations could be explained by a single latent factor. Principal component factor analysis was used to obtain the single sum methylation value METsum. Statistical significance (2-tailed $p<0.05$) between methylation of each five CpG residue and work stress was assessed using the Student's T-test. Associations between work stress, MBI-GS, METsum and 5-HTTLPR were assessed by analysis of covariance (ANCOVA). In the initial multivariable model, we analyzed the main effects of 6 variables and 15 variables in the interaction model. Non-significant variables were then systematically eliminated using a stepwise backward elimination method until all significant variables were left in the final model. The tools used in all analysis were SPSS Statistics 18, PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell et al., 2007) and SAS analytics (SAS Institute, Cary, NC).

(N/A) Unpublished work (related to manuscript I): The DNA fraction from BF, extracted simultaneously with the RNA from the clomipramine model (3.4) was subject to DNA methylation analysis of the BDNF, NTRK2, IGFII and ACATN promoter areas. The protocol was developed from previously published ones (Lewin et al., 2004) and a predecessor of the one described above and thus similar with the exception of primer design and method for estimating the degree of methylation. The primers were designed using MethPrimer (Li & Dahiya, 2002). The estimation of the degree of methylation was done by comparing to a dilution curve of in vitro methylated (using SssI) DNA.

3.7 Genotyping (IV)

5-HTTLPR genotype was assayed in two stages. Amplification of 5-HTTLPR was carried out using PCR using the primers HTTLPR_F (gttgccgctctgaatgccag) and HTTLPR_R (ggataatgggggttgcaggg) that amplify either the long, L allele (280 bp product) or the short, S allele (236 bp product). The reaction was run in a total volume of 20 µl containing 60 ng of genomic DNA, 500 nM primers, 10 mM dNTP, 2.5 mM MgCl2 and 0.7U Dynazyme II Hotstart polymerase (Finnzymes). Thermocycling conditions were 94°C initial denaturing for 10 min followed by 35 cycles of the following: denaturing at 94°C for 15 s, annealing at 65°C for 20 s and extension at 72°C for 25 s. This was followed by a final extension at 72°C for 10 min. 5 µl of PCR product was then mixed with 8 units of Msp I restriction enzyme (Pharmacia Biotech) in

One-Phor-All assay buffer. The reaction mixture was then incubated at 37°C for 16 hours followed by 20 min at 65°C. 5-HTTLPR genotypes were assessed on a 2% gel. Associations between work stress, MBI-GS, METsum and 5-HTTLPR were assessed by analysis of covariance (ANCOVA). In the initial multivariable model, we analyzed the main effects of 6 variables and 15 variables in the interaction model. Non-significant variables were then systematically eliminated using a stepwise backward elimination method until all significant variables were left in the final model. The tools used in all analysis were SPSS Statistics 18, PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell et al., 2007) and SAS analytics (SAS Institute, Cary, NC).

3.8 Pathway analysis (I, II, III)

In the pathway analysis we studied groups of genes, functionally related to each other, rather than single genes. In this approach, an aggregate score (based on the absolute FC of each probeset) for genes in specific categories was computed and the significance of the aggregated change in each pathway was estimated by random sampling, using a custom made program (similar to (Pietilainen et al., 2008; Pontynen et al., 2008; von Schantz et al., 2008)). An input list consisting of the total number of probes on the Affymetrix Rat chip excluding the control probes was used (31,042 target sequences). Rattus Norvegicus core (50.34t in manuscripts I & III, 53.34u in manuscript II) and gene ontology database (50 in manuscripts I & III, 53 in manuscript II) by Ensembl (Hubbard et al., 2005) was the source of choice for retrieving the gene ontology information for the probes. By utilizing the directed topology of the Gene Ontology (GO) tree by traversing all available routes to the root of the GO tree, all encountered vertexes were added as GO annotations for the given gene. The non-parametric iterative cumulative hypergeometric distribution was calculated and further tested using 100,000 permutations:

$$p(j,k,t,n) = 1 - \sum_{c=0}^{k-1} \frac{\binom{j}{c}\binom{n-j}{t-c}}{\binom{n}{t}}$$

where j=absolute rank of the probeset/gene; k=rank of the probeset /gene amongst all the members of the pathway; t=total number of probeset /genes in the pathway; and n=total number of probeset/genes in the experiment. The objective is to find the optimum p-value for a set of genes which belongs to the same annotated GO collection (maximal regulation for the pathway).

By using input lists sorted by absolute FC values this analysis was designed to search for the most differentially expressed pathways and not up- or down- regulated pathways separately (as common praxis).

(II) Inter-tissue pathway analysis

To identify differentially expressed pathways that possibly span more than one tissue, using the same pathway analysis method as described above, we combined the experimental data from the three different tissue studies. We made an input list by combining the total probe set data from the BF, FCX and HIP, and sorting the list according to descending absolute FC (Figure 1). To allow the same gene to appear in the input list from more than one tissue, but to avoid artifacts because of larger genes being detected by multiple probes, we first discarded all but the highest ranking probe for each gene in the input list from the separate tissues, but allowed for duplicates later in the combined analysis.

The output was divided into two: pathways consisting of 50 to 200 genes and pathways of less than 50 genes. In this way the larger pathways, of an often more general nature, were separated from the smaller, more specific pathways.

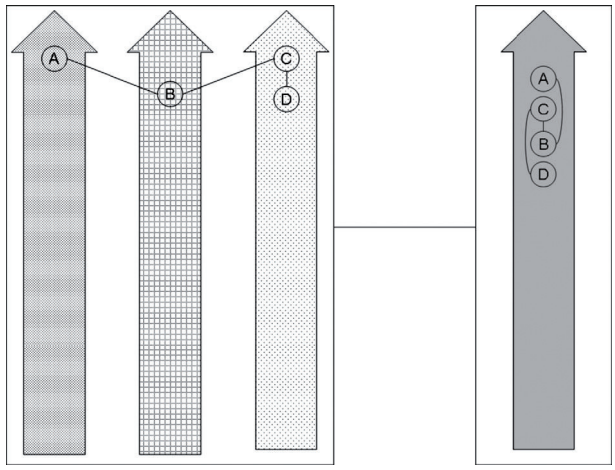

Figure 1. On the left: Example of a pathway that spans three tissues. The arrows each represent the complete gene list from an array experiment in one tissue, sorted according to descending absolute Fold Change (FC) of treated vs. controls. The pathway consists of genes A, B, C and D. Genes A, B and C are connected and within the same pathway, although they are detected (presenting a high FC) in three different tissues.

On the right: By combining the gene lists from the three different array experiments and sorting the combined list according to descending absolute Fold Change (FC) one can easily, by using any regular program for pathway analysis, detect differentially expressed pathways that span one, two, or all three tissues. (Figure used under permission from Journal of Neurogenetics (II))

3.9 Inter-tissue network analysis (II)

To find important interactors and visualize the gene networks involved between the three brain areas (See Figure 2 for a simplified view), the STITCH 2.0 software (Kuhn et al., 2010; Kuhn, von Mering, Campillos, Jensen, & Bork, 2008) was used. The input list consisted of a list combined from the most differentially expressed genes in BF, HIP and FCX (Supplementary Tables 1a, 1b and 1c (in II)). The maximum number of interactors was set to twelve and all prediction methods and databases were activated. The confidence level (score) for edges to qualify was set to 0 in order not to emphasize well studied genes and molecules. The nodes were manually categorized (by colouring) as either belonging to BF expressed, HIP expressed, FCX expressed or interactor.

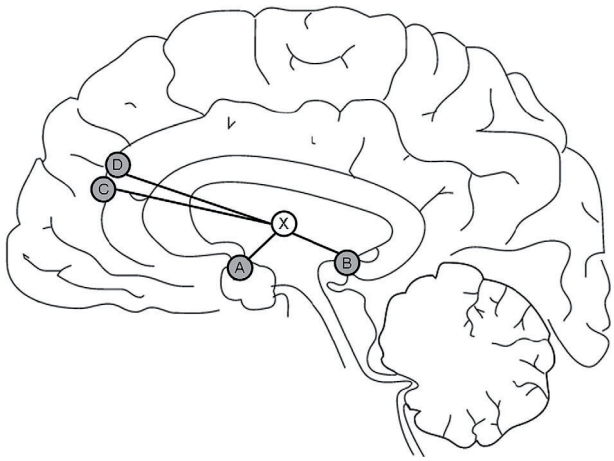

Figure 2. Example of a gene network consisting of four genes A, B, C and D detected in three different tissues. The interactor X, that connects the four genes, has no defined physical location. (Figure used under permission from Journal of Neurogenetics (I))

3.10 Promoter analysis (I)

The most outstanding genes from the array analysis were subject to a promoter analysis.

Using Ensmart (Biomart) a list of the mouse orthologues was created (Durinck et al., 2005). Sequences of +/- 2000 bp from the transcription start site were analyzed for enrichment of binding sites for vertebrate transcription factors using oPOSSUM (www.cisreg.ca/oPOSSUM). Only non – coding areas with 70 % conservation to human were used and the findings were compared to a random background.

3.11 Search for over-represented biological motifs (II)

The DAVID functional annotation tool (Huang da, Sherman, & Lempicki, 2009) was used to search for over-represented biological motifs according to gene ontologies. As input we used a list combined from the most differentially expressed genes in BF, HIP and FCX (Supplementary Tables 1a, 1b and 1c (in II)). FAT GO terms (for each, Biological Process, Cellular Component and Molecular function) were used in order to eliminate the largest gene ontologies, without sacrifying relevant information. The calculation was made using the whole Affymetrix Rat 230.2 chip as background and the p-value cut-off was set at 0.05.

3.12 Behavioural testing - Sucrose preference test (II)

The animals (n=26: 12 CLI and 14 SAL) were housed individually throughout the test session. During the 48h training session, starting at 19:00, each animal was presented with 2 bottles in the cage: one containing 1% sucrose solution and the other containing standard drinking water (Bhagya, Srikumar, Raju, & Shankaranarayana Rao, 2008). The volume of water and sucrose water intake was measured every 24h and bottle positions were exchanged during this period. After an 18h period of food and water deprivation (to increase the efficacy of the test) a 2h test session was conducted (starting at 13:00). The amount of liquid remaining in each bottle was measured at the end of the testing period. The sucrose preference score was expressed as a percentage of total fluid intake (sucrose preference = sucrose water consumed / total liquid consumed x 100), and the data were normalized to animal body weights. During the test period all animals were given a full bottle (250 ml) of both water and sucrose solution. The test was performed at the age of 3 months (all males, to exclude sex differences).

3.13 Sleep deprivation (SD) (V)

To keep the animals awake and to evoke active waking and exploratory behaviour, novel objects (e.g. wooden blocks, Styrofoam objects and stretchable strings) were introduced to the cages. Whenever an animal appeared behaviourally sleepy, or slow waves were visible in the EEG, a new object was introduced or the cages were gently tapped. This method is referred to as sleep deprivation by gentle handling (Franken, Dijk, Tobler, & Borbely, 1991).

Animals were deprived of sleep for either 3 or 6 hours (from 1 P.M. to 4 P.M. or from 10 A.M. to 4 P.M., respectively). This experiment was repeated twice to al-

low for recording of EEG recovery sleep after the first SD, with a minimum 48 hours between the experiments. The animals were then sacrificed by decapitation immediately after the end of the deprivation. An age-matched control group was left undisturbed and sacrificed at the same circadian time point. All animals in the EEG group were deprived of sleep for 6 hours (from 10 A.M. to 4 P.M.).

3.14 Sleep recordings and analysis (II, V)

(II) Six animals treated with CLI and 6 treated with saline were recorded for sleep at the age of 3 months. The rats (all males, to exclude sex differences) were implanted under general anaesthesia (3mg/kg i.p. diapam, 0.5 mg/kg domitor and 50 mg/kg ketalar i.p.) with electrodes for recording of electroencephalogram (EEG) and electromyogram (EMG). Two EEG electrodes were screwed in frontoparietal positions (2mm rostral and 2 mm lateral to bregma; 4 mm rostral and 1 mm lateral to lambda); two silver EMG electrodes were inserted into neck muscles. After one week of recovery rats were connected to counterbalanced recording leads for 48-hour adaptation before the actual 48-hour baseline EEG recording took place. The EEG/EMG signals were amplified (Bioamplifier AC-916, CWE, Inc., Ardmore, PA USA) and sampled at 104 Hz. EEG data were digitally high-pass filtered (0.75 Hz) for removing low-frequency artefacts and divided into 5-s epochs which were manually scored for REM sleep using the program Spike 2 (version 5.11; Cambridge Electronic Devices, Cambridge, UK). Total REM sleep amount, REM sleep fragmentation, and average length of REM sleep bouts were calculated for each hour during the 24 hours of recording. To calculate REM sleep fragmentation and the length of bouts, only REM epochs with duration starting from 10s, were considered.

(V) A group of animals (Y, n=8; M, n=7; O, n=8) were implanted with chronic epidural EEG electrodes and dorsal nuchal EMG electrodes under anaesthesia (2.5 mg/kg, i.p. diazepam; 0.4 mg/kg, i.p. medetomidine; 60 mg/kg, i.p. ketamine) for studying vigilance states and EEG activity. The bipolar screw EEG electrodes were placed contralaterally to the skull (2 mm caudal and 2 mm lateral from bregma; 2 mm rostral and 1 mm lateral from lambda) and fixed to the skull with acrylic dental cement. After surgery, rats were single-housed in Plexiglas boxes and allowed to recover for 1 - 2.5 weeks. After connecting the EEG/EMG cables (Plastics1, Va, USA) the animals were allowed to adapt for one more week, after which a stable 24 hour baseline recording was obtained prior to the experiments. The EEG/EMG signal was amplified (gain 10,000) and filtered (HP 0.3 Hz, LP 100 Hz) using a 16 channel amplifier (Model 3500, A-M Systems, Carlsborg, WA, US). The signal was digitized at 277 Hz with an A/D converter (unit 1401, Cambridge Electronics, Cambridge, UK), monitored on-line during the experiments and stored on a standard computer using Spike2 software (Cambridge Electronics, Cambridge, UK).

The multiple sleep latency test (MSLT), using continuous EEG/EMG recording, adapted from McKenna et al. (McKenna et al., 2008), was performed after a 6-hour baseline period, from 10 AM to 4 PM, during which spontaneous sleep-wake behaviour was allowed to occur, or after 6 hours of SD by gentle handling, from 10 Am to 4 PM. The MSLT consisted of short (5 min) periods of wakefulness induced by gentle handling, followed by longer (25 min) periods during which the animals were free to sleep. This procedure was repeated every 30 min for 3 hours (from 4 PM to 7 PM) and was designed to minimize the amount of sleep lost due to the testing procedure.

The EEG/EMG recordings from the last baseline day and from the two 6-hour SD days were scored in 5 second epochs for wake, NREM sleep and REM sleep with an automated scoring program (Rytkonen, Zitting, & Porkka-Heiskanen, 2011). Sleep scoring was done for the baseline day, the MSLT-baseline day, and the MSLT sleep deprivation day. Baseline NREM delta power (0.75-4 Hz) was calculated from the 24-hour baseline and normalized to total power from the same period. Recovery sleep NREM delta power (0.75-4 Hz) was calculated from the recovery sleep following SD, altogether for 16 hours (4 PM to 8 AM), and normalized to baseline values.

For power spectral analysis the first 25 minutes, from the beginning of the recovery sleep period following 6-hour SD, was manually checked to exclude artefacts and to correct for vigilance state errors. The NREM power spectra was generated in the Spike2 program (Cambridge Electronics) using fast Fourier transform (512 Hanning window; 0.4 Hz resolution) within a frequency range of 0.75–35 Hz, averaged over the time period investigated, and normalized to total power of the same recording period. The amount of recovery NREM sleep delta (0.75-4 Hz), was used as a measure of sleep intensity.

3.15 Work stress, burnout and depression assessment (IV)

From the baseline 5615 female health care professionals, potential subjects (n=422) were chosen to respond to the Karasek's Job Content Questionnaire (JCQ). This was based on two consecutive questionnaires of work stress in 2004 and 2008. Three questions assessed job demand and 9 questions assessed job control. Responses to all questions were given on a 5-point scale (completely agree, somewhat agree, not agree/neither disagree, somewhat disagree, completely disagree). The division into high and low work stress groups was based first on grouping the wards with at least 5 respondents according to the mean score of survey responses to the job demand and job control scales at ward level, using median split to identify high stress (high demands and low control) and low stress (low demands and high control) wards. Using these cut-off points, the nurses from the wards were identified, who belonged to the same high and low stress groups also based on their individual mean score values of job demands and job control. Finally, to increase contrast between the comparison

groups, nurses who belonged to the quartile with least stress in the high stress group and most stress in the low stress group were excluded.

To assess burnout in our study sample, each subject took the Maslach Burnout Inventory General Survey (MBI-GS). MBI-GS is a modified version of the original MBI to measure levels of burnout in occupations that involve working closely with people (Schaufeli, Leiter, Maslach, & Jackson, 1996). The survey covers all the three components of burnout: exhaustion (EX), cynicism (CY), and professional efficacy (PE). Subjects scoring higher than 1.5 in the MBI-GS were considered to have at least moderate burnout.

Depression was measured using the Beck Depression Inventory (BDI) (A. T. Beck, Ward, Mendelson, Mock, & Erbaugh, 1961). The questionnaire is widely used to screen depression in clinical practice and in community samples (Aaron T. Beck, Steer, & Carbin, 1988). Subjects scoring between 10 and 18 were considered to have mild depression while scores of 19 to 29 represent moderate to severe depression. Associations between work stress, MBI-GS, METsum and 5-HTTLPR were assessed by analysis of covariance (ANCOVA). In the initial multivariable model, we analyzed the main effects of 6 variables and 15 variables in the interaction model. Non-significant variables were then systematically eliminated using a stepwise backward elimination method until all significant variables were left in the final model. The tools used in all analysis were SPSS Statistics 18, PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell et al., 2007) and SAS analytics (SAS Institute, Cary, NC).

4 Results

4.1 Findings in the clomipramine (CLI) model

4.1.1 Sleep recordings and Sucrose preference test (II)

The interest in the clomipramine model to this study is its effect on both sleep and mood. According to the proposed mechanism, the neonatal treatment would affect the sleep of the young animals, with the outcome of depressive-like behaviour and sleep pattern at adult age (Feng & Ma, 2002; Vogel, Neill, Kors et al., 1990). The sucrose preference is used to detect signs of anhedonia, which in turn is a symptom of depression (Willner & Mitchell, 2002). The sleep recordings and testing for sucrose preference were performed for two concomitant reasons: 1) to further validate the model itself in order to verify that the neonatal treatment with clomipramine has a permanent effect on both sleep and mood, and 2) to ensure that the introduction of the model to our laboratory has been successful.

The sleep recordings of the three months old animals show that the CLI treated animals present permanent changes in sleep architecture. As compared to the control animals they present an increase in overall REM sleep during the first hour of darkness (20.30-21.30) and REM sleep fragmentation between 19.30-20.30 ($p=0.03$) and 03.30-04.30 ($p=0.048$) (Figure 3). The CLI treated animals also exhibit shorter REM bouts during the four night hours ($p=0.029$) as well as towards the end (19.30-20.30) of the light period ($p=0.014$).

The sucrose preference testing show the CLI treated animals to have a reduced sucrose preference (56.62 +/-9.57%) compared to the control animals (78.83 +/-5.52%), as measured in percentage of sucrose solution consumed out of total fluid consumed ($p=0.048$) (Figure 4).

Additionally, by measuring the animals during both the sleep recordings and the sucrose preference testing, we detected a decreased bodyweight in the animals from the CLI group. A t-test showed ($p=0.028$) that the average adult bodyweight of the CLI treated animals (307.5 ± 8.6 g) was lower than in the control group of CLI treated animals (332.6 ± 5.5 g) during the sleep recordings, whereas the respective weights during the sucrose consumption testing were 370.3 ± 9.9 g for the CLI group and 415.9 ± 10.1 g for the control group ($p=0.004$).

Not only do these findings indicate that permanent changes occur due to neonatal CLI treatment, but they also display the reproducibility of the clomipramine model.

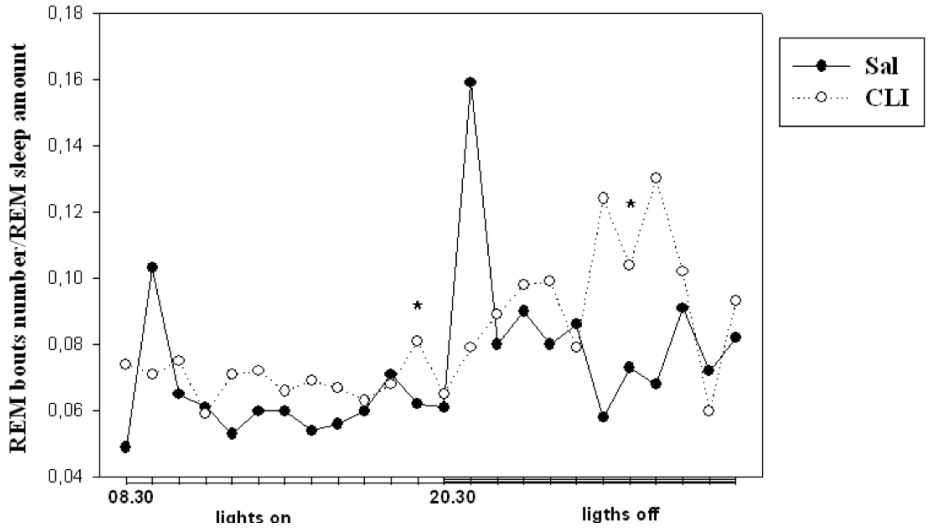

Figure 3. REM sleep fragmentation during 24h in rats treated with clomipramine (CLI). The animals were treated with daily injections of CLI (n = 6) or saline (Sal) (n = 6) for 2 weeks in their early infancy. The sleep was recorded at the age of three months. The CLI animals had increased REM sleep fragmentation at 19.30-20.30 (p=0.03) and 03.30-04.30 (p=0.048). Thus, early administration of CLI leads to permanent disturbance of sleep in the animals. * p<0.05 (Figure used under permission from *Journal of Neurogenetics* (II))

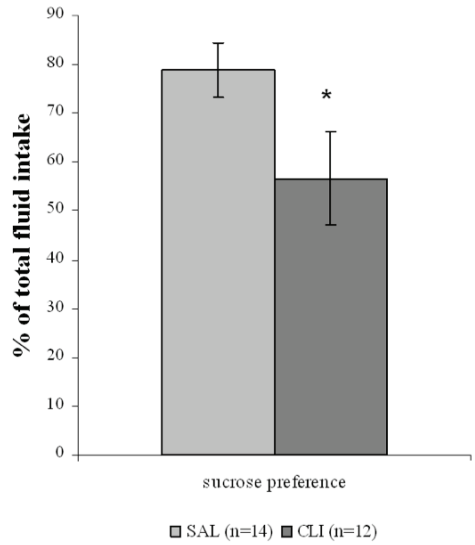

Figure 4. Sucrose preference of three months old rats that have undergone clomipramine vs. saline treatment in early infancy. The sucrose preference score is expressed as a percentage of total fluid intake (sucrose preference = sucrose water consumed / total liquid consumed x 100), and the data were normalized to animal body weights. (Figure used under permission from *Journal of Neurogenetics* (II))

4.1.2 Differentially expressed inter-tissual pathways and networks between basal forebrain (BF), hippocampus (HIP) and frontal cortex (FCX) (II)

The disorders of interest arise due to dysfunction of several brain areas and are not attributed to any exact brain area or cell type. The inter-tissual analyses were designed to detect possible imbalance in networks and pathways that span two or three different brain areas. The results reveal pathways and networks possibly involved in the communication between these areas as well as in the phenomena of interest. The brain areas included in this study were chosen for their previously reported function and interconnectivity.

By the inter-tissue network approach we managed to visualize the involved networks on a molecular level (Figure 5). Furthermore, this analysis revealed: 1) the most interconnected genes of the primary expression analysis, and 2) predicted functional partners, not detected in the primary expression analysis, that are either genes/gene products or other molecules of natural or synthetic origin (Table 1).

1. Transforming protein RhoA (Rhoa), RAS-associated protein Rab-3A (Rab3a) and Carbonic anhydrase IV (Ca4).
2. Gene products: Fibroblast growth factor (Fgf1), Vascular endothelial growth factor A (Vegfa), Rabphilin-3A (Rph3a). Other molecules: serotonin, serotonin agonists 5-carboxamidotryptamine, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane and the serotonin derivative 5-methoxytryptamine, the maleate salt of methylergonovine methylergometrine, the ergot alkaloid ergotamine, lysergic acid diethylamide (LSD), JNK (c-Jun N-terminal kinase) inhibitor SP600125 and manganese Mn(II).

In overall, the most highly interconnected molecules were: Vegfa, Rhoa, serotonin and Mn(II).

The inter-tissue pathway analysis revealed pathways or gene ontology groups that are affected by the CLI treatment. To separate more specific pathways from general ones, pathways of 2) less than 50 genes (Table 2b) were examined separately from the larger pathways 1) consisting of between 50 and 200 genes (Table 2a).

1. The most significantly changed pathways within the Biological Process – category (BP) were those of nerve-nerve synaptic transmission (optimal p=8,84E-10, permuted p=1,00E-06) and the regulation of transmission of nerve impulse (optimal p=9,35E-11, permuted p=1,00E-06), whereas the Molecular Function – category (MF) presented the most significant changes in pathways related to translation activity (starting from optimal p=6,55E-08, permuted p=6,00E-06) and small conjugating protein-specific protease activity, or more precisely, ubiquitin activity (starting from optimal p=3,40E-10, permuted p=1,00E-06). Spatially, the largest changes, as explained by the Cellular Component – category (CC) seem to be taking place in the axons (optimal p<10E-38, permuted p=1,00E-06) and the

melanosomes/pigment granules (optimal p=1,35E-07, permuted p=8,00E-06/1,1E-05).

2. Apart from the pathways for astrocyte development and glutamate signalling, the separation of smaller pathways also revealed pathways directly related to behaviour such as startle response, conditioned taste aversion and visual learning.

The strongest contributors to any of the significantly changed pathways were the precursors for Pro-melanine concentrating hormone (Pmch), Transthyretin (Ttr) and Orexin (Hcrt).

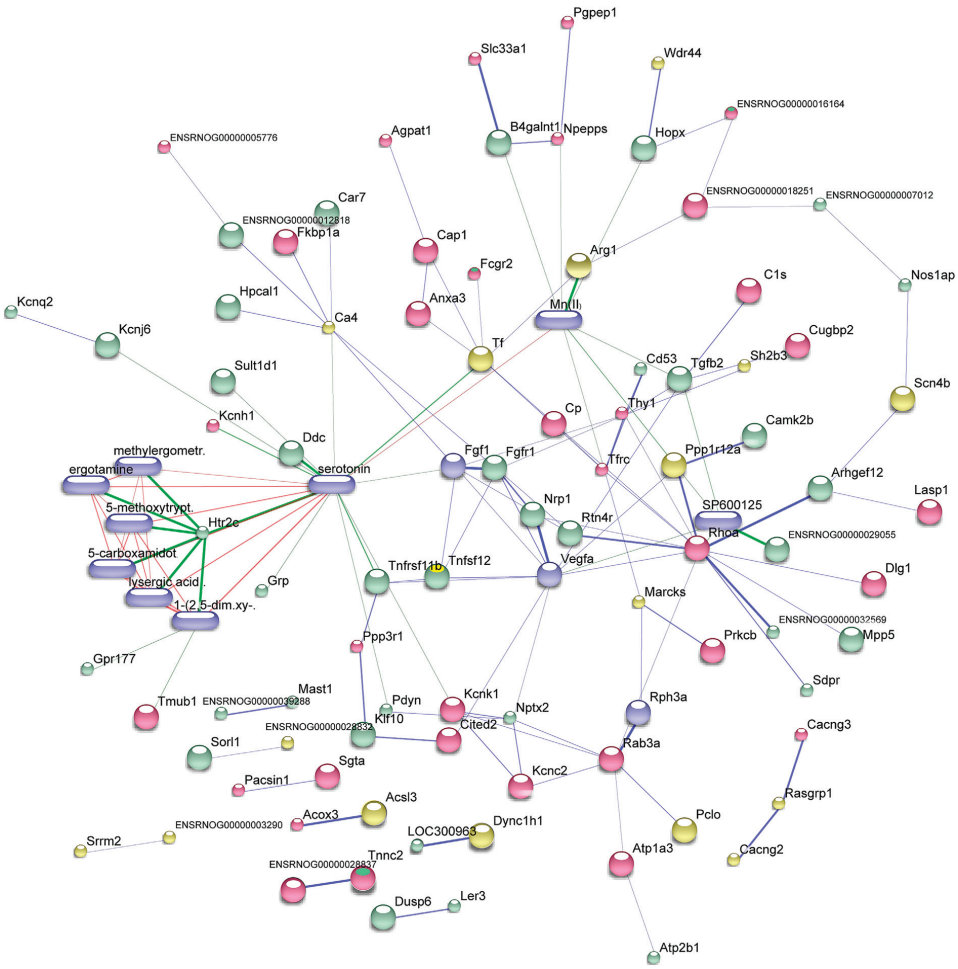

Figure 5. A network view of differentially expressed genes in the basal forebrain, frontal cortex and hippocampus. Circular spheres denote genes/proteins, whereas the pillshaped spheres denote small non-RNA dependent molecules. Colour scheme: red = differentially expressed in the basal forebrain, yellowish = differentially expressed in the frontal cortex, green = differentially expressed in hippocampus, blue = interactor. Genes that are differentially expressed in two tissues are two-coloured. Refer to Table 1 for scores and details of the interactors. (Figure used under permission from *Journal of Neurogenetics* (II))

Table 1. Predicted functional partners (interactors) derived from the network analysis. Refer to Figure 5 for a complete view of the networks involved. (Table adapted from *Journal of Neurogenetics* (II))

Predicted functional partners		Score
Rph3a	Rabphilin-3A (Exophilin-1); Protein transport. Probably involved with Ras-related protein Rab-3A in synaptic vesicle traffic and/or synaptic vesicle fusion. Could play a role in neurotransmitter release by regulating membrane flow in the nerve terminal.	0.999
1-(2,5-dimethoxy-4 iodophenyl)-2-aminopropane	Hallucinogen; Serotonin agonist (321.2 g/mol)	0.999
5-methoxytryptamine	5-methoxytryptamine; Serotonin derivative proposed as potentiator for hypnotics and sedatives. (190.2 g/mol)	0.999
ergotamine	A naturally occurring ergot alkaloid with vasoconstrictor and analgesic property. Ergotamine selectively binds and activates serotonin (5-HT) 1D receptors located on intracranial blood vessels, including those on arterio-venous anastomoses, thereby resulting in vasoconstriction and reducing the blood flow in cerebral arteries that may lead to relieve of vascular headaches. This agent also selectively binds to alpha-adrenergic receptors, thereby stimulating vascular smooth muscle and causing vasoconstriction in both arteries and veins, which also contributes to the relief of headaches. Furthermore, the activation of 5-HT1D receptors on sensory nerve endings of the trigeminal system results in the inhibition of pro-inflammatory neuropeptide release.	0.999
methylergometrine	The maleate salt of methylergonovine, a semi-synthetic ergot alkaloid with vasoconstrictive and uterotonic effects. Methylergonovine stimulates serotoninergic and dopaminergic receptors as well as inhibits the release of endothelial-derived relaxation factor. This results in arterial vasoconstriction and increased uterine smooth muscle contractions.	0.999
serotonin	Serotonin agent (176.2 g/mol)	0.999
SP600125	JNK (c-Jun N-terminal kinase) inhibitor (220.2 g/mol)	0.999
Fgf1	Heparin-binding growth factor 1 precursor (HBGF-1) (Acidic fibroblast growth factor) (aFGF); The heparin-binding growth factors are angiogenic agents in vivo and are potent mitogens for a variety of cell types in vitro. There are differences in the tissue distribution and concentration of these 2 growth factors.	0.999
Vegfa	Vascular endothelial growth factor A precursor (VEGF-A) (Vascular permeability factor) (VPF); Growth factor active in angiogenesis, vasculogenesis and endothelial cell growth. Induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis and induces permeabilization of blood vessels. Binds to the VEGFR1/Flt-1 and VEGFR2/Kdr receptors, heparan sulfate and heparin (By similarity). May play a role in increasing vascular permeability during lactation, when increased transport of molecules from the blood is required for efficient milk protein synthesis.	0.998

Table 1. (continues)

5-carboxamidotryptamine	Serotonin agonist (203.2 g/mol)	0.998
lysergic acid diethylamide	A substance that yields hydrogen ions or protons in aqueous solutions; a substance capable of accepting a pair of electrons for the formation of a coordinate covalent bond; a substance whose hydrogen can be replaced by metals or basic radicals, or which react with bases to form salts and water.	0.998
Mn(II)	An element with atomic symbol Mn, atomic number 25. (54.9 g/mol)	0.998

Table 2a. Differentially expressed inter-tissue pathways, spanning the basal forebrain, frontal cortex and hippocampus. Pathway size: 51-200 genes (Table adapted from *Journal of Neurogenetics* (II)).

Table2a Inter-tissue Pathways 51-200 genes								
Cellular Component								
ID	**Pathway**	**Opt. p-value**	**Permuted**	**Total # genes**	**Top # of genes**	**p<0.0025 in individual tissues**		
			p-value	**in pathway**	**in pathway**	**BF**	**FCX**	**HIP**
GO:0030424	axon	<10E-38	<1,00E-06	184	72	■		■
GO:0030426	growth cone	1,42E-08	4,00E-06	68	38	■	■	■
GO:0030427	site of polarized growth	1,42E-08	4,00E-06	68	38	■	■	■
GO:0008021	synaptic vesicle	2,93E-08	5,00E-06	131	26	■		
GO:0048770	pigment granule	1,35E-07	8,00E-06	140	137	■		
GO:0042470	melanosome	1,35E-07	1,10E-05	140	137	■		
GO:0030136	clathrin-coated vesicle	1,64E-07	2,20E-05	175	115	■		
GO:0030425	dendrite	2,39E-07	2,50E-05	180	37	■		
GO:0031252	leading edge	3,86E-07	2,60E-05	141	124	■		
GO:0005769	early endosome	7,42E-07	2,60E-05	60	49		■	
GO:0030118	clathrin coat	7,87E-07	2,70E-05	67	58			
GO:0019717	synaptosome	4,65E-07	3,70E-05	120	108		■	■
GO:0005770	late endosome	1,91E-06	6,10E-05	62	46			
GO:0030135	coated vesicle	9,34E-07	6,30E-05	199	126	■		
GO:0014069	postsynaptic density	3,10E-06	9,90E-05	65	15			■
GO:0016528	sarcoplasm	3,52E-06	0,0001	58	24			
GO:0048475	coated membrane	1,75E-06	0,000105	117	93			
GO:0030117	membrane coat	1,75E-06	0,000114	117	93			
GO:0030662	coated vesicle membrane	4,05E-06	0,000138	68	66			
Molecular Function								
ID	**Pathway**	**Opt. p-value**	**Permuted**	**Total# genes**	**Top# of genes**	**p<0.0025 in individual tissues**		
			p-value	**in pathway**	**in pathway**	**BF**	**FCX**	**HIP**
GO:0019783	small conjugating protein-specific protease activity	3,40E-10	<1,00E-06	101	86		■	
GO:0004843	ubiquitin-specific protease activity	1,02E-09	2,00E-06	98	94		■	
GO:0004221	ubiquitin thiolesterase activity	3,84E-09	2,00E-06	93	89		■	
GO:0004842	ubiquitin-protein ligase activity	4,29E-09	4,00E-06	157	111		■	■
GO:0003743	translation initiation factor activity	6,55E-08	6,00E-06	105	95	■		

Table 2a. (continues)

GO:0004812	aminoacyl-tRNA ligase activity	1,57E-07	1,30E-05	139	128			
GO:0005543	phospholipid binding	1,05E-07	1,50E-05	166	121			
GO:0045182	translation regulator activity	1,86E-07	1,50E-05	142	127			
GO:0016876	ligase activ. forming aminoacyl-tRNA & related comp.	1,57E-07	1,60E-05	139	128			
GO:0008135	translation factor activity, nucleic acid binding	1,86E-07	1,80E-05	142	127			
GO:0016875	ligase activity, forming carbon-oxygen bonds	1,57E-07	2,00E-05	139	128			
GO:0008080	N-acetyltransferase activity	7,22E-07	2,60E-05	76	59			
GO:0016247	channel regulator activity	9,05E-07	3,00E-05	63	31			
GO:0015457	auxiliary transport protein activity	9,05E-07	3,20E-05	63	31			
GO:0016407	acetyltransferase activity	1,96E-06	8,70E-05	90	67			
GO:0005516	calmodulin binding	3,02E-06	0,000187	189	83			
GO:0016836	hydro-lyase activity	6,99E-06	0,000241	75	7			
Biological Process								
ID	**Pathway**	**Opt. p-value**	**Permuted**	**Total # genes**	**Top# of genes**	**p<0.0025 in individual tissues**		
			p-value	**in pathway**	**in pathway**	**BF**	**FCX**	**HIP**
GO:0051969	regulation of transmission of nerve impulse	9,35E-11	<1,00E-06	194	25			
GO:0007270	nerve-nerve synaptic transmission	8,84E-10	<1,00E-06	116	32			
GO:0051129	negative regulation of cellular component organization	1,95E-09	<1,00E-06	119	90			
GO:0006418	tRNA aminoacylation for protein translation	3,13E-09	<1,00E-06	129	122			
GO:0048489	synaptic vesicle transport	5,47E-09	<1,00E-06	60	17			
GO:0050804	regulation of synaptic transmission	5,78E-10	2,00E-06	191	24			
GO:0007218	neuropeptide signaling pathway	7,63E-10	2,00E-06	126	39			
GO:0043039	tRNA aminoacylation	3,13E-09	2,00E-06	129	122			
GO:0043038	amino acid activation	3,13E-09	2,00E-06	129	122			
GO:0001505	regulation of neurotransmitter levels	3,63E-10	3,00E-06	151	30			

Table 2a. (continues)

GO:0035249	synaptic transmission, glutamatergic	3,34E-08	3,00E-06	65	21			
GO:0008380	RNA splicing	4,35E-08	8,00E-06	165	143			
GO:0007611	learning or memory	1,19E-07	1,10E-05	156	20			
GO:0007269	neurotransmitter secretion	9,24E-08	1,30E-05	100	92			
GO:0022618	ribonucleoprotein complex assembly	3,07E-07	1,70E-05	110	98			
GO:0042417	dopamine metabolic process	4,58E-07	1,70E-05	58	10			
GO:0006584	catecholamine metabolic process	5,48E-07	1,80E-05	75	9			
GO:0051494	Neg. reg. of cytoskeleton organization and biogenesis	1,37E-06	3,10E-05	54	43			
GO:0031646	positive regulation of neurological system process	8,83E-07	3,80E-05	70	11			
GO:0033043	regulation of organelle organization and biogenesis	6,49E-07	4,10E-05	165	116			
GO:0010639	Neg. reg. of organelle organization and biogenesis	1,37E-06	4,10E-05	54	43			
GO:0043087	regulation of GTPase activity	6,49E-07	4,90E-05	117	82			
GO:0032269	Neg. reg. of cellular protein metabolic process	8,60E-07	5,30E-05	140	97			
GO:0016567	protein ubiquitination	8,14E-07	6,20E-05	124	85			
GO:0055114	oxidation reduction	2,04E-06	6,70E-05	60	59			
GO:0032535	regulation of cellular component size	1,67E-06	7,40E-05	78	44			
GO:0051493	regulation of cytoskeleton organization and biogenesis	1,68E-06	8,90E-05	162	113			
GO:0051248	negative regulation of protein metabolic process	1,36E-06	9,00E-05	146	100			
GO:0031109	microtubule polymerization or depolymerization	3,17E-06	0,000106	71	53			
GO:0050767	regulation of neurogenesis	1,69E-06	0,000119	171	114			
GO:0022900	electron transport chain	4,35E-06	0,000123	57	56			
GO:0022904	respiratory electron transport chain	4,35E-06	0,000125	57	56			

Table 2a. (continues)

GO:0031110	Reg. of microtubule polymerization / depolymerization	4,62E-06	0,000126	65	49			
GO:0032446	protein modification by small protein conjugation	1,94E-06	0,000131	133	107			
GO:0006413	translational initiation	4,19E-06	0,000155	86	64			
GO:0050770	regulation of axonogenesis	3,32E-06	0,000157	86	63			
GO:0007631	feeding behavior	4,22E-06	0,00019	104	12			
GO:0007613	memory	6,66E-06	0,000191	61	10			
GO:0007411	axon guidance	3,73E-06	0,000196	166	71			
GO:0006403	RNA localization	4,40E-06	0,000203	91	82			

Table 2b. Differentially expressed inter-tissue pathways, spanning the basal forebrain, frontal cortex and hippocampus. Pathway size: 0-50 genes (Table adapted from *Journal of Neurogenetics* (II)).

Table2b Inter-tissue Pathways 0-50 genes								
Cellular Component								
ID	**Pathway**	**Opt. p-value**	**Permuted**	**Total # genes**	**Top# of genes**	**p<0.0025 in individual tissues**		
			p-value	**in pathway**	**in pathway**	**BF**	**FCX**	**HIP**
GO:0030665	clathrin coated vesicle membrane	2,32E-07	8,00E-06	49	45			
GO:0060053	neurofilament cytoskeleton	1,50E-06	1,60E-05	19	19			
GO:0005883	neurofilament	3,37E-06	3,10E-05	16	16			
GO:0031091	platelet alpha granule	7,04E-06	3,10E-05	6	2			
GO:0042734	presynaptic membrane	2,80E-06	6,50E-05	47	14			
Molecular Function								
ID	**Pathway**	**Opt. p-value**	**Permuted**	**Total # genes**	**Top# of genes**	**p<0.0025 in individual tissues**		
			p-value	**in pathway**	**in pathway**	**BF**	**FCX**	**HIP**
GO:0004089	carbonate dehydratase activity	4,02E-08	3,00E-06	36	7			
GO:0017067	tyrosine-ester sulfo-transferase activity	7,70E-06	1,20E-05	3	3			
GO:0016151	nickel ion binding	2,82E-06	2,40E-05	9	3			
GO:0017034	Rap guanyl-nucleotide exchange factor activity	1,94E-05	4,90E-05	3	3			
GO:0070052	collagen V binding	2,82E-05	5,50E-05	3	3			
GO:0004827	proline-tRNA ligase activity	1,48E-05	7,30E-05	6	5			
GO:0015066	alpha-amylase inhibitor activity	4,94E-05	0,00011	3	3			
GO:0015459	potassium channel regulator activity	1,11E-05	0,000112	12	4			
GO:0015349	thyroid hormone transmembrane transporter activity	2,10E-05	0,000117	6	2			
GO:0004691	cAMP-dependent protein kinase activity	3,13E-05	0,000128	6	5			
GO:0004712	protein serine/threonine/ tyrosine kinase activity	2,00E-05	0,000194	12	11			

Table 2b. (continues)

Biological Process								
ID	**Pathway**	**Opt. p-value**	**Permuted**	**Total # genes**	**Top# of genes**	**p<0.0025 in individual tissues**		
			p-value	**in pathway**	**in pathway**	**BF**	**FCX**	**HIP**
GO:0007215	glutamate signaling pathway	1,33E-07	2,00E-06	24	13			
GO:0016079	synaptic vesicle exocytosis	6,35E-08	4,00E-06	34	12	■		
GO:0007598	blood coagulation, extrinsic pathway	1,41E-06	6,00E-06	3	2			
GO:0046655	folic acid metabolic process	2,62E-06	6,00E-06	3	2			
GO:0051346	negative regulation of hydrolase activity	5,50E-07	7,00E-06	25	15			
GO:0042706	eye photoreceptor cell fate commitment	3,08E-05	2,60E-05	2	2			
GO:0051966	regulation of synaptic transmission, glutamatergic	2,19E-06	3,30E-05	40	14	■		
GO:0046552	photoreceptor cell fate commitment	3,08E-05	3,40E-05	2	2			
GO:0051968	pos. regulation of synaptic transmission, glutamatergic	5,67E-06	4,60E-05	14	5	■		
GO:0016338	calcium-independent cell-cell adhesion	4,85E-06	5,70E-05	18	6			
GO:0006433	prolyl-tRNA aminoacylation	1,48E-05	6,20E-05	6	5		■	
GO:0030514	negative regulation of BMP signaling pathway	5,90E-06	7,70E-05	19	2			
GO:0014002	astrocyte development	8,37E-06	9,40E-05	15	5	■		■
GO:0001661	conditioned taste aversion	9,32E-06	9,50E-05	12	6	■		
GO:0008542	visual learning	4,16E-06	0,0001	49	12	■		
GO:0016188	synaptic vesicle maturation	1,30E-05	0,000101	9	4	■		
GO:0001964	startle response	7,11E-06	0,00012	33	7	■		
GO:0007632	visual behavior	5,22E-06	0,000124	50	12	■		
GO:0051261	protein depolymerization	6,67E-06	0,000147	50	45			
GO:0017157	regulation of exocytosis	8,14E-06	0,000171	38	31			
GO:0007217	tachykinin signaling pathway	3,67E-05	0,000172	6	5			■
GO:0030510	regulation of BMP signaling pathway	1,30E-05	0,000215	28	2			
GO:0060052	neurofilament cytoskeleton organization & biogenesis	1,81E-05	0,000229	21	16			

4.1.3 Differentially expressed pathways within the basal forebrain (BF) (I) and the hypothalamus (HYP) (III)

The BF and HYP are important structures related to the regulation of both sleep and mood. Detecting pathways, within these tissues, that are in inequilibrium would explain the function of these areas in the clomipramine model and thereby possibly elucidate the involvement of these brain areas in the regulation of sleep and mood.

Within the BF (Table 2 in (I)) we detect changes in pathways or gene ontology groups related to the cellular component – category (CC) of the synapse (optimal p=1.7E-07, permuted p=0.00001) and the biological process – category (BP) of synaptic transmission (optimal p=9.3E-09, permuted p=0.00001), regulation of neurotransmitter levels (optimal p=3.92E-07, permuted p=0.00002) and the more specific pathway for serotonin receptor phospholipase C activation (optimal p=4.89E-05, permuted p=0.00004). The most differentially expressed pathway within the molecular function – category (MF) is the gamma-aminobutyric acid (GABA) signalling pathway (optimal p=7.6E-05, permuted p=0.00099), emphasizing the role of BF in GABAergic neurotransmission.

The pathways presenting the statistically most significant changes in the HYP (Table 1 in (III)) are of a more general nature. The changes are located to (CC) synaptic vesicles (optimal p=7.81E-07, permuted p=3.00E-05) and related to (BP) macromolecular (RNA and protein) metabolism (starting from optimal p=3.74E-11 and permuted p=1.00E-05). Molecular functions (MF) such as GTPase activity (optimal p=7.07E-08, permuted p=1.00E-05) and protein ligase activity (starting from optimal p=2.23E-07 and permuted p=1.00E-05) are altered. Nevertheless, also more precise (MF) pathways such as extracellular-glycine-gated chloride channel activity and GABA-A receptor activity are detected at reasonable level of statistical significance. Unfortunately the HYP had to be excluded from the inter-tissue analysis as the samples consist of 3-month old females, and can therefore not be normalized together with the samples from BF, FCX and HIP of three week old animals.

4.1.4 Promoter analysis of the differentially expressed genes in basal forebrain (BF) (I)

By analysing the promoter areas of the most differentially expressed genes within the BF (Table 1 in (I)), for an enrichment of common regulatory motifs, important transcription factor binding sites (TFBS) were detected. The over-represented TFBS, and their respective transcription factors (TF) could be responsible for the changes in gene expression within the tissue. The number of analysed genes was reduced to 48 due to insufficient sequence data and the criterias set for the promoter areas.

In the analysis five TFBS qualified at p<0.0025 (Table 3). The top TFBS was found in 23 of the investigated promoters, and binds the cAMP response element-binding

protein 1 (CREB1) (see Discussion for a background on this molecule). This CREB1 binding site is over-represented amongst the promoters at a Z-score of 9.113, generating a Fisher score of p=0.000059.

Table 3. Search for enriched binding sites for transcription factors (TF; TFBS) among differentially expressed genes in basal forebrain of animals treated with clomipramine (n=6) as compared to those treated with saline (n=7) at the age of 3 weeks. (Table adapted from European Journal of Neuroscience (I))

TF	TF Class	Background genes		Target genes		Background TFBS		Target TFBS		Z-score	Fisher score
		Hits	Non-hits	Hits	Non-hits	Hits	Rate	Hits	Rate		
CREB1	bZIP	3892	11258	23	19	5673	0,0055	33	0,0086	9,113	5,90E-05
Ddit3-Cebpa	bZIP	2749	12401	16	26	3888	0,0038	19	0,005	4,214	1,90E-03
MIZF	ZN-FINGER, C2H2	1298	13852	10	32	1531	0,0012	11	0,0024	7,048	2,47E-03
TLX1-NFIC	HOMEO/CAAT	435	14715	5	37	456	0,0005	5	0,0015	9,461	7,01E-03
Pdx1	HOMEO	9899	5251	35	7	54092	0,0261	242	0,0317	7,404	8,41E-03

TF = Transcription factor, TFBS = Transcription factor binding site,IC = The information content or specificity of this TFBS profile's position weight matrix, Z–Score = The likelihood that the number of TFBS nucleotides detected for the included target genes is significant as compared with the number of TFBS nucleotides detected for the background set. Z-score is expressed in units of magnitude of the standard deviation, Fisher Score = The probability that the number of hits vs. non-hits for the included target genes could have occurred by random chance based on the hits vs. non-hits for the background set (table adapted from (I) European Journal of Neuroscience)

4.1.5 DNA methylation analysis of *Brain-derived neurotrophic factor* (BDNF) , *Neurotrophic tyrosine kinase receptor, type 2* (NTRK2), *Insulin-like growth factor 2* (IGFII) and *Acetyl-Coenzyme A transporter 1* (ACATN) promoters in basal forebrain (BF)

The promoters of *BDNF, NTRK2, IGFII* and *ACATN* show no significant difference in DNA methylation, between the two groups (clomipramine treated / saline treated), at any of the CpG sites investigated (unpublished data). This implies that neither gene is under transcriptional regulation by DNA methylation in the BF.

4.2 Age dependent changes in sleep (V)

The NREM sleep, slow wave activity (SWA) and the sleep latency were analyzed in relation to age, and considered as measures of sleep need, recovery sleep respectively sleep pressure. Reduced effects according to age, after sleep deprivation, would indicate an age dependent decrease in sleep pressure buildup during restricted sleep.

The reason for analysing the expression of BDNF together with the sleep data is to investigate the relation between plasticity genes and sleep pressure.

4.2.1 Sleep latency (SL)

The normal SL for the three age groups was tested after a baseline period. An age related reduction in SL can be observed between the young and the old animals (Two-Way Repeated Measures ANOVA, followed by Holm-Sidak post hoc test, $p<0.05$) (Figure 6). After a 6h sleep deprivation (SD) all age groups present a reduction in SL. The magnitude of the reduction in SL is similar in all age groups, with the exception of the last hour, when the old animals present a significantly higher SL, as compared to the two other groups (Figure 6b).

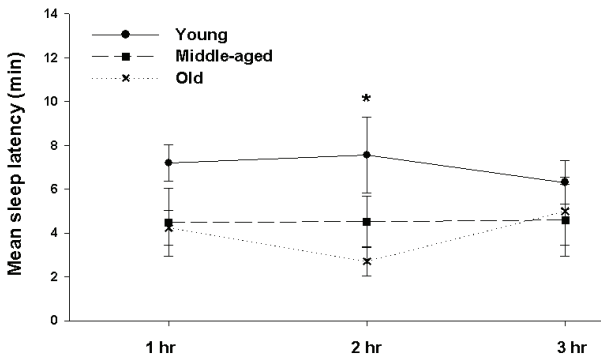

Figure 6a. Mean latency (averaged per hour) to non-rapid eye movement (NREM) sleep after a baseline period during which spontaneous sleep-wake behaviour occurred. The old rats have a significant decrease in sleep latency compared to young rats in the second hour after onset of test (Figure adapted from (V)).

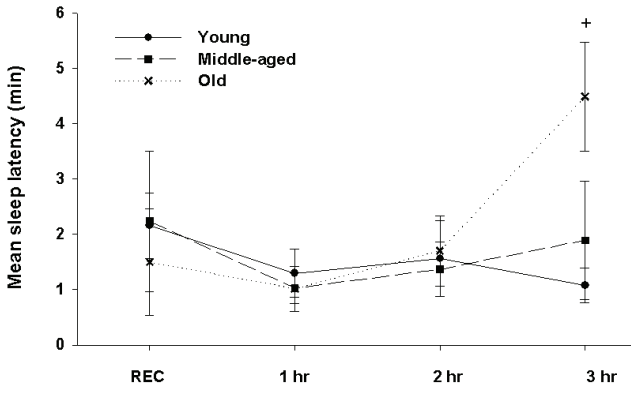

Figure 6b. Latency to non-rapid eye movement (NREM) sleep after 6 h sleep deprivation. Old rats have a significant increase in sleep latency compared to young in the third hour after the onset of test (REC= start of recovery sleep). Number of animals: young, n=7; middle-aged; n=6, old, n=9. Significances: between age-group significance denoted with an asterisk (*); near significant denoted with a plus (+) (Figure adapted from (V)).

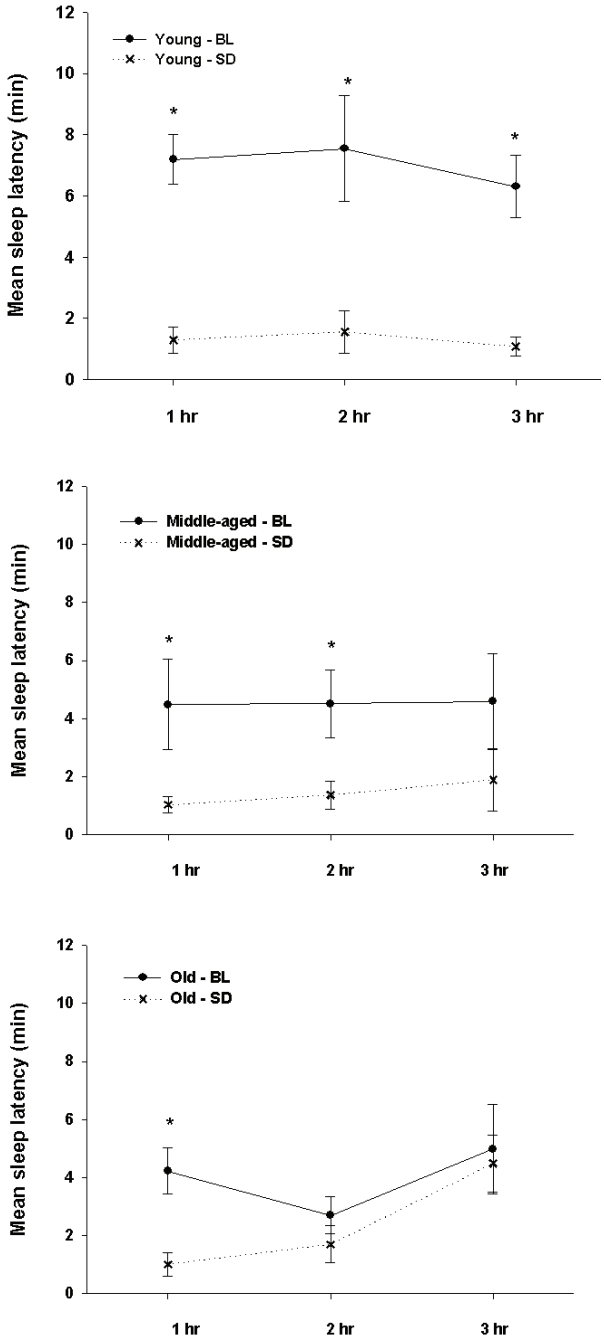

Figure 7. Mean latency (averaged per hour) to non-rapid eye movement (NREM) sleep after a baseline period during which spontaneous sleep-wake behaviour occurred and after 6 h sleep deprivation in young (A), middle-aged (B) and old (C) rats. Both young and middle rats exhibited significantly decreased sleep latencies following sleep deprivation (as compared to baseline values) during the whole 3 hour test period, while the old rats the sleep latency was significantly decreased only during the first hour. Number of animals: see Figure . Significances: inside age group significance denoted with an asterisk (*) (Figure adapted from (V)).

The SL after SD compared to the SL after the baseline period, for each animal, show that the latency stays lower during the whole test (3 hours) only in the young rats (Figure 7A), whereas the aged animals have a significant decrease in SL only during the first two hours (middle-aged rats, Figure 7B) or the first hour (old rats, Figure 7C) of testing.

The average number of interventions during the whole 6 hour SD in the different age groups was: Young, 31 ± 4; Middle-aged, 53 ± 7; Old 111 ± 13 (One-Way ANOVA, p =< 0.001 between all groups) (Figure 8).

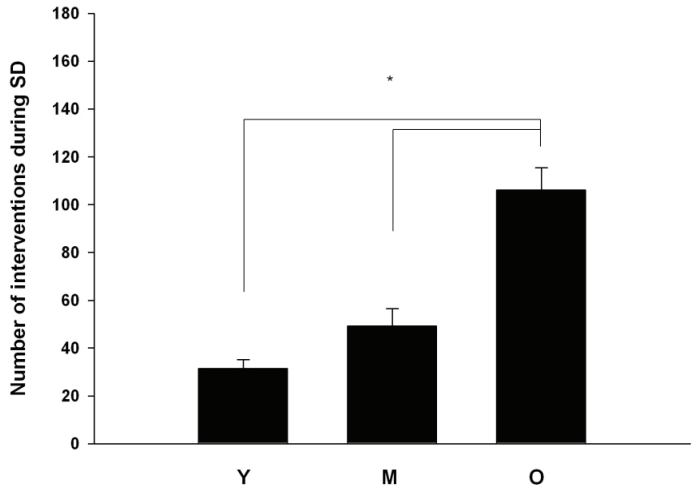

Figure 8. Number of interventions during the 6 hour sleep deprivation (SD). Old rats had significantly more interventions during SD than either young or middle-aged rats (Figure adapted from (V)).

4.2.2 Non-REM (NREM) sleep

During the baseline period the young animals present higher NREM delta (0.75-4 HZ) values compared to both old and middle-aged rats during the first 6 hours of lights on (8.30 AM to 2.30 PM). Also between hours 7 and 13 (2.30 PM to 9.30 PM) the young rats have higher delta values compared to the old animals, but no difference can be detected between the young and the middle aged, nor between the middle aged and the old (Two-Way Repeated Measures ANOVA followed by Holm-Sidak post hoc test, $p<0.05$) (Figure 9A).

During the first three hours of recovery sleep a rise in delta power (0.75-4 HZ) in the young animals, as compared to both the middle-aged and the old, can be observed after normalization to baseline levels. Between hours 7 and 13 (2.30 PM to 9.30 PM) this rise is only significant when compared to the old animals (Two-Way Repeated Measures ANOVA followed by Holm-Sidak post hoc test, $p<0.05$) (Figure 9b).

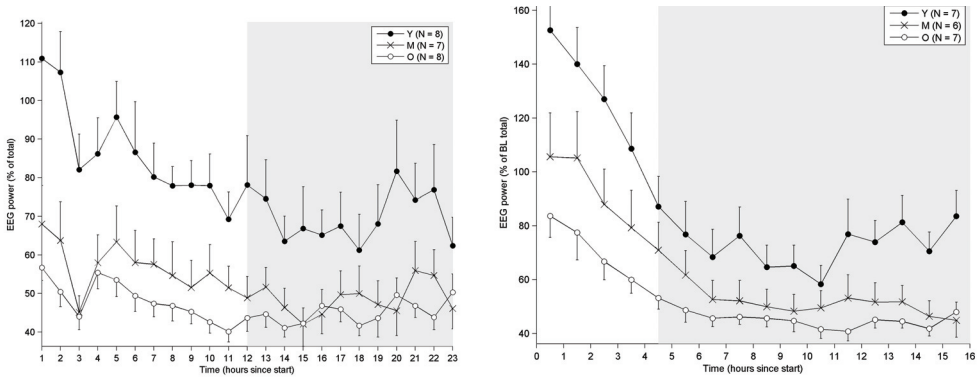

Figure 9a. Baseline NREM delta power (0.75-4 Hz) expressed as percentage of total power. The young rats have significantly higher NREM delta values compared to both old and middle-aged rats during the first 6 hours of lights on (8.30 AM to 2.30 PM), and significantly higher NREM delta values compared to old rats between hours 7 and 13 (2.30 PM to 9.30 PM) (Two-Way Repeated Measures ANOVA followed by Holm-Sidak post hoc test, $p<0.05$) (Figure adapted from (V)).

Figure 9b. Recovery NREM delta power (0.75-4 Hz) normalized to baseline values. The young rats have significantly higher NREM delta values compared to both old and middle-aged rats during the first 3 hours of recovery sleep (4 PM to 7 PM), and significantly higher NREM delta values compared to old rats between hours 7 and 13 (2.30 PM to 9.30 PM) (Two-Way Repeated Measures ANOVA followed by Holm-Sidak post hoc test, $p<0.05$) (Figure adapted from (V)).

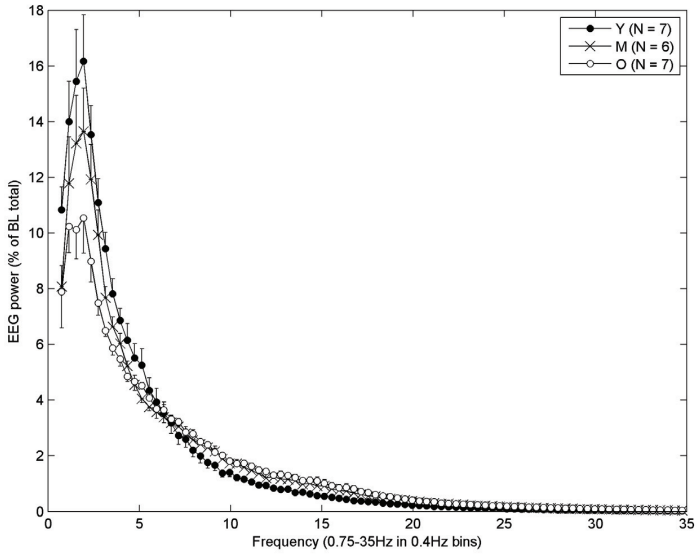

Figure 10. NREM EEG power (0.75-35 Hz) during the first 25 minutes of recovery sleep after sleep deprivation (before first MSLT activation), normalized to baseline total power. Significant differences between aged groups were found in the 0.75 Hz to 4.75 Hz frequencies with young rats showing consistently higher power in these frequencies (Two-Way Repeated Measures ANOVA followed by Holm-Sidak post hoc test, $p<0.05$) (Figure adapted from (V)).

4.2.3 Response of Brain-derived neurotrophic factor (BDNF) and slow wave activity (SWA) to sleep deprivation (SD)

At the level of mRNA, the expression of BDNF in the cortex is increased in all age groups after both 3h and 6h of SD. The magnitude of the increase in BDNF expression at mRNA level, after SD, is significantly smaller in the aged animals than in the young animals (p=0.015) (Figure 11a). These changes correlate positively with SWA during the first 25 min of NREM recovery sleep as aged rats have lower BDNF mRNA expression and lower SWA and young rats have higher BDNF expression and respectively higher SWA (Pearson's product moment, p<0.05) (Figure 12).

The concentration of the BDNF protein, during the SD, acts completely differently. BDNF protein expression is slightly increased during the 3h SD (not statistically significant) in all age groups, whereas a clear drop in BDNF protein concentration can be observed in all age groups after the 6h SD (p<0.05, for all) (Figure 11b).

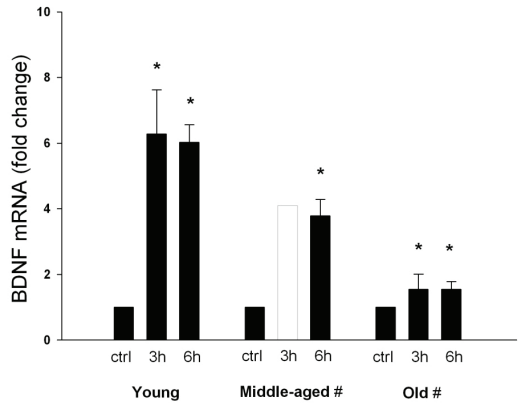

Figure 11a. BDNF mRNA expression (fold change) after sleep deprivations (SD). BDNF mRNA expression increased significantly in all aged groups following either 3 hour or 6 hour SD. (note the missing middle-aged 3h SD group, replaced by a white bar depicting the expected value). There was a significant difference between the young and aged (middle-aged and old) animals during the 3h and 6 SD (One-Way ANOVAs followed by Holm-Sidak post hoc tests, p<0.05, t-test was used to compare 6h SD between young and old, p=0.015). Number of animals: young control, n=7; young 3hsd, n=6; young 6hsd, n=12; middle-aged ctrl, n=5, middle 3hsd, n=0, middle-aged 6hsd, n=13; old ctrl n=5, old 3hsd, n=5; old 6hsd, n=5). Significances: inside age group denoted with an asterisk (*); between age groups denoted with a hash (#) (Figure adapted from (V)).

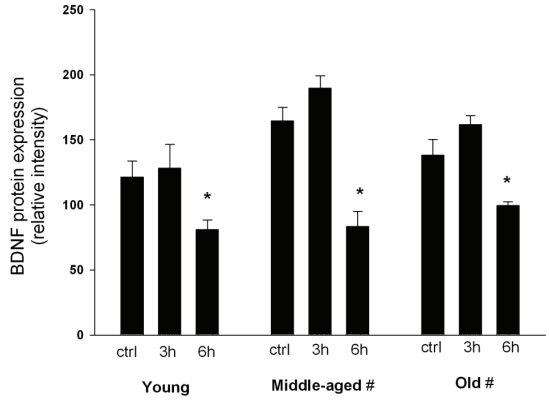

Figure 11b. BDNF protein expression decreased following 6 hours of sleep deprivation (6hsd) compared to control conditions (ctrl) in all age groups. BDNF expression tended to increase following 3 hours of sleep deprivation (3hsd) compared to control conditions (ctrl) in all age groups, but this increase did not reach significance. Overall, BDNF expression in young rats significantly differed from the BDNF expression in middle-age and old rats (Two Way ANOVA followed by Holm-Sidak post-hoc test, $p<0.05$. Number of animals: young control, n=13; young 3hsd, n=11; young 6hsd, n=14; middle-aged ctrl, n=11, middle 3hsd, n=6, middle-aged 6hsd, n=12; old ctrl n=13, old 3hsd, n=9; old 6hsd, n=5). Significances: inside age group denoted with an asterisk (*); between age groups denoted with a hash (#) (Figure adapted from (V)).

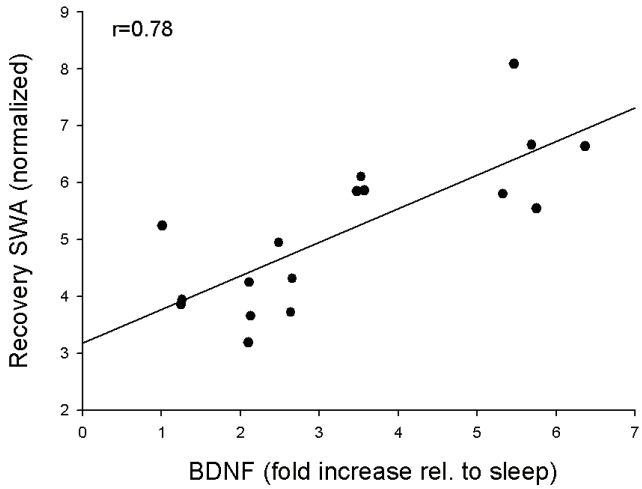

Figure 12. Significant correlation between cortical BDNF mRNA levels (frontal cortex) and the increase in slow-wave activity (SWA, normalized) during the first 25 minutes of non-rapid eye movement (NREM) sleep. Number of animals: young, n= 7; middle-aged, n=5; old n=5) (Figure adapted from (V)).

4.3 The effect of environmental stress on the serotonergic system (IV)

The DNA methylation analysis was performed in order to detect possible differences in the DNA methylation of the SLC6A4 promoter between the high- and low-workstress groups. Such differences would suggest a mechanism for the regulation of SLC6A4 in relation to work stress. Furthermore, the method used in the analysis would also expose the exact locations for the DNA methylation. Through the questionnaire we wanted to ascertain that high work stress actually correlates with burnout and/or depression in the material used. The genotyping of the subjects was performed in order to investigate if either genotype was predisposed to the negative effects of workstress. A secondary analysis was performed to detect if differences in DNA methylation, between the two genotypes, exist. In case of such differences, the relevance of the differences in DNA methylation could fall under dispute.

4.3.1 Self assessed burnout and depression in relation to work stress

According to Karaseks Model (Karasek, 1998), 24 of the subjects (nurses) are categorized to work in a high stress environment and 25 in a low stress environment. Based on scores from the Maslach Burnout Index General Survey (MBI-GS), half of the subjects (n=12) belonging to the high stress group report at least moderate burnout symptoms (MBI-GS scores higher than 1.5), whereas none of the subjects belonging to the low stress group report any symptoms of burnout (MBI-GS scores lower than 1.5). The mean score is 1.3(±0.76) for the high stress group and 0.72(±0.49) for the low stress group (Table 4). This difference qualifies as statistically significant (p=2.3 x 10E-5, t-test).

To detect signs of depression the Beck Depression Index (BDI) was used. In the high stress group 12 subjects are rated with mild depression (BDI 10-18) and two subjects are rated with moderate to severe depression (BDI >18). In the low stress group three subjects rate as mildly depressed, whereas none are rated as moderately or severely depressed. The mean BDI scores are 8.36(±6.31) and 4.37(±3.36) in the high and low stress groups respectively (Table 4). This difference in mean BDI scores proved to be statistically significant (p=2x10E-3, t-test), thereby identifying the nurses in the high works stress group to be more vulnerable to depression. By using categorical variables according to the three assigned categories (0=no depression, 1=mildly depressed, 2=moderate to severe depression) the t-test p-value is further reduced to 2.1x10E-4.

Table 4. Demographic data of the nurse groups by work stress. (Table adapted from (IV) PLOS ONE)

Work Stress Environment	N	Age	MBI-GS	BDI
High work stress	24	49 (± 6.02)	1.33 (±0.760)	8.36 (±6.31)
Low work stress	25	48 (±6.78)	0.723 (±0.487)	4.37 (±3.36)

4.3.2 CpG methylation of the *Serotonin transporter 6A4* (SLC6A4) promoter

The methylation levels of the SLC6A4 promoter are found to be lower in the high work stress group compared to the low work stress group, suggesting that the activity of SLC6A4 is regulated by DNA methylation. This finding is consistent for all five CpG dinucleotides investigated. The methylation levels of the five CpG sites are; CpG1 = 5.5±2.7, CpG2 = 5.9±3.5, CpG3 = 9.8±3.7, CpG4 = 3.7±2.8, CpG5 = 4.2±3.0 in the high stress group and CpG1 = 13±6.7, CpG2 = 12±5.7, CpG3 = 15±5.4, CpG4 = 8.8±4.3, CpG5 = 7.8±3.5 in the low work stress group. A t-test calculation show these changes to be highly significant (p=7.10E-06, p=2.50E-05, p=0.000292, p=4.37E-06, and p=0.000289 respectively) (Figure 13). As the investigated CpG sites are located close together (Figure 14), it is likely that they share a common function and that their methylation/demethylation is concerted. This is also the case as all five sites are detected in a principal component analysis (PCA). A sum score for methylation was then calculated by multiplying the values for each individual CpG residue by its factorial loading obtained in the first component of PCA. These weighted values were then summed up to create a weighted sum score, referred to as ”METsum”. The mean METsum in the high work stress group is 25.7±10.6 (95% confidence interval 21.2-30.1) and 50.7±21 (95% confidence interval 42.0-59.4) in the low work stress group. The mean METsum in the total sample is 38.4±20.8. In order to determine the effect size between work stress groups, the Cohen's d for the difference between the means was calculated (50.7-25.7)/20.8=1.2. The effect size of 1.2 indicates that the difference is notable. These results are summarized in Figure 15 and Table 5.

Table 5. METsum in high and low work stress groups and work stress environment as a whole. The effect size can be calculated using Cohen's d, defined as the difference between the two means of high and low work stress groups divided by the standard deviation for the complete data. (Table adapted from (IV) PLOS ONE)

Work stress environment	Mean	SD	Lower 95% CL for Mean	Upper 95% CL for Mean
High work stress	50.7	21.0	42.0	59.4
Low work stress	25.7	10.6	21.2	30.1
Work stress environment	38.4	20.8	32.5	44.4

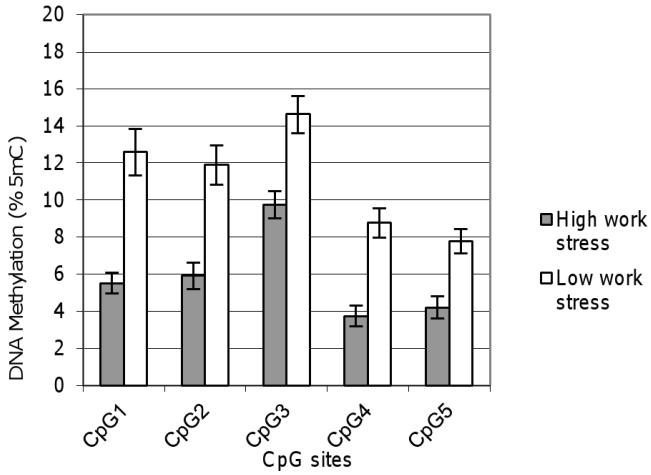

Figure 13. Methylation in the promoter of SLC6A4 at five CpG sites in high (grey) and low (white) work stress environments. Coordinates for each residue are 28 563 120 (CpG5), 28 563 109 (CpG4), 28 563 107 (CpG3), 28 563 102 (CpG2), and 28 563 090 (CpG1) as per hg19 assembly of the UCSC Genome Browser. Differences between work stress environments were significant as per t-test (p=7.10E-06, p=2.50E-05, p=0.000292, p=4.37E-06, and p=0.000289 respectively). Standard errors are indicated. (Figure used under permission from (IV) PLOS ONE)

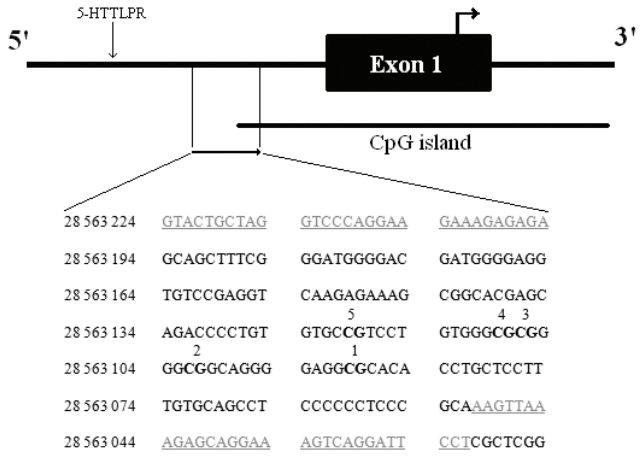

Figure 14. Reresentation of the SLC6A4 gene promoter region for bisulfite sequencing analysis. Analyzed CpG sites are in bold and numbered from 1 through 5. Coordinates are based on the hg19 assembly of USCS Genome Browser. (Figure used under permission from (IV) PLOS ONE)

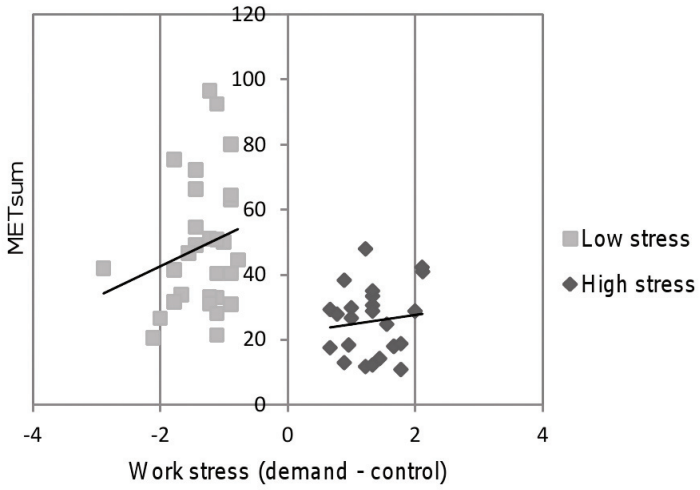

Figure 15. Distribution of methylation levels in the high and work stress environments. Work stress is defined according to Karasek's Model by the difference between averages of work demand and control. (Figure used under permission from (IV) PLOS ONE)

A linear regression analysis was performed by analysis of covariance (ANCOVA) to assess the association of the calculated METsum (as dependent variable) with work stress environment, burnout, work control, work demand, age, and 5-HTTLPR (as explanatory variables). When the main effects of all explanatory variables are included into the model, only work stress is statistically significant and independently ($F=5.7$; $p=0.022$) associated with METsum (Table 6). After elimination of non-significant effects from the model, both work stress ($p<0.0001$) and MBI-GS ($p=0.038$) are independently associated with the methylation levels and together they explain 43% of the variance of METsum ($R2=0.43$, $p<0.0001$).

Table 6. Results of the initial main effects model using METsum as dependent variable. (Table adapted from (IV) PLOS ONE)

Variable	df	Type III SS	F	P
Work stress	1	1480.196769	5.66	0.0221
5-HTTLPR	2	685.987277	1.31	0.2806
MBI-GS	1	695.005206	2.66	0.1108
Control	1	222.192198	0.85	0.3621
Demand	1	0.01083	0	0.9949
Age	1	448.159783	1.71	0.1979

4.3.3 Effects of the *5-HTTLPR* genotype

No significant association between the different genotypes (La/La, La/S and S/S) and work stress or burnout is present. The METsum scores show no correlation with genotype (Table 7) and after testing the interactions for the genotype and work stress or burnout (5-HTTLPR x work stress or 5-HTTLPR x MBI-GS) with METsum in a multivariable model, no statistically significant (p=0.58 and p=0.082, respectively) interactions are found.

Table 7. Distribution of the 5-HTTLPR genotype. (Table used under permission from PLOS ONE)

Genotype	La/La	La/S	S/S
N (%)	21 (39.6%)	19 (35.8%)	9 (17.0%)
Mean burnout scores (SD)	0.889 (±0.764)	1.02 (±0.604)	0.951 (±0.499)
Mean depression scores (SD)	6.48 (±6.32)	6.05 (±4.94)	6.89 (±4.34)
METsum (SD)	35.3 (±16.2)	36.5 (±22.3)	49.9 (±25.3)

5 Discussion

Seldom is a disease restricted to only one certain tissue or celltype and the need for tools to investigate disorders as a whole grows stronger as more pathway and network information is being gathered (Dobrin et al., 2009).

The inter-tissue pathway analysis and the network analysis are attempts to model a more complete molecular picture of disease.

It is evident that this kind of study represents the disorder of interest more completely, compared to investigating tissues separately (as implied by the field of neuroimaging (Rigucci, Serafini, Pompili, Kotzalidis, & Tatarelli, 2010)). Studies conducted using neuroimaging, lesion analysis, and post mortem methodologies, all support the model in which the pathophysiology of depression is due to a dysfunction in an extended network involving the FCX and anatomically-related limbic, striatal, thalamic and BF structures (Price & Drevets, 2012). Lately, the importance of multidiciplinary studies on the function of different brain areas has been emphasized and extended to include genome-wide gene expression analysis (Stansberg et al., 2007). Some of the studies performed have been further refined to monitor, not only the healthy brain, but also include detection of differences in gene expression related to phenotype or disorder (Letwin et al., 2006). Despite the amazing achievements of work such as the Allen Brain Atlas –project (Lein et al., 2007), it is still, for the time being, practically impossible to reliably monitor global three (not to mention four or five) dimensional gene expression without spatial interruptions, which means that tools are needed to estimate the interactivity and the situation in the "space" in between the measuring points.

In the inter-tissue pathway and network analyses two already existing tools were used, and by careful planning of the input data, intricate hidden networks affected by the clomipramine treatment, were revealed.

In both analysis methods a large number of findings were previously well known to the field of psychiatry. These changes may lack novelty value, but instead they confer a certain reliability to the methods. The fact that changes in sleep pathways were detected and qualified near the top list of disrupted pathways (not present in Table 2a or 2b due to p-value cut-off at permuted p≤2,5E-04 in the tables) indicates not only that the CLI model really affects sleep (GO:0030431, optimal p=6,61E-04, permuted p=0,00723) and the regulation of circadian rhythm (GO:0042752, optimal p=0,00164, permuted p=0,0161), but it also shows that the present GO databases are becoming extensive and precise enough to distinguish pathways involved in very intricate phenomena.

In addition, the most interesting of the novel findings from the two methods consistently combine as the melanosome, detected in the inter-tissue pathway analysis, is the primary reservoir of Mn(II) (Cotzias, Papavasiliou, & Miller, 1964; Van Woert, Nicholson, & Cotzias, 1965) that was detected in the inter-tissue network analysis.

At first glance the proposed central role of melanosomes in the regulation of sleep and mood alerts of a secondary effect or of deficiencies in pathway databases. Nevertheless, melanosomes are existent in the brain, where their function is still quite unknown, making the finding even more exciting. Lately melanocytes have been shown capable of producing classical stress neurotransmitters, neuropeptides and hormones, which are secreted to either act locally in a neurotransmitter-like manner, or to enter the circulation and affect systemic homeostasis according to endocrine model. Actually, many of the products of melanocytes have been previously linked to the regulation of sleep, mood and stress. Corticotropin releasing factor (CRF) and proopiomelanocortin (POMC) that is processed to adrenocorticotropic hormone (ACTH), α-MSH and β-endorphin, have both been shown to be central to all three phenomena (Plotsky, 1995). Additional examples of melanocyte neuroendocrine activity are their ability to produce and secrete catecholamines, L-DOPA, lipocalin-type prostaglandin D synthase (L–PGDS) and the capability to transform L-tryptophan to serotonin, N-acetylserotonin and melatonin with its further metabolism (Slominski, 2009). Because melanocytes are able to produce L–PGDS, a potent inducer of sleep, and produce opioids that regulate respiratory rhythm, it suggests that brain melanocytes produce these molecules in vivo to regulate sleep (Colombo, Berlin, Delmas, & Larue, 2011).

Furthermore, as the melanosomes are the primary reservoirs of Mn(II) (Cotzias et al., 1964; Van Woert et al., 1965), a molecule that was found to have a central role in the networks involved in the clomipramine model. Mn(II) is involved in the control of mood and sleep, and has been linked to psychiatric traits such as increased anxiety, insomnia and irritability (Calne, Chu, Huang, Lu, & Olanow, 1994; Pal, Samii, & Calne, 1999). Further clues of the behavioural effects of Mn(II) can be acquired by observing manganism (Mn intoxication). In the initial stage of manganism the neurological symptoms consist of reduced response speed, irritability, intellectual deficits, mood changes, and compulsive behaviours, but after a prolonged state of manganism the symptoms start to resemble those observed in Parkinsons disease (Roth, 2006).

The research in the cofactors stress and age and their contribution to the vulnerability to changes in mood and sleep, is paradigmatically more conventional and the experimental designs are not as multidimensional as in the clomipramine model. Nevertheless, as the experimental designs were based to a higher degree on previous findings and assumptions, the outcomes were, although not completely unforeseen, very precise and add up to the growing knowledge of these phenomena.

The diminished increase in the expression of the plasticity gene BDNF, during sleep deprivation, observed in the old animals, correlated well with the age dependent increase in sleep latency and reduced SWA response after the prolonged wakefulness. This supports not only the plasticity hypothesis, according to which the homeostatic sleep need is build up due to a plasticity related time dependent increase in synaptic strength, but it also explains the reduced sleep need that is accompanied by age. The reduction in the SWA response and the higher sleep latency, in the old animals, being indicators of a reduced sleep need.

The investigations of regulatory mechanisms, related to sleep deprivation and adaptation to stress, were also very productive. The most outstanding genes from the experiment in BF were subject to a promoter analysis that revealed a highly significant enrichment of the binding site for CREB1. CREB1 has previously been linked to both sleep regulation (Graves et al., 2003) and depression (Zubenko et al., 2003). The differences in methylation, at the SLC6A4 promoter, between the low work stress group and the high stress group, were significant. The results suggest that this coping mechanism is responsible for the increased susceptibility for depression observed during prolonged periods of stress. Furthermore, during the initial introduction of the DNA methylation analysis, in form of direct bisulfite sequencing, two major improving factors were discovered. After the bisulfite conversion the DNA is highly degraded and includes mismatches, due to the C→U/T conversion. By using a polymerase that lacks the proofreading domain (3'- 5' exonuclease activity) the PCR of the highly degraded DNA can be highly enhanced. Another major improvement, at the level of sequence quality, can be achieved by using tailed primers in the PCR and the use of primers targeted to these overhangs in the sequencing reaction.

In addition to the results presented here, the studies yielded a vast amount of useful knowledge, especially the studies in the clomipramine model, as each tissue was also investigated separately on a single gene level, and the results, although not directly presented here, are available in the original publications (I, II & III).

6 Conclusions

This study evinces that stress, sleep and mood are highly interconnected phenomena that affect each other and, furthermore, their properties change with age. The results demonstrate that the regulatory mechanisms of these phenomena have extensive overlap on a molecular level. In addition, these findings also suggest that coping mechanisms exist for the body to deal with extreme situations of high stress and restricted sleep. Whereas these mechanisms have a short-term benefit, prolonged exposure to any of these conditions result in the same mechanisms giving rise to increased susceptibility for disorder.

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