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SLEEP DEPRIVATION AND BRAIN ENERGY METABOLISM

in vivo studies in rats and humans

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ACADEMIC DISSERTATION

To be publicly discussed
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SUMMARY

Sleep deprivation leads to increased subsequent sleep length and depth and to deficits in cognitive performance in humans. In animals extreme sleep deprivation is eventually fatal. The cellular and molecular mechanisms causing the symptoms of sleep deprivation are unclear. This thesis was inspired by the hypothesis that during wakefulness brain energy stores would be depleted, and they would be replenished during sleep. The aim of this thesis was to elucidate the energy metabolic processes taking place in the brain during sleep deprivation. Endogenous brain energy metabolite levels were assessed *in vivo* in rats and in humans in four separate studies (Studies I-IV).

In the first part (Study I) the effects of local energy depletion on brain energy metabolism and sleep were studied in rats with the use of *in vivo* microdialysis combined with high performance liquid chromatography. Energy depletion induced by 2,4-dinitrophenol infusion into the basal forebrain was comparable to the effects of sleep deprivation: both increased extracellular concentrations of adenosine, lactate, and pyruvate, and elevated subsequent sleep. This result supports the hypothesis of a connection between brain energy metabolism and sleep.

The second part involved healthy human subjects (Studies II-IV). Study II aimed to assess the feasibility of applying proton magnetic resonance spectroscopy (^{1}H MRS) to study brain lactate levels during cognitive stimulation. Cognitive stimulation induced an increase in lactate levels in the left inferior frontal gyrus, showing that metabolic imaging of neuronal activity related to cognition is possible with ^{1}H MRS.

Study III examined the effects of sleep deprivation and aging on the brain lactate response to cognitive stimulation. No physiologic, cognitive stimulation-induced lactate response appeared in the sleep-deprived and in the aging subjects, which can be interpreted as a sign of malfunctioning of brain energy metabolism. This malfunctioning may contribute to the functional impairment of the frontal cortex both during aging and sleep deprivation.

Finally (Study IV), ^{1}H MRS major metabolite levels in the occipital cortex were assessed during sleep deprivation and during photic stimulation. N-acetyl-aspartate (NAA/H_2O) decreased during sleep deprivation, supporting the hypothesis of sleep deprivation-induced disturbance in brain energy metabolism. Choline containing compounds (Cho/H_2O) decreased during sleep deprivation and recovered to alert levels during photic stimulation, pointing towards changes in membrane metabolism, and giving support to earlier observations of altered brain response to stimulation during sleep deprivation.

Based on these findings, it can be concluded that sleep deprivation alters brain energy metabolism. However, the effects of sleep deprivation on brain energy metabolism may vary from one brain area to another. Although an effect of sleep deprivation might not in all cases be detectable in the non-stimulated baseline state, a challenge imposed by cognitive or photic stimulation can reveal significant changes. It can be hypothesized that brain energy metabolism during sleep deprivation is more vulnerable than in the alert state. Changes in brain energy metabolism may participate in the homeostatic regulation of sleep and contribute to the deficits in cognitive performance during sleep deprivation.

TIIVISTELMÄ

Valvotus lisää korvausunen määrää ja johtaa ihmisillä kognitiivisen suorituskyvyn heikkenemiseen. Eläimillä on havaittu äärimmilleen pitkitetyn valvotuksen johtavan lopulta kuolemaan. Unen puutteen aiheuttamien oireiden taustalla olevat solu- ja molekyylitason mekanismit tunnetaan puutteellisesti. Tämä väitöskirja on saanut innoituksensa hypoteesista, jonka mukaan aivojen energiavarastot ehtyisivät valveen ja palautuisivat ennalleen unen aikana. Työssä tutkittiin aivojen energia-aineenvaihdunnan muutoksia valvotuksen aikana. Neljässä osatyössä (I-IV) mitattiin rotilla ja ihmisillä *in vivo* aivojen sisäsyntyisiä energia-aineenvaihduntatuotteita.

Ensimmäisessä osatyössä tutkittiin *in vivo* -mikrodialyysillä ja korkean erotuskyvyn nestekromatografialla paikallisen energiavajeen vaikutuksia rottien uneen ja aivojen energia-aineenvaihduntaan. 2,4-dinitrofenoli-infuusiolla aikaansaatu kokeellinen energiavaje etuaivojen pohjaosissa oli verrattavissa unen puutteen vaikutuksiin: molemmat aiheuttivat adenosiinin, laktaatin ja pyruvaatin solunulkoisten pitoisuuksien kasvua sekä korvausunen lisääntymistä. Tulos tukee aiemmin esitettyä hypoteesia energia-aineenvaihdunnan ja unen yhteydestä.

Osatöissä II–IV tutkittiin ihmisiä. Osatyössä II arvioitiin protonispektroskopian (^{1}H MRS) soveltuvuutta terveiden ihmisaivojen laktaattipitoisuuden mittaamiseen kognitiivisen tehtävän suorittamisen aikana. Tehtävän suorittamisen todettiin nostavan aivojen laktaattipitoisuutta paikallisesti vasemmassa otsalohkossa. Tulos osoittaa, että korkeampiin aivotoimintoihin liittyvän energia-aineenvaihdunnan kuvantaminen ^{1}H MRS:lla on mahdollista mittaamalla ns. laktaattivastetta.

Osatyössä III tutkittiin unen puutteen ja ikääntymisen vaikutusta kognitiivisen tehtävän suorittamisen aikaansaamaan aivojen laktaattivasteeseen. Fysiologinen laktaattivaste kognitiivisen tehtävän suorittamiselle ei tullut esiin ikääntyvillä eikä valvotetuilla koehenkilöillä. Voidaan tulkita, että laktaattivasteen puuttuminen johtuu normaalin energia-aineenvaihdunnan häiriintymisestä. Pitkittyneen valveen sekä ikääntymisen aikana havaitut otsalohkon toiminnan häiriöt saattavat osin selittyä tämän havainnon pohjalta.

Viimeisessä osatyössä (IV) tutkittiin aivojen aineenvaihduntatuotteiden muutoksia näköaivokuorella unen puutteen ja näköärsytyksen aikana. N-asetyyliaspartaatti (NAA/H_2O) laski unen puutteen aikana, mikä tukee hypoteesia unen puutteen aikaisesta aivojen energia-aineenvaihdunnan häiriöstä. Myös koliiniyhdisteiden (Cho/H_2O) määrä laski unen puutteen aikana mutta palautui lähtötasolle näköärsytyksen myötä. Havainto viittaa solukalvojen aineenvaihdunnan muutoksiin ja tukee aiempia havaintoja aivojen ärsytysvasteen muuttumisesta valvotuksen aikana.

Tulosten perusteella voidaan päätellä, että valvominen muuttaa aivojen energia-aineenvaihduntaa. Unen puutteen vaikutus aivojen energia-aineenvaihduntaan voi kuitenkin vaihdella eri aivoalueiden välillä. Vaikka muutos ei aina tulekaan ilmi lepotilassa, se voi ilmetä kognitiivisen tehtävän suorittamisen tai näköärsytyksen aikana. Voidaan olettaa, että aivojen energia-aineenvaihdunta on unen puutteen aikana haavoittuvaisempi kuin virkeänä. Aivojen energia-aineenvaihdunnan muutokset saattavat osallistua unen homeostaattiseen säätelyyn sekä vaikuttaa kognitiivisen suorituskyvyn heikkenemiseen unen puutteen aikana.

ABBREVIATIONS

Ac-CoA	Acetyl-coenzyme A
aCSF	artificial cerebrospinal fluid
ADP	adenosine diphoshate
AMP	adenosine monophosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BF	basal forebrain
BL	baseline
BOLD	blood oxygenation level-dependent
cAMP	cyclic adenosine monophosphate
CHESS	chemical shift selective
Cho	choline-containing compounds
Cr	creatine
CSF	cerebrospinal fluid
CMRglc	cerebral metabolic rate of glucose
CNS	central nervous system
DNP	2,4-dinitrophenol
EEG	electroencephalography
EMG	electromyography
EOG	electro-oculography
$FADH_2$	flavin adenine dinucleotide (reduced form)
fMRI	functional magnetic resonance imaging
GABA	gamma-aminobutyric acid
^{1}H MRS	proton magnetic resonance spectroscopy
HAROLD	hemispheric asymmetry reduction in older adults
HPLC	high performance liquid chromatography
KCN	potassium cyanide
LDH	lactate dehydrogenase
MCT	monocarboxylate transporter
MR	magnetic resonance
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
NA	N-acetyl containing compounds
NAA	N-acetyl aspartate
NAAG	N-acetylaspartyl glutamate
NADH	nicotinamide adenine dinucleotide (reduced form)
NMR	nuclear magnetic resonance
non-BF	non-basal forebrain
non-REM	non-rapid-eye-movement
^{31}P	phosphorus
PCr	phosphocreatine
PET	positron emission tomography
P_i	inorganic phosphate
PRESS	point-resolved spectroscopy
PS	photic stimulation
PSG	polysomnography
PVT	psychomotor vigilance task

REM	rapid-eye-movement
RF	radio frequency
S1-S4	sleep stage 1-4
SNR	signal-to-noise ratio
STEAM	stimulated echo-acquisition mode
SWG	silent word-generation
SWS	slow-wave sleep
TCA	tricarboxylic acid
tCr	total creatine
TE	echo time
TR	repetition time
UV	ultraviolet
VLPO	ventrolateral preoptic area
VOI	volume of interest

ORIGINAL PUBLICATIONS

The thesis is based on the following publications referred to in the text by their Roman numerals:

I. Kalinchuk AV, Urrila AS, Alanko L, Heiskanen S, Wigren H-K, Suomela M, Stenberg D, Porkka-Heiskanen T (2003) **Local energy depletion in the basal forebrain increases sleep.** *European Journal of Neuroscience. 17(4):863-9.*

II. Urrila AS, Hakkarainen A, Heikkinen S, Vuori K, Stenberg D, Häkkinen A-M, Lundbom N, Porkka-Heiskanen T (2003) **Metabolic imaging of human cognition: an fMRI/^{1}H-MRS study of brain lactate response to silent word generation.** *Journal of Cerebral Blood Flow & Metabolism. 23(8):942-8.*

III. Urrila AS, Hakkarainen A, Heikkinen S, Vuori K, Stenberg D, Häkkinen A-M, Lundbom N, Porkka-Heiskanen T (2004) **Stimulus-induced brain lactate: effects of aging and prolonged wakefulness.** *Journal of Sleep Research. 13(2):111-9.*

IV. Urrila AS, Hakkarainen A, Heikkinen S, Huhdankoski O, Kuusi T, Stenberg D, Häkkinen A-M, Porkka-Heiskanen T, Lundbom N (2006) **Preliminary findings of proton magnetic resonance spectroscopy in occipital cortex during sleep deprivation.** *Psychiatry Research – Neuroimaging. 147(1):41-6.*

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1. INTRODUCTION

Humans spend asleep one-third, and rats over half their lives. Sleep is widely spread in the animal kingdom and has been well preserved in the course of evolution, regardless of the apparent danger of recurrent periods of reduced awareness. Despite the fact that sleep seems to be vitally important, the physiological function of sleep remains unknown.

A commonsense explanation for the function of sleep is restoration: we sleep in order to rest and to recover, and to prepare to be awake again. It is unclear, however, what exactly is restored by sleep. A multitude of theories have been proposed concerning such processes as thermoregulation, immune function, and also learning and memory. It has also been suggested that sleep is needed to restore brain energy reserves, which are depleted during wakefulness.

Sleep deprivation, the removal of sleep from an organism, is a major methodological approach in studies aiming at understanding the function or the functions of sleep. What happens when an animal or a human is deprived of sleep? Sleep pressure builds up, and wake-time behavior and health are affected adversely. In rats, chronic sleep deprivation is eventually fatal, whereas in humans, sleep deprivation of shorter duration affects mood and leads to impaired cognitive performance. The biochemical events underlying the symptoms of sleep deprivation remain unclear.

Sleep deprivation is not merely a tool to study the function of sleep. It has also become a common phenomenon affecting the life and work of millions of people in modern society, where people tend to work prolonged hours and engage in late-night web-surfing and social programs at the expense of a good nights' sleep.

The aim of this thesis was to elucidate in further detail the energy metabolic processes taking place in the brain during sleep deprivation. A deeper knowledge of the molecular and cellular mechanisms behind the symptoms of sleep deprivation is necessary for better evaluation of the potentially harmful effects of sleep deprivation on health – and also for understanding of why we actually sleep.

2. REVIEW OF THE LITERATURE

2.1 Sleep

2.1.1 Normal human sleep

Normal human sleep consists of two main states: rapid-eye-movement (REM) sleep, and non-rapid-eye-movement (non-REM) sleep. Non-REM sleep can be further divided into light (S1-S2) and deep (S3-S4) stages. Each sleep stage can be recognized by its distinct characteristics in polysomnography (PSG): electroencephalography (EEG), electro-oculography (EOG), and electromyography (EMG) recordings. S3 and S4 together are called slow-wave sleep (SWS) or delta sleep, based on the presence of high-amplitude, slow (2 Hz or slower) delta waves. During the night, the non-REM and REM sleep phases alternate cyclically: Sleep normally begins with S1, progresses through S2 and S3 to S4, and finally to REM sleep. This cycle is repeated every 70 to 110 mins, four to six times a night. SWS predominates during the first third of the night, whereas REM sleep predominates during the last third (Rechtschaffen and Kales, 1968; Turek and Zee, 1999).

2.1.2 Normal rat sleep

In rats, like in humans, sleep is divided into non-REM and REM sleep, but rat non-REM sleep is usually not subdivided into stages S1-S4, and the rat sleep-cycle length is only ~10 to 12 mins. Rats spend asleep ~50 to 65% of their time, and their sleep occurs primarily during the light period of the day (80% of the day and 20% of the night is spent asleep). Sleep does not occur in a single consolidated phase, but is interrupted by activity bouts (polyphasic sleep) (Tobler, 1995). Similarly as in humans, slow-wave activity is highest shortly after sleep onset and then in the course of the main sleep period declines progressively.

2.1.3 Regulation of sleep

According to the current and widely accepted model of sleep regulation, sleep is controlled by two separate components: a circadian component C and a homeostatic component S (Borbely, 1982). Process C affects the appropriate timing of sleep, and is mainly controlled by the genetically driven rhythmic activity of the suprachiasmatic nucleus in the hypothalamus. Process S accounts for a sufficient amount of sleep: it accumulates during wakefulness and declines during sleep. It is controlled by several interacting neural systems in the hypothalamus, basal forebrain (BF) and brainstem nuclei (Borbely, 1982; Pace-Schott and Hobson, 2002; Espana and Scammell, 2004).

A number of different neurotransmitters participate in the regulation of sleep. The wake-promoting system, which shows high activity levels during wakefulness, consists of neuronal networks producing acetylcholine, amines, and orexins. The sleep-promoting neurons located in the anterior hypothalamus ventrolateral preoptic area (VLPO) contain the inhibitory transmitters gamma-aminobutyric acid (GABA) and galanin, and these produce sleep by inhibiting the wake-promoting brain regions. REM sleep is controlled mainly by an interaction of cholinergic and aminergic brainstem neurons (Hobson *et al.*, 1975; Espana and Scammell, 2004; Jones, 2005).

The complexity of sleep regulation is further increased by the actions of somnogens, sleep-promoting endogenous substances that accumulate in the brain during wakefulness. The putative somnogens include at least several inflammatory factors and adenosine (Espana and Scammell, 2004; Taber and Hurley, 2006). Adenosine is a neuromodulator which is ubiquitously present in the central nervous system (CNS) and can inhibit both excitatory and inhibitory neurons (Porkka-Heiskanen *et al.*, 2002). Adenosine and its analogs induce sleep, whereas the adenosine antagonist caffeine is a powerful and widely consumed stimulant (Fredholm *et al.*, 1999).

2.2 Sleep deprivation

2.2.1 Effects on subsequent sleep

Sleep deprivation affects the homeostatic component S of sleep regulation. It impairs the ability to maintain consolidated waking (Bonnet and Arand, 1999), and is followed by a rebound increase in sleep. The increases in SWS and delta power during recovery sleep correlate with the duration of the preceding sleep-deprivation period (Franken *et al.*, 1991; Turek and Zee, 1999). SWS is thus thought to represent the vitally important component of sleep, which has to be primarily replenished after sleep deprivation.

2.2.2 Effects on wake-time behavior

Sleep deprivation has marked effects also on wake-time behavior and health. In rats, sleep deprivation impairs temperature control, dietary metabolism, and immune function. Extreme sleep deprivation of 2 to 3 weeks leads ultimately to death (Rechtschaffen and Bergmann, 2002).

In humans, a large number of studies performed since the end of the 19th century show sleep deprivation of shorter duration to have a negative impact on mood, subjective alertness, and performance in a wide variety of tasks (Patrick and Gilbert, 1896; Pilcher and Huffcutt, 1996; Jones and Harrison, 2001; Van Dongen *et al.*, 2003).

During sleep deprivation, cognitive performance becomes unstable (Doran *et al.*, 2001), reaction times are prolonged (Adam *et al.*, 2006), and the number of produced errors is increased (Smith *et al.*, 2002). Recent evidence suggests substantial inter-individual differences in this performance decline (Leproult *et al.*, 2003; Van Dongen *et al.*, 2004; Van Dongen *et al.*, 2005). Usually, however, 24 h of sleep deprivation is long enough to result in decreased performance in both long and simple as well as short and complex cognitive tasks (Pilcher and Huffcutt, 1996; Harrison and Horne, 1998; Thomas *et al.*, 2000; Van Dongen *et al.*, 2003).

Sleep deprivation affects cognitive performance task-specifically. Tasks that require attention and reaction speed (like simple reaction time or psychomotor vigilance tasks) have been observed to be very sensitive to sleep deprivation (Gillberg and Åkerstedt, 1998; Van Dongen *et al.*, 2003; Loh *et al.*, 2004). These tasks are often simple, repeatable, and easy to apply, the learning curve is short, and the tasks can be practiced before the start of sleep deprivation. These tasks may, however, become monotonous and dull when repeatedly performed during

the sleep-deprivation period. Thus, part of the performance decrement observed during sleep deprivation probably arises from undesired motivation effects.

At the same time, it has been proposed that tasks requiring more "creative" thinking or executive functions will be particularly susceptible to the effects of sleep deprivation (Horne, 1988; Harrison and Horne, 1998; Harrison *et al.*, 2000; Muzur *et al.*, 2002; Nilsson *et al.*, 2005). These more complex tasks include verbal learning and fluency (Horne, 1988; Harrison and Horne, 1997, 1998; Drummond *et al.*, 2000) and arithmetic tasks (Drummond *et al.*, 1999; Thomas *et al.*, 2003). Their performance relies mainly on the activation of the prefrontal cortex, shown by imaging methods to be vulnerable to the effects of sleep deprivation (Drummond *et al.*, 1999; Thomas *et al.*, 2000, 2003). The prefrontal cortex-oriented tasks are often short, stimulating, and relatively difficult, and normally lead neither to boredom nor to loss of motivation. However, the analysis procedures involved may be relatively burdensome, and the tasks cannot be practiced beforehand or repeated in the course of the sleep-deprivation period without a considerable learning effect. These task-specific limitations need to be taken into account in planning of cognitive tests and in interpreting cognitive performance in sleep-deprivation studies.

Despite intensive research, the exact cellular and molecular mechanisms causing the rebound increase in sleep and the symptoms of sleep deprivation are as yet unclear. In addition, knowledge as to the effects of sleep deprivation on female subjects remains scarce, since the effects of sleep deprivation have been mainly studied in men (Dzaja *et al.*, 2005).

2.3 Brain energy metabolism

2.3.1 Introduction

The brain accounts for about 2% of an individual's body weight, but in rest it accounts for approximately one-fifth of total body oxygen utilization (Clarke and Sokoloff, 1994). The majority of the energy used by the brain is required for physiological functioning, including neurotransmission, intracellular messaging, and the maintenance of ionic gradients across plasma membranes (Ames, 2000). The regional metabolic needs of the brain are highly variable, and may increase many-fold during local functional activity (Clarke and Sokoloff, 1994; Ames, 2000).

Under normal conditions, the brain derives its energy almost entirely from oxidation of glucose: The principal energy substrates for the brain are glucose and O_2, and the principal end products are CO_2 and H_2O (Ames, 2000). Glucose is either taken up directly from the blood, or is derived from brain glycogen, which is located mainly in astrocytes and forms the brain's only significant energy reserve (Clarke and Sokoloff, 1994; Benington and Heller, 1995).

The free energy liberated in the combustion of glucose is trapped as adenosine triphosphate (ATP), which functions as the main source of chemical energy in cells. Additionally, when ATP generation is separated from consumption, creatine kinase and adenylate kinase can act to buffer the ATP/adenosine diphosphate (ADP) ratio and to facilitate the transfer of the P_i (inorganic phosphate) (Ames, 2000).

Glucose combustion combined with ATP production occurs in two phases: glycolysis and oxidative phosphorylation (Fig. 1). Glycolysis occurs in the cytosol, and produces 2 mol of ATP per mol of glucose. In glycolysis, glucose is converted to pyruvate in a sequence of reactions, with hexokinase, phosphofructokinase, and pyruvate kinase as the main regulatory enzymes. The resulting pyruvate can either be converted by lactate dehydrogenase (LDH) to lactate, or by pyruvate dehydrogenase to acetyl coenzyme A (Ac-CoA), which then enters the tricarboxylic acid cycle (TCA cycle; also: Krebs cycle, citric acid cycle) in mitochondria. The TCA cycle generates the reducing equivalents nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) that are utilized to produce ATP in the electron transport-oxidative phosphorylation sequence. In addition, the TCA cycle functions as a source of various amino acids such as glutamate and aspartate (Devlin, 1997).

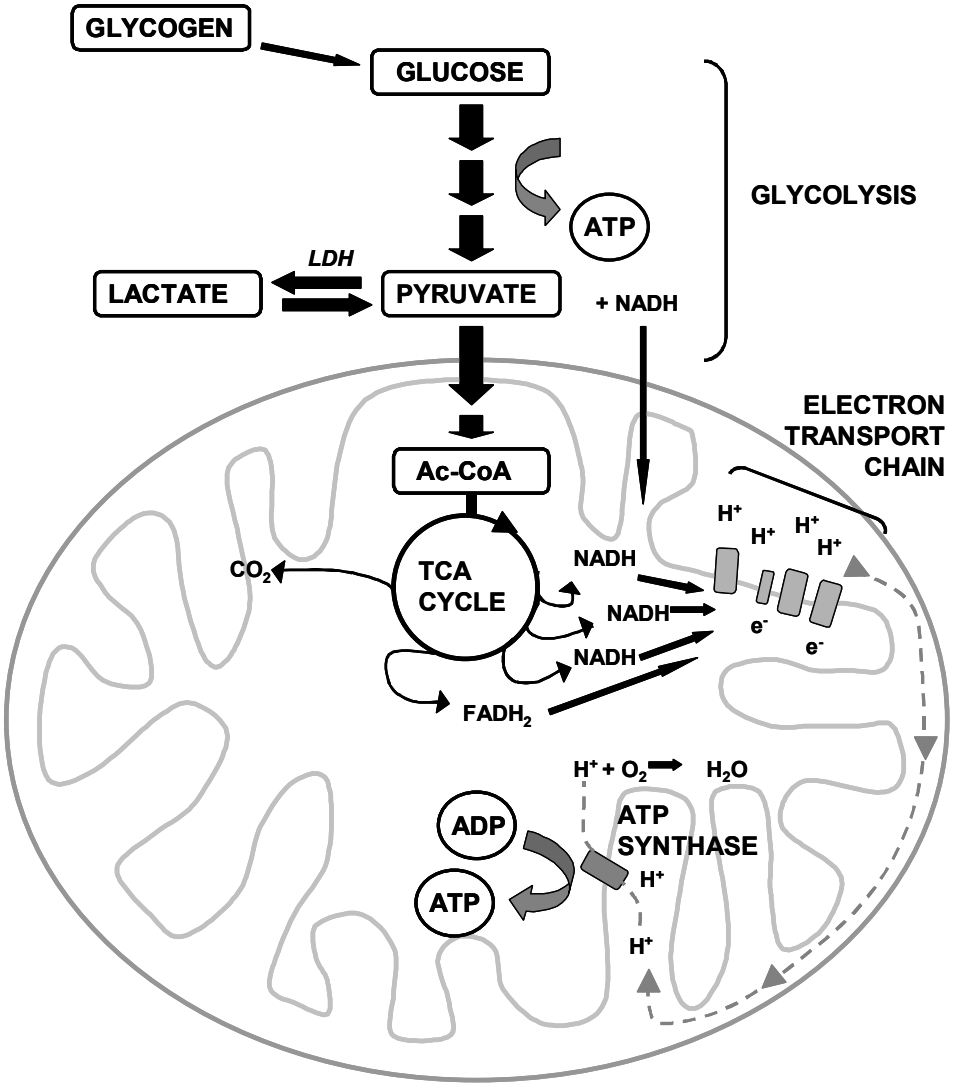

Figure 1. Glucose combustion and ATP production inside the cell.
Glycolysis occurs in the cytosol and leads to pyruvate or lactate production. The tricarboxylic acid (TCA) cycle, electron transport, and oxidative phoshorylation occur in mitochondria. Stoichiometric relations are not shown. LDH = lactate dehydrogenase; ATP = adenosine triphosphate; Ac-CoA = acetyl-coenzyme A; NADH = nicotinamide adenine dinucleotide; $FADH_2$ = flavin adenine dinucleotide.

The electron transfer chain and oxidative phosphorylation together yield 36 mol of ATP per mol of glucose. They convert NADH and $FADH_2$ into utilizable energy in the presence of sufficient oxygen (Erecinska and Silver, 1989). The electron transport chain consists of sequentially arranged electron carriers in the mitochondrial inner membrane. During oxidation-reduction reactions, protons are pumped from the mitochondrial matrix into the intramembrane space to establish an electrochemical gradient across the mitochondrial membrane. This gradient is converted into chemical energy (ATP) by ATP synthase, through which the protons flow back into the mitochondrial matrix.

2.3.2 Lactate in brain energy metabolism: two theories

The conventional assumption is that the brain would generate virtually all of its ATP from the direct oxidation of glucose, and that lactate would be a metabolic end-product generated from pyruvate only in pathological conditions (Najm *et al.*, 1998) or when oxygen availability for oxidative phosphorylation is insufficient.

Recently, a more beneficial and active role for lactate in brain energy metabolism has been proposed. This new theory, the astrocyte-neuron lactate shuttle hypothesis, was initially proposed by Pellerin and Magistretti in 1994, and has later been redefined according to more recent evidence (Pellerin and Magistretti, 2003, 2004). The hypothesis has raised an intensive debate during the past two decades (Pellerin and Magistretti, 1994; Chih *et al.*, 2001; Hertz, 2004; Schurr, 2006), with no consensus reached on the matter thus far.

According to the astrocyte-neuron lactate shuttle hypothesis, a substantial portion of the brain's energy comes from the conversion of glucose to lactate in astrocytes, followed by the oxidation of lactate in neurons (Fig. 2); this production of lactate is triggered by the uptake of glutamate into astrocytes during increased neuronal activity (Pellerin and Magistretti, 1994),

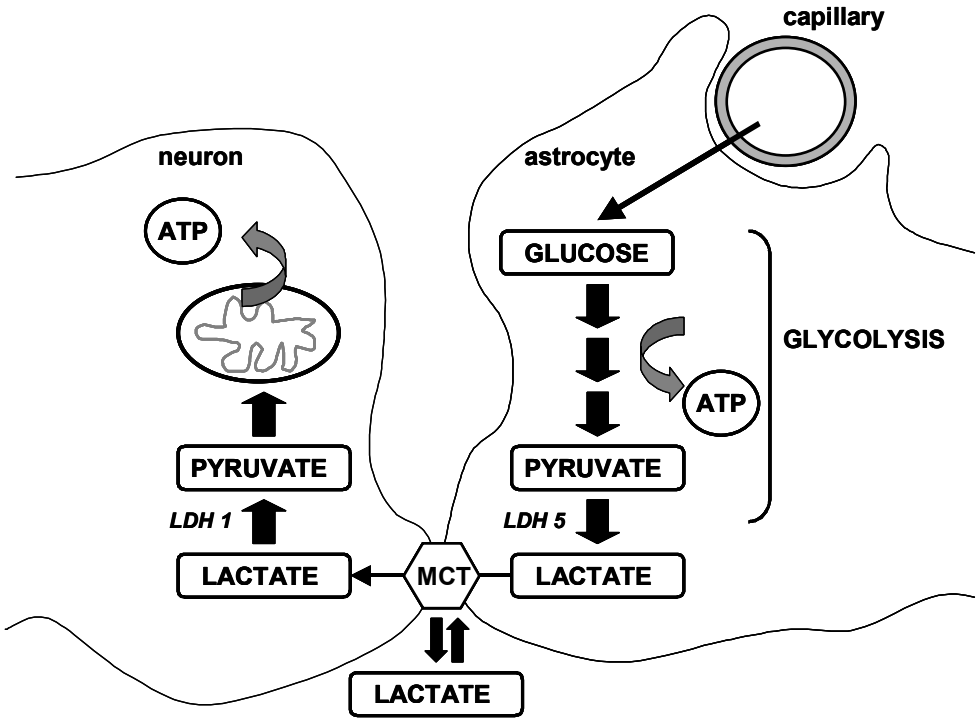

Figure 2. A simplified model of the astrocyte-neuron lactate shuttle hypothesis. LDH = lactate dehydrogenase; MCT = monocarboxylate transporter.

with the total amount of produced ATP in these reactions being the same as when glucose is oxidized more directly. This hypothesis has been supported by a number of *in vitro* and *in vivo* studies, the main evidence for which is outlined below.

The formulation of the astrocyte-neuron lactate shuttle hypothesis was initially based on the observation that glutamate uptake into cultured astrocytes stimulates glucose utilization and lactate release by a mechanism involving activation of the Na+/K+-ATPase (Pellerin and Magistretti, 1994). According to this model, one glutamate is taken up from the synaptic cleft with three Na+ ions, whereas one glucose is consumed through glycolysis in astrocytes, producing two ATPs. One of these ATPs is used for the extrusion of the three Na+ ions by the Na+/K+-ATPase, the other ATP for the synthesis of glutamine from glutamate by glutamine synthase. Together with the two ATPs, glycolysis also produces two lactate molecules (Magistretti and Pellerin, 1999a), which are transported to neurons for oxidation. A 1:1 stoichiometry between glutamate cycling and glucose utilization has been observed (Sibson *et al.*, 1998) and interpreted, although disputably (Chih *et al.*, 2001), as supporting the tight coupling between glutamate-mediated synaptic activity and glucose utilization (Magistretti and Pellerin, 1999a).

In the mid-1980s, before the formulation of the astrocyte-neuron lactate shuttle hypothesis, the conventional view of brain energy metabolism had already attracted controversy based on findings from imaging studies. With positron emission tomography (PET), Fox and colleagues (1988) observed that glucose use and blood flow (Fox and Raichle, 1986) increased in excess of oxygen consumption during stimulation. A later discovery was that increases in blood oxygenation (the blood oxygenation level-dependent BOLD signal) could be detected with functional magnetic resonance imaging (fMRI), only because cerebral blood flow increases beyond oxygen-consumption needs during neuronal activity (Ogawa *et al.*, 1990). Together, these important results pointed towards the occurrence of activation-induced glycolytic processing of glucose.

Further, before the formulation of this hypothesis, proton magnetic resonance spectroscopy (^{1}H MRS) showed that visual stimulation in humans induced transient lactate production during neuronal activation (Prichard *et al.*, 1991; Sappey-Marinier *et al.*, 1992). In successive human studies, such activity-induced increase in lactate was confirmed by use of various types of stimuli in successive human studies (Kuwabara *et al.*, 1995; Frahm *et al.*, 1996; Richards *et al.*, 1997), although some discrepant findings have also been reported (Merboldt *et al.*, 1992; Boucard *et al.*, 2005). In animals, several techniques have revealed an activity-related increase in brain lactate (Richter and Dawson, 1948; Ueki *et al.*, 1988; Kuhr and Korf, 1988; Hu and Wilson, 1997; Madsen *et al.*, 1999), although in anesthetized cats no increase was observed (Kauppinen *et al.*, 1997).

Lactate added to *in vitro* preparations appears to be consumed preferentially to glucose, particularly during periods of intense neuronal activity (Schurr *et al.*, 1988; Bouzier-Sore *et al.*, 2003). The hypothesis of a regulated flux of lactate from astrocytes to neurons has been supported by the observation of a differential distribution of LDH isoforms between astrocytes and neurons. LDH-1, the form better suited to oxidize lactate to pyruvate, is the predominant isoform in neurons, but astrocytes also contain LDH-5, which preferentially converts pyruvate to lactate (Bittar *et al.*, 1996). The monocarboxylate transporters (MCTs), responsible for lactate transport across cell membranes, also differ in their distribution between neurons and glia. MCT2 (high-affinity, Km ~0.7mM) is predominantly expressed in neurons, whereas MCT1 and MCT4 (low-affinity, Km ~3-5mM) are found in astrocytes

(Hertz and Dienel, 2005). The functional roles of the different MCTs remain, however, to be discovered.

In short, lactate appears to be produced at least to some extent in the brain *in vivo*, and the cells responsible for lactate production are probably astrocytes. The brain lactate production (by astrocytes?) and oxidation (by neurons?) seem, however, to be temporally dissociated phenomena, as suggested by the mismatch between the use of glucose and oxygen consumption during brain activation (Fox and Raichle, 1986; Fox *et al.*, 1988).

The astrocyte-neuron shuttle hypothesis mainly concerns lactate formed within the brain parenchyma. However, peripheral lactate has been observed to penetrate the blood-brain barrier to a considerable extent during both hypo- (Thurston *et al.*, 1983; Maran *et al.*, 1994) and euglycemia (Smith *et al.*, 2003). This is in contrast with earlier observations (Pardridge and Oldendorf, 1977) and further emphasizes the need to re-evaluate the role of lactate in brain energy metabolism.

2.3.3 Brain energy metabolism during sleep deprivation

Recent human studies performed mainly with PET techniques have yielded quite consistent results on global brain glucose utilization, oxygen consumption, and cerebral blood flow during the normal sleep-wake cycle: the levels are high during wakefulness and REM sleep, and low during SWS (Buchsbaum *et al.*, 1989; Madsen *et al.*, 1991; Maquet *et al.*, 1990; Maquet, 2000; Nofzinger, 2005). The decline in brain glucose metabolism during sleep is a significant determinant of the falling rates of systemic glucose utilization during sleep (Boyle *et al.*, 1994). In animals, the levels of brain glucose (Netchiporouk *et al.*, 2001), creatine phosphate, and ATP (Reich *et al.*, 1972) are lower, whereas those of lactate (Richter and Dawson, 1948; Reich *et al.*, 1972; Shram *et al.*, 2002) and adenosine (Porkka-Heiskanen *et al.*, 1997) are higher during normal wakefulness than during sleep.

During sleep deprivation, similar changes indicating further diminished brain energy stores occur in animals (Van den Noort and Brine, 1970). Mitochondrial gene expression is also altered (Cirelli and Tononi, 1998), and it has been proposed that sleep deprivation should lead to oxidative stress (Gopalakrishnan *et al.*, 2004; Ikeda *et al.*, 2005). Furthermore, changes in brain glycogen levels as well as accumulation of adenosine occur in the course of sleep deprivation, which will be discussed below in more detail.

The replenishment of brain glycogen stores was proposed as the function of sleep by Benington and Heller in 1995, a hypothesis tested in subsequent studies with controversial results. On the one hand, brain glycogen stores are depleted (Kong *et al.*, 2002), and the expression of genes related to glycogen metabolism is changed during sleep deprivation (Petit *et al.*, 2002). On the other hand, glycogen depletion occurs site-specifically only in the cerebellum, not the cortex (Gip *et al.*, 2002), and depends also on strain of rat, some strains failing to show a depletion (Franken *et al.*, 2003). Further, rest deprivation in *Drosophila melanogaster* has elicited a biphasic glycogen response: initial decline followed by later recovery (Zimmerman *et al.*, 2004).

Benington and Heller also hypothesized that adenosine acts as a feedback signal whereby depletion of cerebral glycogen stores results in increased sleep. Adenosine is a by-product of energy metabolism: it is produced from ATP degradation both intra- and extracellularly (Fig. 3), and its levels increase when energy availability and need do not match. Adenosine levels

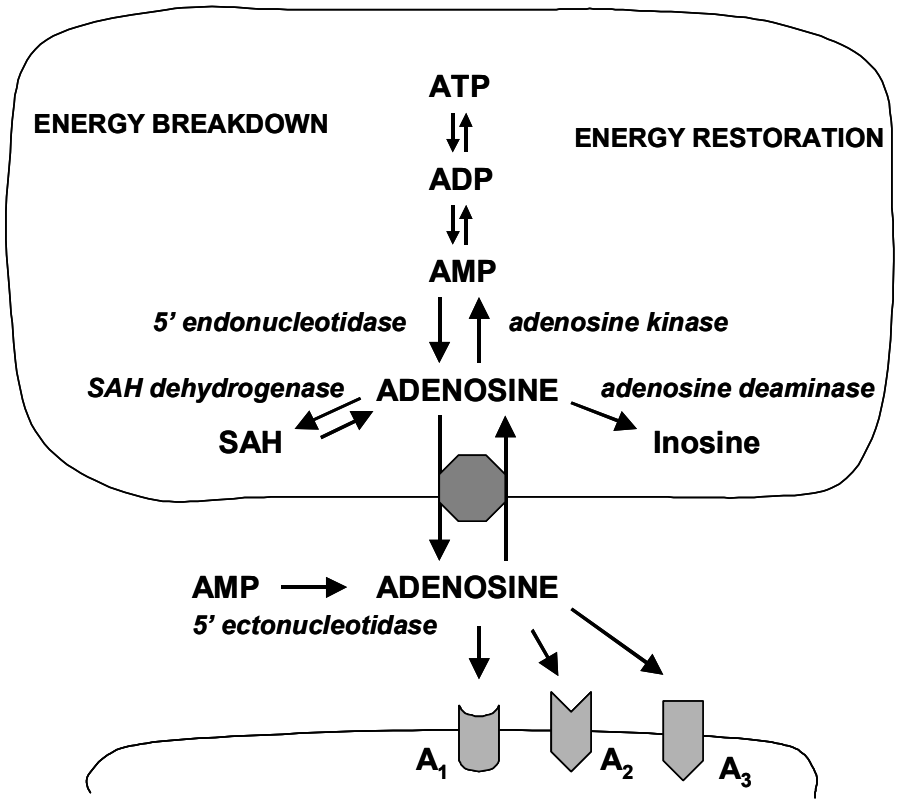

Figure 3. A simplified scheme of the main intra- and extracellular metabolic pathways of adenosine. Adenosine is formed from ATP intracellularly by the enzymes ATP-ase, ADP-ase, and 5'-endonucleotidase and in the extracellular space by their respective ectoenzymes. Adenosine is degraded by adenosine kinase to adenosine monophosphate (AMP), and by adenosine deaminase to inosine, and to a lesser extent by S-adenosylhomocysteine hydrolase to S-adenosylhomocysteine (SAH). Adenosine is transported through the cell membrane by nucleoside transporters, and its effects are mediated through specific receptors A_1, A_{2A}, A_{2B} and A_3.

are higher during active periods and wakefulness than during rest and sleep (Huston *et al.*, 1996; Porkka-Heiskanen *et al.*, 1997), and adenosine concentrations rise progressively with each hour of sleep deprivation and slowly decline during recovery sleep (Porkka-Heiskanen *et al.*, 1997; Porkka-Heiskanen *et al.*, 2000). The accumulation of adenosine during sleep deprivation is, however, a site-specific phenomenon encountered only in the basal forebrain (BF) and to lesser extent in the cortex (Porkka-Heiskanen *et al.*, 2000; Zeitzer *et al.*, 2006). In the BF, adenosine may exert its somnogenic actions by acting through A_1 receptors and by reducing the activity of the wake-active neurons which send widespread projections to the cerebral cortex and limbic structures and are important in creating cortical arousal (Basheer *et al.*, 2004). The adenosine A_{2A} receptors are involved in the regulation of sleep outside the BF: infusion of A_{2A} agonists into the subarachnoid space under the rostral basal forebrain induces sleep (Satoh *et al.*, 1999), and adenosine disinhibits the sleep-active neurons in the VLPO (Basheer *et al.*, 2004; Morairty *et al.*, 2004).

Studies on human brain energy metabolism during sleep deprivation are less numerous. In two PET studies, both global and regional decreases in the cerebral metabolic rate of glucose (CMRglc) occurred. The earlier study of these reported a ~6% decline in global CMRglc during sleep deprivation, although this decrease was not statistically significant (Wu *et al.*, 1991). Significant regional decreases occurred in the thalamus, basal ganglia, temporal lobes, and cerebellum, with an increase seen in the visual cortex (Wu *et al.*, 1991). More recently,

significant decreases have been reported also in global CMRglc: a decrease of ~8% after 24 h (Thomas *et al.*, 2000), and 6% after both 48 and 72 h of sleep deprivation, when compared to the alert state (Thomas *et al.*, 2003). The most prominent regional decreases in CMRglc occurred in the fronto-parietal cortices and the thalamus. As the decreases during sleep deprivation and during normal sleep (Nofzinger *et al.*, 2002) occur in roughly the same brain areas, those noted during sleep deprivation have been interpreted as representing the brain's involuntary progression toward sleep onset (Thomas *et al.*, 2003).

Despite the detectable changes in brain energy metabolism with PET, phosphorus (^{31}P) magnetic resonance spectroscopy (MRS) studies in humans have failed to show any significant changes in high-energy phosphate metabolism during sleep deprivation (Murashita *et al.*, 1999b; Dorsey *et al.*, 2003).

When cerebral metabolic responses to stimulation during sleep deprivation were studied with fMRI in humans, verbal learning (Drummond *et al.*, 2000) and divided-attention (Drummond *et al.*, 2001) tasks elicited increased activation (BOLD responses) in the bilateral prefrontal cortex and parietal lobes. In contrast, in these areas arithmetic task performance showed decreased activation (Drummond *et al.*, 1999), pointing towards task-specific changes in activation patterns during sleep deprivation.

Several findings point towards a decrease in brain energy availability during sleep deprivation, which can result in an inability to provide working neurons with sufficient energy. Yet, the number of studies is still limited and their findings not entirely consistent. Further studies and the application of new methods are needed to clarify the brain energy metabolic processes during sleep deprivation in more detail.

2.3.4 Brain energy metabolism during aging

The effects of aging on resting overall brain energy metabolism have been best characterized in human beings. During aging, either no change (de Leon *et al.*, 1987; Ernst *et al.*, 1998) or a decrease (Leenders *et al.*, 1990; Eustache *et al.*, 1995; Petit-Taboue *et al.*, 1998; Willis *et al.*, 2002) occurs in brain glucose and oxygen consumption, as well as in cerebral blood flow. These decreases vary in amount, but generally range from 10 to 30% during adult life. Regionally, the most prominent decreases in brain energy metabolism with age have been found in the cortical areas, particularly in the frontal lobes (Martin *et al.*, 1991; Salmon *et al.*, 1991). Individual variations, as well as variations caused by undiagnosed brain pathologies, may exceed those caused by the aging process itself (Finch, 1994), and partially account for the varying results. No study has, however, reported an increase in brain energy metabolism during aging, and it thus seems likely that aging has a preferably attenuating effect on resting brain energy metabolism.

Less is known about the effects of aging on brain metabolic response to stimulation. In ^{31}P MRS, occipital lobe phosphocreatine decreases in older subjects during photic stimulation, with no differences in the young (Murashita *et al.*, 1999a). Recent evidence suggests that during cognitive stimulation older subjects use both of their hemispheres in task performance, whereas in young subjects only one hemisphere is involved (hemispheric asymmetry reduction in older adults, HAROLD) (Cabeza *et al.*, 1997; Cabeza, 2002; Cabeza *et al.*, 2004).

The actual cause for the age-dependent reduction in brain energy metabolism is unclear, and can be associated with many different factors. During aging, brain volume and weight decline, accompanied by increased ventricular volume (Anderton, 2002; Scahill *et al.*, 2003). Controversy exists as to whether actual neuronal loss occurs or only a decline in neuronal volume (Anderton, 2002). The relative number of glial cells, however, seems to increase in the aging brain, although knowledge on the functional aspects of aged astrocytes is still very limited (Cotrina and Nedergaard, 2002). At the subcellular level, changes related to aging include decreased levels of neurotransmitters (Miguez *et al.*, 1999) and impaired maintenance of intracellular ATP levels (Joo *et al.*, 1999). Furthermore, calcium dysregulation (Toescu *et al.*, 2004), mitochondrial dysfunction (Melov, 2004), and the production of reactive oxygen species (Sohal and Weindruch, 1996; Lee *et al.*, 2000) have been described. In addition, in women, the declining levels of estrogen in menopause can have negative effects on brain energy metabolism (Norbury *et al.*, 2003; Rasgon *et al.*, 2005).

2.4 In vivo microdialysis

2.4.1 The principle of microdialysis

Brain microdialysis, first introduced in 1966 (Bito *et al.*, 1966), is a powerful sampling technique for neuroscience research and pharmacokinetic studies. It is based on the passive transfer of substances across a dialysis membrane according to concentration gradient. A small segment of semipermeable membrane is implanted at the tip of a probe into the target tissue (Fig. 4). When the probe is perfused slowly with a solution matching the ionic composition of the extracellular fluid, substances with a molecular weight less than the cut-off of the membrane cross the membrane to enter the dialysate. The dialysate is collected at set intervals, and, once collected, can be assayed by a wide number of chemical assays or high performance liquid chromatography (HPLC) (Benveniste, 1989; Boutelle and Fillenz, 1996).

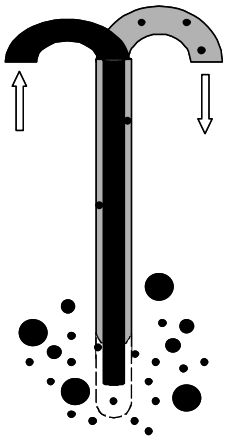

Figure 4. Schematic diagram of a microdialysis probe. The probe consists of two concentric tubes. Molecules diffuse across the membrane at the probe tip from the extracellular fluid into the fluid pumped through the probe.

Microdialysis has been successfully applied to study the effects of sleep and sleep deprivation on brain chemistry (Iwakiri *et al.*, 1993; Portas and McCarley, 1994; Williams *et al.*, 1994; Porkka-Heiskanen *et al.*, 1997; Porkka-Heiskanen *et al.*, 2000). By coupling microdialysis with EEG recording techniques, it is possible to monitor levels of chemical compounds region-specifically in animals whose behavioral state is polygraphically determined. Microdialysis can also be used for local delivery of drugs into brain tissue.

2.4.2 2,4-dinitrophenol infusion

One member of the group of uncouplers of oxidative phosphorylation is 2,4-dinitrophenol (DNP). The term "uncoupler" refers to dissociation of oxidation and phosphorylation: whereas DNP depresses high-energy phosphate bond formation, it has no effect on oxygen consumption (Heytler, 1979). DNP exerts its effects on ATP production by shortcutting the inner mitochondrial membrane and thus preventing the development of the proton gradient that drives ATP synthesis (Kariman *et al.*, 1986) (Fig. 5).

Potassium cyanide (KCN) inhibits ATP production by binding to cytochrome c oxidase (Devlin, 1997) (Fig. 5). The KCN experiments in Study I confirmed that the changes in sleep after DNP infusion were due to energy depletion as opposed to nonspecific effects of the DNP molecule.

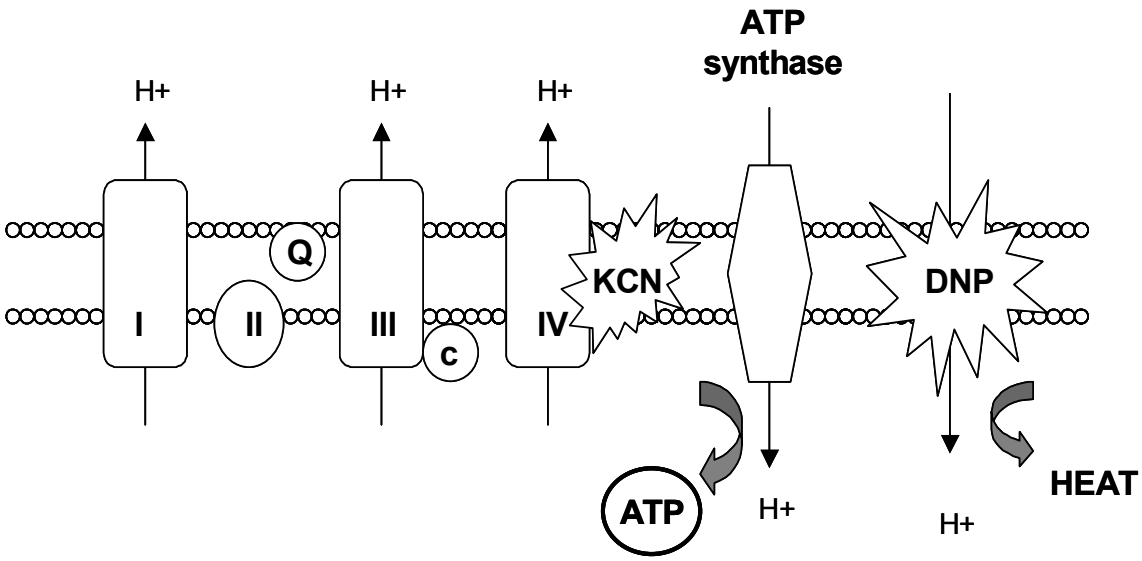

Figure 5. Mitochondrial electron transfer chain and the inhibitors of ATP production used in Study I. The four complexes of the electron transfer chain are numbered I to IV: I = NADH dehydrogenase; II = succinate dehydrogenase; III = cytochrome bc complex; IV = cytochrome oxidase. Q = coenzyme Q; c = cytochrome c. DNP = 2,4-dinitrophenol; KCN = potassium cyanide.

2.5 Magnetic resonance imaging and magnetic resonance spectroscopy

2.5.1 Introduction

Magnetic resonance imaging (MRI) and proton (^{1}H) magnetic resonance spectroscopy (MRS) are non-invasive techniques which rely on the nuclear magnetic resonance (NMR) phenomenon. Felix Bloch and Edward Mills Purcell described the NMR phenomenon independently in 1946, and shared the Nobel Prize in physics in 1952 for their discovery. NMR techniques have been used in basic research for decades prior to the development of the more current *in vivo* and clinical applications. In MRI, tissue water protons are detected to create a structural image, whereas in ^{1}H MRS, protons from molecules other than water are of interest.

Hydrogen nuclei (single protons) possess a magnetic property called the nuclear spin. Normally, the direction of the nuclear spin vectors in tissue is randomly distributed, and the sum of the spins gives a null net magnetization. When a powerful external magnetic field (B_o, strength usually 0.1-3 Tesla in human studies) is applied, the nuclear spins align with the external field and start precessing (rotating) about the axis of B_o (z-axis). Since slightly more nuclei are aligned parallel (low energy state) than antiparallel (high energy state) to B_o, the net effect is a weak magnetic vector aligned with B_o, a phenomenon called longitudinal magnetization. As the spins do not precess in phase, no net transverse magnetization occurs.

In magnetic resonance (MR) protocols, however, the equilibrium of the spins in B_o can be modified by radiofrequency (RF) pulses, during which electromagnetic energy is absorbed by the nuclei (excitation). For this excitation to occur, the radiofrequency pulse must be given at a frequency inherent to the nucleus in question (Larmor frequency). During excitation in general, longitudinal magnetization decreases, and a transverse magnetization appears as the nuclei start to precess synchronically. When the RF pulse is switched off, the system returns from the imbalanced state back to equilibrium (relaxation). The energy absorbed by the nuclei during excitation is emitted back as electromagnetic radiation and induces a voltage in the receiver coil. The signal is collected in the time domain and is Fourier transformed to yield an MR spectrum or with phase-encoded acquisitions and a 2D Fourier transformation an MR image.

The relaxation rate of the protons is characterized by two time constants, T1 and T2. After time T1, longitudinal magnetization has regained 63% of its equilibrium value (=longitudinal relaxation). The time constant T2 describes transverse relaxation, which results from dephasing of spins. After time T2, 63% of the synchronic precession (and transverse magnetization) has disappeared. T1 depends on spin-surroundings (spin-lattice), and T2 on spin-spin interactions, whereas the so-called T2* also takes into account the inhomogeneities in magnetic field which accelerate spin dephasing. Transverse relaxation is always faster than longitudinal relaxation.

2.5.2 The physiological basis and principle of functional MRI

Functional MRI (fMRI) can be used to map brain activity during task performance. The most commonly used fMRI technique is blood oxygenation level-dependent (BOLD) imaging which is based on the coupling between increased brain activity and local cerebral blood flow (Roy and Sherrington, 1890; Ogawa *et al.*, 1990). During neuronal activity, blood flow increases out of proportion to tissue oxygen extraction (Fox and Raichle, 1986), leading to a local increase in the amount of oxygenated hemoglobin on the venous side. The balance between oxygenated and deoxygenated hemoglobin serves as the source of the signal for fMRI: oxyhemoglobin is diamagnetic; deoxyhemoglobin is paramagnetic. When the amount of oxyhemoglobin is locally increased during brain activation, the paramagnetic deoxyhemoglobin alters less the magnetic field, T2* is prolonged, and the signal on the T2*-weighted image will increase.

For BOLD fMRI, data is most commonly acquired slice-wise by use of echo planar imaging (Mansfield, 1977). In the presence of a static magnetic field gradient, a frequency-selective RF pulse is applied to excite nuclear spins in a virtual slice. The slice-select gradient is then turned off, and the signals from these spins are encoded by use of rapidly switched magnetic field gradients. This is repeated for all the brain slices of interest.

In its simplest form, an fMRI experiment consists of acquiring a large series of images during two alternating states during each of which the subject maintains a specified cognitive state (block design). The voxels that show signal changes varying with the alternating brain states (task under study vs. control condition) are identified by postprocessing algorithms to yield the fMRI image.

2.5.3 The principle of ^{1}H MRS

^{1}H MRS was for the first time used to study the adult human brain *in vivo* over 20 years ago (Bottomley *et al.*, 1983), and has been applied since both in basic research and in clinical settings. It offers a unique possibility to study endogenous brain metabolites both in animals and in humans without any radioactive load. In MRS, differences in the frequencies of precession (Larmor frequencies, ω_o) serve to distinguish different chemical compounds. The Larmor frequency of a proton is proportional to the magnetic field that it experiences. It thus depends on the large externally applied field (B_o), but to a lesser extent also on the small electronic field produced by the electrons surrounding the nucleus (nuclear shielding). The electronic structure around the nucleus in turn depends on the molecule the nucleus is in, i.e., its chemical environment. As the protons experience a different local field in different molecules (and in different positions in a molecule), they resonate at different frequencies, and thus appear in different positions on the x-axis of the spectrum (Fig. 6). This difference is called the chemical shift. Chemical shift is usually expressed as parts per million (ppm) of B_o (de Graaf, 1998). For protons, the chemical shift range is quite narrow (~12 ppm), and it in fact encompasses all biologically relevant chemical compounds. The peaks in spectra are slightly broadened because the resonant frequency is somewhat modified by local magnetic fields produced by neighboring protons. The area of the peak, however, remains unaffected and proportional to the total number of protons and is usually determined with lineshape fitting.

The metabolites of interest have a much lower concentration (in the mmol/l range) than does water (>80 mol/l). This results in a lower signal-to-noise ratio (SNR), lower spatial resolution, and longer acquisition time in MRS as compared to water-based imaging (MRI). In order to obtain signals from protons of the metabolites of interest, the large signals from water and lipid protons have to be suppressed.

<u>^{1}H MRS data acquisition</u>
Different methods are available for obtaining localized ^{1}H MRS data. With single-voxel methods, it is possible to measure MR signals originating from one region of interest (a cubical volume of typically ~1-10 ml). The most frequently used sequences for single-voxel studies are stimulated echo acquisition mode (STEAM) (Frahm *et al.*, 1987), and point-resolved spectroscopy (PRESS) (Bottomley, 1987).

In the PRESS sequence (used in Studies II-IV; Bottomley, 1987), a selective 90^o RF pulse is first applied. The 90^o pulse is followed by two 180^o pulses, which rephase the spins. These three pulses (90^o-180^o-180^o) are then successively repeated. Repetition time (TR) refers to the time interval between two 90^o pulses. The time between the 90^o pulse and signal sampling is called echo time (TE) (Tofts and Waldman, 2003).

In STEAM (Frahm *et al.*, 1987), all three pulses are 90^o. A stimulated echo is produced with intrinsically only half the amplitude of a conventional echo. With STEAM, shorter TE times can be achieved, but the SNR is not as good as with PRESS (Keevil, 2006).

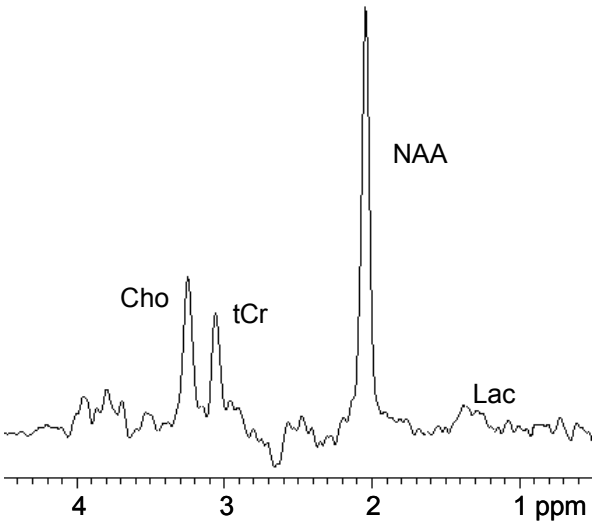

Figure 6. Example of a ^{1}H MRS spectum with and echo time of 288 ms. NAA = N-acetyl aspartate, tCr = total creatine, Cho = choline-containing compounds, Lac = lactate. On the x-axis is the chemical shift in parts per million (ppm).

TR is the time allowed for longitudinal relaxation. If TR is much (generally 6 to 8 times) longer than T1, the longitudinal magnetization will have time to fully recover (fully relaxed condition). This condition requires long data collection times and can rarely be achieved for *in vivo* measurements. T2 governs transverse relaxation, and therefore, with very long TEs, transverse magnetization (the synchronic precession of the spins) has time to disappear completely. Conversely, for the transverse magnetization to remain fully intact, TE should be close to zero. This condition is never achieved *in vivo*, since the spatial localization schemes require a spin echo. Due to these relaxation issues, commonly accepted acquisition parameters (TR and TE) are preferred to enable systematic comparison of *in vivo* results.

The local environment of the proton affects its T2 and visibility with ^{1}H MRS. The protons that are reasonably mobile have T2 values long enough for their transverse magnetization to remain for tens of milliseconds after excitation, and they will be detected by the spectroscopy sequence. Conversely, protons that are less mobile (e.g. protons in large protein molecules or in lipid) have a shorter T2. Thus, their transverse magnetization decays quickly, and will not be observed by the *in vivo* spectroscopic sequences, which use TEs of at least 20 to 30 milliseconds. As a consequence, the spectra acquired with long TEs have well-defined peaks and a flat baseline, but the T2 losses are comparably large. Use of short TEs allows observance of a larger number of metabolites, but the peak area definition is more complex, as peaks overlap, and the baseline may become irregular. The SNR of the peaks can be improved by raising voxel size (which reduces spatial resolution and leads to increased SNR in a directly proportional manner) or by raising the number of excitations (which prolongs the measurement and increases SNR only by square root) (Kreis, 1997; Tofts and Waldman, 2003).

Quantitation in ^{1}H MRS

Determination of the absolute concentrations of metabolites in ^{1}H MRS spectra is not straightforward. Although the spectroscopic peak area is proportional to the concentration of a metabolite, it is also dependent on receiver coil sensitivity, coil loading (which depends on the subject or sample), voxel size, and temperature. In general, all quantitation methods utilize an internal or external reference to which the metabolite signals are compared. Each quantitation method has its strengths and limitations.

Peak ratios can serve for quantitation, when one metabolite in the spectrum is known not to vary in concentration. The ratio method eliminates the need for coil load- and instrument-

performance corrections, and does not require additional measurements. The body has, however, no reliable internal standard: The concentration of the reference compound is rarely both sufficiently stable and precisely known. Changes in the ratio can thus originate either from a change in the numerator or a change in the denominator, or both, and the direction of the change is unknown (Kreis, 1997; Tofts and Waldman, 2003).

The large and relatively stable water signal is easily collected and can also serve as an internal reference for other metabolites. Although the physiological water content in the brain varies only within narrow limits (Kreis *et al.*, 1996; Kreis, 1997), the use of metabolite ratios to water has limitations similar to those in the use of other metabolite ratios: Both the exact water concentration, and any minor changes in water content in the target area are unknown (Kreis, 1997; de Graaf, 1998; Tofts and Waldman, 2003).

External standard solutions have a precisely determined concentration of the reference compound, but their use requires additional measurements. Either a same-time standard (positioned outside the target tissue but inside the sensitive volume of the coil) or a same-place standard (placed at the same location as the center of the brain) can be used. For the same-time standard, coil and receiver performance are the same as for the target tissue, but RF non-uniformity and temperature variations may occur. The same-place standard has to be scanned at a different time, and is thus vulnerable to changes in receiver sensitivity (Kreis, 1997; Tofts and Waldman, 2003).

2.5.4 ^{1}H MRS metabolites

The number of different molecules detectable with ^{1}H MRS is limited, and the compounds yielding the optimal MR signals are not always those that would be of major interest for neuroscientists or sleep researchers (Ross and Bluml, 2001; Hutchinson *et al.*, 2002). The main metabolites detected with long TE (used in Studies II-IV) are described below.

N-acetyl-aspartate

N-acetyl-aspartate (NAA) has a chemical shift of 2.02 ppm and is the most prominent peak in the ^{1}H MRS spectrum of the brain (normal concentration 7.8 mM; range 6.5-9.7 mM; Fig. 6). The peak contains at least two chemical moieties, N-acetyl aspartate and N-acetylaspartyl glutamate (NAAG; normal concentration 0.3-3 mM), which are difficult to distinguish spectroscopically and are normally grouped together as NAA or NA (N-acetyl-containing compounds).

N-acetyl aspartate is a free amino acid localized primarily in the nervous tissue. It has originally been considered a neuronal marker, but recent evidence suggests a more dynamic role for it in the CNS. NAA synthesis occurs primarily in neuronal mitochondria and is dependent on energy metabolism (Bates *et al.*, 1996; Moreno *et al.*, 2001; Signoretti *et al.*, 2001) (Fig. 7). Among other functions, NAA is believed to act as an osmolyte, a storage form of aspartate, and a precursor of NAAG (Tsai and Coyle, 1995). NAAG is synthesized in neurons and cleaved at the cell surfaces of astrocytes to NAA and glutamate. NAA has a turnover rate of ~17 h (Baslow, 2002), and is broken down into aspartate and acetate by the enzyme asparto-acylase, which is localized in oligodendrocytes (Baslow, 2000; Govindaraju *et al.*, 2000). The intercompartmental cycling of NAA has been proposed to serve primarily as a mechanism to remove large amounts of the metabolic water generated in neuronal glucose metabolism (Baslow, 2002).

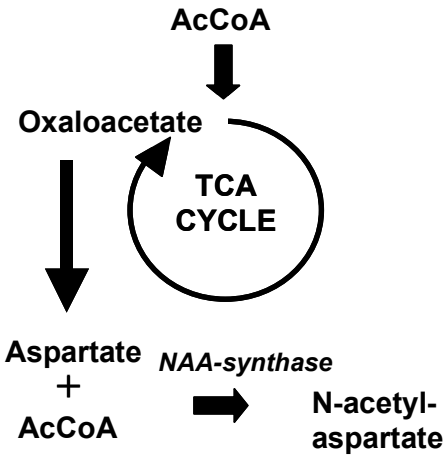

Figure 7. N-acetyl-aspartate (NAA) synthesis and its relationship to the tricarboxylic acid (TCA) cycle. NAA synthesis from aspartate and acetyl-coenzyme A (Ac-CoA) occurs in mitochondria and is catalyzed by the enzyme NAA-synthase (l-aspartate-N-acetyltransferase).

Low NAA levels have appeared in a wide range of situations involving neuronal loss or dysfunction, such as during ischemia, trauma, dementia, gliosis, or infection. In various psychiatric disorders, NAA levels also decrease (Tsai and Coyle, 1995), as well as in the course of aging (Angelie *et al.*, 2001; Brooks *et al.*, 2001). A case report has described complete absence of NAA in a 3-year-old boy with neurodevelopmental retardation and moderately delayed myelination (Martin et al., 2001). Elevated NAA levels are encountered in Canavan's disease, a rare inborn error of NAA metabolism (Barker *et al.*, 1992; Wittsack *et al.*, 1996; Tofts and Waldman, 2003).

Choline-containing compounds

Choline-containing compounds (Cho) include free choline, glycerophophorylcholine, and phosphorylcholine (concentration normally 1.3mM, range 0.8-1.6mM) and give rise to the peak at 3.22 ppm (Fig. 6). Choline is required for synthesis of the neurotransmitter acetylcholine, and of phosphatidylcholine, a major constituent of cell membranes. The membrane-bound choline-containing compounds are usually invisible with ^{1}H MRS (Miller *et al.*, 1996). The biochemical interpretation of alterations in Cho is complicated by uncertainty as to the metabolites contributing to the signal. Changes in Cho are, however, generally associated with acute or chronic alterations in membrane metabolism, or in membrane integrity. Both enhanced cell proliferation (anabolism) and membrane degradation (catabolism) can raise Cho levels. Accordingly, elevated Cho signals have been observed in tumors, ischemia, and multiple sclerosis. In chronic stroke and liver diseases, Cho has been observed to decrease (Govindaraju *et al.*, 2000; Tofts and Waldman, 2003). During aging, Cho levels have been observed to increase (Chang *et al.*, 1996; Leary *et al.*, 2000), although the findings are not entirely consistent (Brooks *et al.*, 2001).

Total creatine

The total creatine (tCr) peak at 3.03 ppm (Fig. 6) represents the sum of creatine (Cr) and phosphocreatine (PCr), which play important roles in cellular energy storage and transmission. Total Cr levels (normal concentration 4.5 mM, range 3.4-5.5 mM) increase in trauma and hyperosmolar states, and decrease in states like hypoxia and stroke (Tofts and Waldman, 2003). During aging, some studies have reported increased tCr levels (Chang *et al.*, 1996; Leary *et al.*, 2000), while others have found no age-related change (Saunders *et al.*, 1999; Brooks *et al.*, 2001).

Lactate

The lactate doublet at 1.33 ppm is best observed with TEs around 288 ms (positive; Fig. 6) or 144 ms (inverted), due to its spin-spin coupling constant (J) of ~7 Hz. In baseline conditions,

brain lactate levels are generally below the ~1mM detection limit of lactate. Lactate levels increase in differing states like ischemia and impaired tissue metabolism, but also in the healthy brain during physiological activation (see p.15, section 2.3.2) (Tofts and Waldman, 2003). In the aging brain, some evidence exists of lactate being detectable in baseline conditions (Sijens *et al.*, 2001a), but findings are not consistent (Grachev and Apkarian, 2001), and may actually indicate the presence of hypoperfusion (van der Grond *et al.*, 1995) or some other asymptomatic pathologic process not detected with MRI (Sijens *et al.*, 2001a). The role of lactate in brain energy metabolism has been described in section 2.3.2 (p.15).

2.5.5 ^{1}H MRS in sleep research

The relatively underdeveloped status of NMR techniques in the field of sleep research and sleep medicine has recently raised some concerns (Otte *et al.*, 2002; Nofzinger, 2004; Maquet, 2005). Among all NMR studies, those using ^{1}H MRS have been particularly scarce. ^{1}H MRS has been used to study brain metabolism in sleep disorders such as sleep apnea (Kamba *et al.*, 1997, 2001, 2003; Alchanatis *et al.*, 2004; Bartlett *et al.*, 2004, Halbower *et al.*, 2006), narcolepsy (Ellis *et al.*, 1998; Lodi *et al.*, 2004), and REM sleep behavioral disorder (Iranzo *et al.*, 2002; Hanoglu *et al.*, 2006). ^{1}H MRS has not, however, been applied to study the normal sleep-wake cycle. Further, effects of sleep deprivation on healthy human brain ^{1}H MRS metabolites have been explored in one pilot study only (Murck *et al.*, 2002). There, an increase in unresolved Glx (comprising glutamine, glutamate, and GABA) was observed in the pons after sleep deprivation.

3. AIMS OF THE STUDY

The major aim of this thesis was to assess *in vivo* the energy metabolic processes taking place in the rat and human brain during sleep deprivation.

The specific aims of the studies were:

I. To compare the effects of sleep deprivation and experimentally induced local brain energy depletion on sleep and on the levels of markers of brain energy metabolism: adenosine, lactate, and pyruvate.

II. To study brain lactate levels in the healthy human brain with ^{1}H MRS during a cognitive task.

III. To study the effects of sleep deprivation and of aging on the brain lactate response to cognitive stimulation.

IV. To study levels of the main metabolites of the brain proton spectrum in the occipital cortex during photic stimulation and sleep deprivation.

4. MATERIALS AND METHODS

Details of the materials and methods are given in the original publications (I-IV). The main principles are described below.

4.1 Study subjects (I-IV)

4.1.1 Rats (I)

Adult male rats (weight 300-400 g) were kept under constant temperature in a 12/12 light/dark rhythm; standard food and water were provided *ad libitum*.

Under general anesthesia the animals were each fitted with two EEG and two EMG electrodes, and with a microdialysis guide cannula (CMA 11 guide, CMA/Microdialysis, Stockholm, Sweden) aimed unilaterally 4 mm above the basal forebrain (BF) area (AP, -0.3; ML, 2.0; V, 5 mm). After 1 week of recovery from surgery, the rats were adapted to the recording chambers and recording leads for 3 to 4 days until the start of the experiments.

After experiments, the rats were killed with a lethal dose of pentobarbital. Their brains were removed, frozen, sectioned on a freezing microtome, stained, and inspected under a microscope to define the location of the probe tip. According to probe location, the animals were divided into two groups: the basal forebrain (BF) group (probe tip in the basal forebrain cholinergic area), and the non-basal forebrain (non-BF) group (probe tip in noncholinergic brain areas adjacent to the basal forebrain).

The study was approved by the University of Helsinki Ethics Committee for Animal Experiments.

4.1.2 Humans (II-IV)

The subjects were healthy, non-smoking, drug-free female volunteers who spoke Finnish as their mother tongue. During an on-site visit, these women were examined and interviewed in detail; those with major somatic, sleep, or psychiatric disorders or unusual sleep habits were excluded.

The subjects belonged to two age groups: 19 to 25 years (young), and >60 years (older). The young subjects were studied during the early phase of their menstrual cycle. The older subjects were postmenopausal and had used no hormone replacement therapy for at least one year prior to the study.

Before the experiments, the subjects went through a one-week period of adaptation to a regular sleep and nutrition schedule. Sleeping hours were fixed (sleep between 22:00 and 7:00), and the nutrition schedule consisted of a regular and balanced daily energy intake and set meal hours. No use of alcohol, caffeine, or medication, except for oral contraceptives for the younger group, was allowed. The subjects spent 2 nights with EEG recordings in the sleep laboratory prior to the actual experiment in order to exclude sleep disorders and to become adapted to the surroundings.

The subjects gave their informed consent to participate in the study, which was approved by the Ethics Committee of the Helsinki University Central Hospital.

4.2 Assessment of sleep (I-IV)

4.2.1 EEG recording and analysis (I-IV)

Rat EEG (I)
In rats, EEG was recorded with a polygraph (Beckman type T, time constant 0.1 s). The signal was brought to a CED 1401-plus interface (Cambridge Electronics, Cambridge, UK), and sampling was performed at 104 Hz with the Spike 2 program into a PC.

During the experiments, EEG and EMG were recorded continuously for 24 h. EEG recordings were scored manually in 30 s epochs as wakefulness, non-REM sleep, and REM-sleep. Initial quantitative analysis was performed in 30-min bins, and the bins between 17:00 and 02:00 h were chosen for the final sleep analysis.

Power spectral analysis was performed with a script for the Spike 2 program and was postprocessed in Microsoft Excel using custom-made macros. The signal was analyzed for delta power (0.5-4 Hz) during sleep relative to total power (0.5-35 Hz) of the recording period.

Human EEG (II-IV)
In humans, PSG registration (ambulatory recording; sampling rate 100 Hz; filters: HP 0.5 Hz, LP 45 Hz; Embla, Flaga hf. Medical Devices, Reykjavik, Iceland) was obtained continuously throughout the sleep-deprivation experiment, except during fMRI and ^{1}H MRS. The electrodes were placed according to standard electrode placements (C3-A2 and C4-A1; EMG and EOG) (Rechtschaffen and Kales, 1968), and the recordings were scored visually in 30-s epochs according to standard criteria (Rechtschaffen and Kales, 1968).

4.2.2 Actigraphy and sleep diaries (II-IV)

Actigraphic registrations (Actiwatch-L, Cambridge Neurotechnology Ltd, epoch length 1 min) combined with a sleep diary served to confirm the regular sleep rhythm during the adaptation period. Actigraphy data were analyzed with Actiwatch Sleep Analysis software, the Sleep-Wake Scoring Algorithm, for the following parameters: sleep start, sleep end, period between sleep start and sleep end, sleep period corrected for nocturnal awakenings (in % of period between start and end of sleep).

4.2.3 Assessment of sleep during fMRI/^{1}H MRS (II-IV)

During fMRI/^{1}HMRS, the director of the experiment was in contact with the subject via microphone, and the subject was immediately awakened if any sign of falling asleep occurred.

4.3 Sleep deprivation (I, III, IV)

4.3.1 Sleep deprivation of rats (I)

Sleep deprivation (see section 4.5.1 and Fig. 8 for experimental schedule) was performed with the gentle handling method (Franken *et al.*, 1993). Objects were introduced into the rats' cages and replaced by new ones when the rats appeared to become sleepy.

4.3.2 Sleep deprivation of humans (III-IV)

The subjects were sleep-deprived for 40 h from day I 6:00/7:00 h until day II 22:00/23:00 h (III/IV). No alcohol or caffeine-containing drinks were allowed during the wakefulness period; light controlled snacks were allowed during the sleep-deprivation night in addition to the adaptation period's nutrition schedule. The subjects were allowed to move freely, read, watch TV in the laboratory while wearing the ambulatory EEG recording device. To ensure wakefulness, the subjects were under close supervision by the sleep laboratory staff at all times.

4.3.3 Sleepiness and vigilance during sleep deprivation (III)

The subjects' vigilance state was assessed by means of a psychomotor vigilance task (PVT) (Dinges and Powell, 1985), which has been shown to be sensitive to sleep loss (Doran *et al.*, 2001; Van Dongen *et al.*, 2003). The 10-min visual PVT task was performed at 2-h intervals with a PVT-192 unit (Ambulatory Monitoring, Inc. of Ardsley, NY, USA). During this task, subjects had to respond to visual stimuli (inter-stimulus interval 2-10 s) displayed on a small computer screen as fast as they could by pushing a button.

A scale (1-10) for self-rating of sleepiness was displayed directly before and after the reaction-time measurement.

4.4 Cognitive and photic stimulation (II-IV)

4.4.1 Silent word-generation (II-III)

The silent word-generation task belongs to the group of verbal fluency tasks that are widely used neuropsychological tests assessing predominantly frontal-lobe function. The task can be easily performed inside the MR scanner without use of special equipment, and its performance produces relatively robust and sustained focal brain activation (Yetkin *et al.*, 1995; Pujol *et al.*, 1996; Friedman *et al.*, 1998; Brannen *et al.*, 2001).

In Studies II and III, the silent word-generation task was performed during fMRI and ^{1}H MRS, with subjects following instructions via headphones. They were instructed to retrieve within a given time-period as many words as possible according to two alternating rules: words belonging to a certain category (e.g., animals, furniture, and plants; category task) and words beginning with a specified letter (letter task). The letter and category tasks were used alternately to avoid motivation loss and to minimize the learning effect. A selection of equally common letters was used in all alertness states. The silent word-generation task was contrasted with the baseline task, which consisted of silently listing numbers upwards from number one (number-listing task).

These tasks were explained in detail to the subjects who practiced them before the start of the experiment. Their ability to perform these tasks during the experiment was tested with a 2-min tape-recorded verbal fluency test session with one letter- and one category task directly after the fMRI/^{1}H MRS.

4.4.2 Photic stimulation (IV)

Photic stimulation involved a strobe light flashing at 8 Hz right outside the magnet room. The light was reflected toward the subject's eyes by a mirror attached to the head coil. The magnet room was darkened so that the strobe light provided the only illumination for the subject's eyes.

4.4.3 Localization of activation with fMRI (II-IV)

At the beginning of the fMRI/^{1}H MRS measurements, the brain areas activated with silent word-generation were determined individually for each subject by functional magnetic resonance imaging (fMRI) in order to position the MRS voxel optimally (II-III; Fig. 9). The fMRI was performed with a T_2^*-weighted gradient-recalled echo planar imaging sequence (TR=3000 ms, TE =60 ms, matrix size 64 x 64). The field of view, slice positioning, and thickness were the same as for the T_1-weighted anatomical images. During an fMRI, 72 volumes of 7 slices were acquired.

The fMRI analysis was performed with FuncTool software (GE Medical Systems, Milwaukee, WI, USA), and later confirmed by analysis of the data using the SPM99 package (Wellcome Department of Cognitive Neurology, London, UK).

In Study IV, the exact location of the brain activation during photic stimulation was examined by fMRI in pilot experiments.

4.5 Assessment of brain energy metabolism (I-IV)

Brain energy metabolism in rats was assessed by microdialysis combined with HPLC (I), and in humans by ^{1}H MRS (II-IV).

4.5.1 Microdialysis and HPLC (I)

Three different kinds of experiments were performed: sleep deprivation, DNP-infusion, and KCN-infusion. For each animal, after a baseline EEG recording, the experiments started with sleep deprivation and were followed by infusions of three different doses of DNP, starting with the lowest dose. KCN infusion, when performed (n=6), was the last experiment. The minimum period between any two consecutive experiments was 48 h.

For all experiments, the microdialysis probe (CMA 11, CMA/Microdialysis) was lowered through the guide cannula at least 20 h before the start of the experiments. The rats were connected to the microdialysis leads between 8:30 and 09:00, and the leads were removed at 16:00 to 16:30 h.

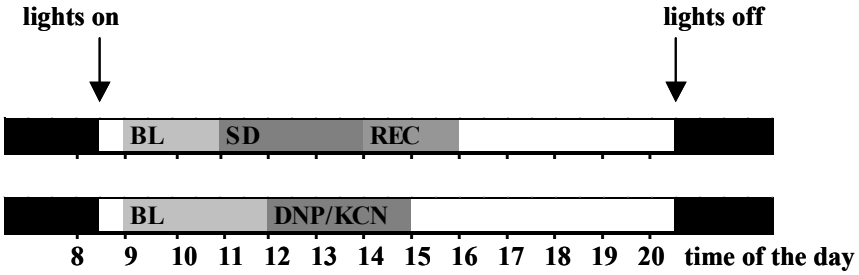

Figure 8. Experimental schedule of Study I. Upper diagram: timing of the baseline (BL), sleep deprivation (SD), and recovery sleep (REC) in the sleep-deprivation studies. Lower diagram: timing of the baseline (BL) and DNP/KCN infusions in the drug-infusion studies. The schedules are shown relative to the 12/12 light-dark cycle.

Sleep-deprivation experiment

The sleep-deprivation experiment consisted of a 2-h baseline artificial cerebrospinal fluid (aCSF) infusion, followed by a 3-h prolonged wakefulness period, and 2 h of recovery sleep (Fig. 8). During the experiment, aCSF was pumped through the microdialysis probe at a rate of 1 μL/min.

DNP and KCN experiments

The drug experiments consisted of a 3-h baseline collection period with aCSF infusion (1 μL/min) followed by a 3-h infusion of either DNP (Sigma Chemical Co., St Louis, MO, USA; product D-7004; 1μL/min) in different doses (0.5, 1.0, and 1.5 mM), or KCN (0.075mM; 1μL/min) (Fig. 8).

HPLC coupled to UV detection

High-performance liquid chromatography (HPLC) coupled to ultraviolet (UV) detection was used to analyze adenosine (Porkka-Heiskanen *et al.*, 2000), lactate, and pyruvate (Hallström *et al.*, 1989) concentrations in the microdialysis samples collected at 30-min intervals as previously described. The detection limits of the assays were 0.8 nM (SNR 2:1) for adenosine, 0.6 μM (3:1) for pyruvate, and 10 μM (3:1) for lactate (Grob, 1985).

4.5.2 ^{1}H MRS (II-IV)

Three ^{1}H MRS experiments were performed (Studies II, III, and IV; for the experimental schedules, see Fig. 9). Study II assessed the effects of cognitive stimulation on lactate levels in the frontal cortex. Study III assessed the effects of sleep deprivation and aging on this local brain lactate response. Study IV assessed the effects of sleep deprivation and photic stimulation on brain NAA, tCr, and Cho ratios to water in the occipital cortex.

All ^{1}H MRS measurements were performed with a 1.5 T whole-body MR system equipped with a standard head coil (II and III: GE Signa Horizon LX EchoSpeed scanner, GE Medical Systems; IV: Siemens Magnetom Sonata, Erlangen Germany). A T_1-weighted image set served as an anatomical reference to position the spectroscopic volumes-of-interest (VOIs).

^{1}H MRS was performed in the morning after standard hours of sleep and a standard meal. In the sleep-deprivation studies (III-IV), the ^{1}H MRS protocol was repeated after 36 h of sleep deprivation.

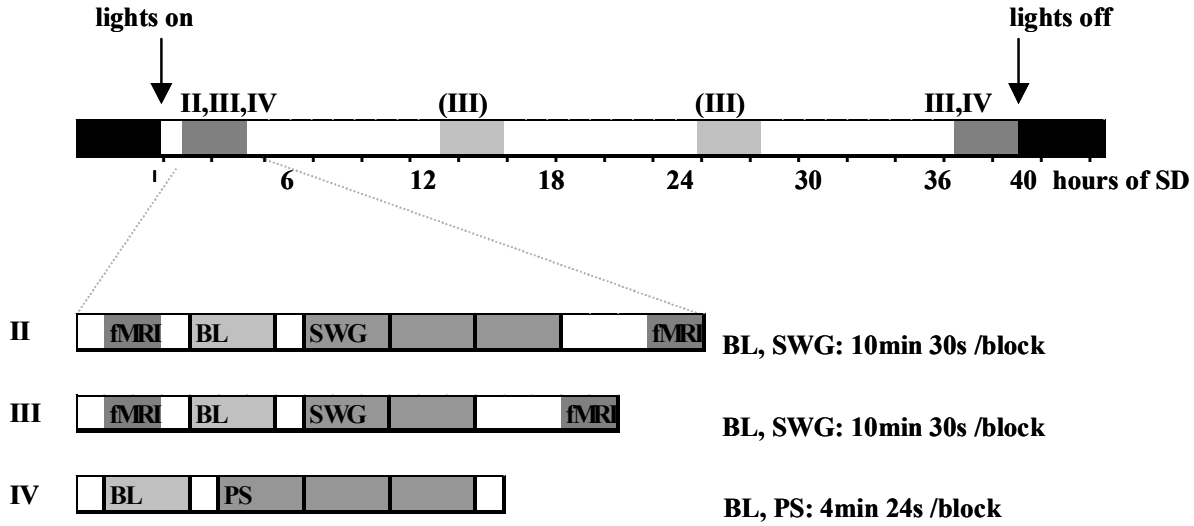

Figure 9. Experimental schedules of the Studies II-IV. Upper panel: Timing of the fMRI/^{1}H MRS measurements in relation to the sleep-deprivation period. In all Studies, fMRI/^{1}H MRS (dark grey bar) was performed in the morning after a night of baseline sleep. In Studies III and IV, the same measurement was repeated after 36 h. In addition, data were also collected 12 and 24 h after the first measurement from the young subjects in Study III (unpublished data; light grey bars (study number)). Lower panels: A more detailed description of the measurements performed in each of the three studies. fMRI = functional MRI; BL = ^{1}H MRS baseline; SWG = ^{1}H MRS silent word-generation; PS = ^{1}H MRS photic stimulation. Duration of the ^{1}H MRS measurements is at the right.

Lactate measurements (II-III)

Lactate was measured in the inferior frontal gyrus during the baseline condition, and during different durations of silent word-generation (Fig. 9). The choice of voxel location and dimensions were based on the results of fMRI (described in section 4.4).

Single-voxel lactate measurements were performed with a PRESS sequence with outer volume saturation pulses and chemical shift-selective (CHESS) water suppression (TR = 1500 ms; TE = 288 ms; acquisition time 10.5 min).

The spectra were processed and analyzed with SAGE software (GE Medical Systems, Fremont, CA, USA). The amplitude of the lactate peak was determined with a coupling constant of 7 Hz, a chemical shift of 1.33 ppm, and a fixed line width. To avoid fitting the noise, the root mean square noise served as the lactate value if the lactate SNR was below two.

NAA, Cho, tCr, and H_2O measurements (II-IV)

In Studies II and III, NAA (2.02 ppm), tCr (3.03 ppm), and Cho (3.22 ppm) were measured in the left inferior frontal gyrus with a data acquisition procedure identical to that of lactate (described above). The signal intensities for each of the metabolites were determined by Lorentzian lineshape fitting.

In Study IV, NAA, Cho, and tCr levels were measured during baseline and different durations of photic stimulation (Fig. 9) in the occipital lobe (VOI size 20 x 20 x 25 mm^3) with a PRESS sequence (TE = 288 ms; TR = 2000 ms; 128 acquisitions; acquisition time 4 min 24 s). CHESS water suppression and an autoshim procedure were performed before data

acquisition. For H_2O (4.75 ppm) determination, the protocol was performed without water suppression.

The spectra in Study IV were processed and analyzed with an MR-spectroscopy package Mrease software (Siemens, Erlangen, Germany). The metabolite intensities (i.e., peak areas) were determined by Gaussian lineshape fitting, and H_2O by Lorentzian lineshape. The Lorentzian lineshape was used for H_2O since it gave a better fit than the Gaussian lineshape. Metabolite ratios were used to adjust for potential variations in the MR system and for differences in the coil loading between individuals.

4.6 Statistical analyses (I-IV)

The statistical analyses were performed with Sigma Stat Statistical Software (SPSS, Chicago, IL, USA).

In Study I, the metabolite levels during treatments (sleep deprivation, DNP, or KCN infusions) vs. the baseline aCSF infusion (directly before the respective treatment) were compared with paired t-tests. The EEG measures after the treatments were compared to the EEG during the baseline day using Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks, followed by Dunn's test.

In Studies II and III, lactate levels during baseline vs. cognitive stimulation were compared with paired t-tests. T-tests for independent samples served for lactate level comparisons between the two age groups (III). Sleepiness, PVT, and verbal fluency performance during baseline vs. sleep deprivation were assessed by repeated measures ANOVA: one-way ANOVA followed by Dunnett's test for within-groups effects, and two-way ANOVA followed by the Student-Newman-Keuls test for between-group effects (III).

In Study IV, metabolite ratios to water during baseline vs. sleep deprivation were compared with paired t-tests. The effects of different durations of photic stimulation (vs. the respective baseline) on metabolite ratios were assessed by repeated measures ANOVA followed by Dunnett's test.

If the data were non-normally distributed, the corresponding nonparametric tests were used. All results were considered statistically significant when $p < 0.05$.

5. RESULTS AND DISCUSSION

5.1 Effects of sleep deprivation and DNP-infusion on brain adenosine, lactate, and pyruvate levels (I)

5.1.1 Adenosine

At the time this study was undertaken, it was known that adenosine levels rise in response to sleep deprivation in a site-specific manner in the BF, and to a lesser extent in the cortex, whereas in other subcortical structures, adenosine levels tend to decline (Porkka-Heiskanen *et al.*, 1997, 2000). The results of Study I confirmed these findings: During its 3-h sleep-deprivation period, significantly increased extracellular adenosine levels occurred only in the BF group (adenosine increase by 297.0 ± 66.3%; mean ± SEM), while the non-BF group showed no significant increase (5.2 ± 34.2%).

In previous studies, DNP treatment has raised adenosine levels in brain tissue, more specifically in hippocampal slices (Doolette, 1997). Further, i.c.v. administration of DNP reduced ADP and ATP, with a simultaneous increase in adenosine monophosphate (AMP) concentrations, which strongly suggests an increase in adenosine (Fujibayashi *et al.*, 1993). Consistent with these findings, DNP infusion in Study I raised adenosine concentrations in a dose-dependent manner irrespective of the location of the probe. The effects of the 0.5-mM DNP infusion on brain adenosine (BF group: an increase of 281.1 ± 72.3%; non-BF group: 265.4 ± 119.8%) were comparable to those of sleep deprivation of the BF group (see Study I, Fig.3A).

The increase in extracellular adenosine may result from changes in the intra- or extracellular adenine nucleotide metabolism. In the extracellular space, adenine nucleotide dephosphorylation may be enhaced in particular during neuronal activity, since ATP is co-released with excitatory neurotransmitters during neuronal activation (White, 1977), and since cyclic AMP (cAMP) is released in conjunction with adenylyl cyclase-coupled receptor activation (Rosenberg and Li, 1995). Even a small shift in the intracellular ATP/AMP ratio towards AMP would result in a considerable increase in both intra- and extracellular adenosine concentrations, since adenosine is rapidly transported out of the cell in order to equilibrate the concentration difference (Basheer *et al.*, 2004).

5.1.2 Lactate and pyruvate

Brain lactate levels are higher during wakefulness than during sleep (Van den Noort and Brine, 1970; Shram *et al.*, 2002). After i.c.v. administration of DNP, an increase in brain lactate occurred (Kuhr and Korf, 1988).

In line with these findings, during the 3-h sleep-deprivation period, extracellular concentrations of lactate and pyruvate increased significantly in the BF group (lactate by 35.2 ± 8.5%; pyruvate by 37.5 ± 9.6%;). However, in the non-BF group, no significant increases occurred (respective percentages 0.2 ± 9.0; -7.2 ± 9.4%). The DNP infusion increased lactate and pyruvate concentrations in a dose-dependent manner both in the BF and in the non-BF groups. For the BF group, lactate increased by 37.7 ± 10.0%, and pyruvate by 50.2 ± 19.1% during the 0.5-mM DNP infusion. Respective increases for the non-BF group were 28.2 ± 7.6, and 26.4 ± 12.1% (see Study I, Figs.3B and C).

The source of the extracellular lactate is disputable. According to the conventional view, lactate results from the oxidation of glucose in the neurons during conditions of inadequate oxygen supply. A reduction in the ATP/AMP ratio would thus induce the activation of hexokinase and phosphofructokinase, resulting in increased glycolysis, and in increased concentrations of pyruvate, which enters the citric acid cycle. In conditions of an insufficient supply of oxygen for oxidative phopshorylation, pyruvate would be metabolized to lactate. According to a recently promoted view, however, lactate would also be produced under normoxic conditions upon brain activation (Prichard *et al.*, 1991; Magistretti *et al.*, 1999), and serve as the preferred energy source over glucose during increased neuronal activity (Shulman *et al.*, 2001). The present findings cannot distinguish between the classical and new theories.

In sum, the increases in adenosine, lactate, and pyruvate in the BF group during sleep deprivation can be interpreted as a sign of local energy depletion during sleep deprivation. The effects of sleep deprivation on brain energy metabolism seem to be site-specific, since no significant effects of sleep deprivation on brain energy metabolism appeared in the non-BF group. The energy depletion in the BF could possibly result from the sustained activity of the wakefulness-promoting cells in the BF area. The increases in adenosine, lactate, and pyruvate during DNP infusion irrespective of probe location was expected with regard to the effect of DNP in uncoupling mitochondrial ATP production.

5.2 Effects of sleep deprivation and DNP-infusion on sleep (I)

5.2.1 Effects of sleep deprivation

The effects of sleep deprivation on subsequent sleep have been well documented (Turek and Zee, 1999). As expected, sleep deprivation was followed by a significant increase in non-REM sleep (53.4 ± 16.4%) and in delta-power (118.7 ± 57.2%) in both BF- and non-BF groups.

5.2.2 Effects of DNP infusion

The effects of local brain energy depletion on subsequent sleep have not been studied prior to our work. Indirect evidence, however, indicates that energy depletion causes increased sleep or fatigue. The intraperitoneal infusion of 2-deoxy-D-glucose, a glucose antimetabolite, leads, in cats, to increased sleep dose-dependently (Panksepp *et al.*, 1973), and mild hypoglycemia in diabetic patients during the night has been observed to generate subjective feelings of fatigue during the following day (King *et al.*, 1998).

The effects of DNP infusions on sleep differed between the two groups: In the BF group, non-REM sleep and delta power were significantly higher, while in the non-BF group the changes were non-significant (see Study I, Fig. 5). The DNP infusion of 0.5 to 1.0 mM into the BF generated a quantitatively similar increase in non-REM sleep to that of sleep-deprivation period (increase in non-REM sleep with the 0.5-mM dose was 43.0 ± 17.4%; with the 1.0-mM dose 105.6 ± 27.0%). For delta power, the 1.5-mM dose caused an increase of 109.2 ± 55.2%, comparable to the effects of sleep deprivation. After KCN infusion, the increase in non-REM sleep corresponded to that with the 1.0 to 1.5 mM DNP dose.

The mechanisms by which sleep deprivation and DNP infusion induce sleep are unclear. However, since adenosine has been recognized as a sleep-inducing agent, and adenosine increased in the BF group during both sleep deprivation and DNP infusion, this increase could at least partly account for the increased sleep amount and depth subsequent to the treatments. The increase in adenosine in the BF can be hypothesised to act as a mechanism to save other brain areas from the injurious effects of energy deficit.

Adenosine may affect sleep through several mechanisms. In the BF, the somnogenic effects of adenosine may be due to its A_1 receptor-mediated inhibition of neuronal activity of wakefulness-promoting BF neurons (Porkka-Heiskanen *et al.*, 1997; Basheer *et al.*, 2004). The long-term effects of adenosine are most probably mediated through transcription: increased levels of c-Fos protein, activator protein-1 (Basheer *et al.*, 1999), and nuclear factor-kB DNA binding (Basheer *et al.*, 2001a), as well as increased expression of A_1 receptor mRNA in the BF (Basheer *et al.*, 2001b). The somnogenic effects of adenosine after sleep deprivation have been connected in particular to the BF cholinergic neurons, although non-cholinergic cells may also be inhibited by adenosine (Basheer *et al.*, 2004; Blanco-Centurion *et al.*, 2006).

Interestingly, in Study I, the increase in sleep in the BF group was noted only after, and not during, the DNP infusion, although very high adenosine concentrations were attained during the infusion. It is possible that the treatment was unable to increase sleep any further at the time of the day when the animals were already sleeping maximally.

Despite the strong evidence of adenosine's acting as a sleep-inducing factor, based on our findings, the possibility of lactate and pyruvate participating in sleep regulation cannot be excluded.

5.3 Effects of sleep deprivation and age on subjective sleepiness, psychomotor performance, and verbal fluency (II, III)

To assess subjects' behavioral state during the sleep-deprivation period, subjective sleepiness scales, psychomotor vigilance tasks, and tape-recorded verbal fluency tests were performed. The activated brain areas during silent word-generation were mapped prior to ^{1}H MRS for each subject to position the MRS voxel optimally.

5.3.1 Subjective sleepiness and psychomotor vigilance (III)

As expected, both subjective sleepiness and psychomotor vigilance scores were affected by sleep deprivation. Subjective sleepiness increased significantly in the course of the prolonged wakefulness period in both age groups (Fig. 10; significant increases in each datapoint after 18.5 h of sleep deprivation as compared to the first measurement; see also Study III, Fig. 2). The young subjects rated themselves as significantly sleepier than did the older subjects at two data points (19.5 and 27.5 h of sleep deprivation). The mean reaction time in the PVT increased significantly during the prolonged wakefulness period in both age groups (significant increases at each data point after 22.5 h of sleep deprivation as compared to the first measurement; see Study III, Fig. 3). The young performed better than the older group after 23.5 h of sleep deprivation.

More detailed descriptions of the effects of age and sleep deprivation on women's PVT performance and subjective sleepiness have recently been published (Karakorpi *et al.*, 2006; Urrila *et al.*, 2006).

5.3.2 Verbal fluency (II, III)

In fMRI, the largest and most consistent activation during the silent word-generation appeared in the left inferior frontal gyrus (Brodmann area 44), extending posteriorly up to the precentral gyrus (BA 6) (see Study II, Fig.1). These areas have also been repeatedly activated in studies using word-generation tasks (Yetkin *et al.*, 1995; Phelps *et al.*, 1997; Friedman *et al.*, 1998; Pihlajamäki *et al.*, 2000; Brannen *et al.*, 2001). Accordingly, the inferior frontal gyrus was chosen as the volume of interest for ^{1}H MRS data collection in Studies II and III.

In the tape-recorded word-generation sessions, no statistical difference appeared between age groups in the number of words produced in the alert state. Age-related differences were noted in the sleep-deprived state: the young subjects' performance remained almost at their initial level (alert vs. sleep-deprived 48 ± 13 vs. 47 ± 11 words), while the older subjects' performance declined abruptly (43 ± 9 vs. 28 ± 8 words, respectively) compared with their alert state.

5.4 Effects of silent word-generation on brain lactate (II-III)

5.4.1 Alert young subjects (II)

At the time this study was undertaken, several types of stimuli had been reported to induce temporary increases in local brain lactate levels. In humans, visual stimulation had been observed to induce a 50 to 150% increase in lactate (Prichard *et al.*, 1991; Sappey-Marinier *et al.*, 1992; Frahm *et al.*, 1996). In rats, brain lactate levels increased during electrical stimulation by 30 to 100% (Kuhr and Korf, 1988; Ueki *et al.*, 1988; Hu and Wilson, 1997), and during gentle stroking of the face, whiskers, and different parts of the body by 90% (Madsen *et al.*, 1999). The effects of cognitive stimulation on brain lactate levels have been documented only in dyslexic children (Richards *et al.*, 1999, 2000, 2002), not in healthy adults.

In Study II, silent word-generation by healthy alert subjects produced a 50% increase in lactate in their left inferior frontal gyrus when compared to their baseline lactate levels. The magnitude of this increase is reasonable when compared to increases reported for specific sensory or motor functions. While the specific sensory or motor functions can be associated with well-defined anatomical loci, the silent word-generation task most probably involves more extensive parallel processing; auditory, phonemic, visual, orthographic, and semantic processes as well as attention, memory, and motor programming of subvocal speech can all be involved (Friedman *et al.*, 1998), possibly resulting in a more diffuse and locally more modest lactate increase. Our results concerning local lactate levels cannot be directly compared to those obtained by Richards and colleagues, who focused on the regional distribution of lactate in dyslexic children (Richards *et al.*, 1997, 1999, 2000).

The increase in lactate was noted in all subjects (n=12) during the first 10.5-min block of silent word-generation. During the second and the third cognitive stimulation blocks, lactate levels remained on average elevated from baseline, but this elevation was non-significant (see

Study II, Fig. 6). Based on these results concerning the time course of the lactate elevation, only two subsequent blocks of silent word-generation were carried out in Study III. The maximal lactate peak during photic stimulation has been reported to occur earlier, at 2.5 mins after start of stimulation (Frahm *et al.*, 1996). It remains to be clarified whether this difference in timing of the maximal lactate reponse is due to the complex nature of the cognitive stimulus, to a lack of adaptation as compared with stimulation of the sensory system (Sappey-Marinier *et al.*, 1992), or to some other mechanism such as individual fluctuations in the performance of the task due to changes in attention and motivation.

The time window during which a lactate peak can be detected by MRS may also depend on the rate and degree of recoupling between astrocytic glycolysis and neuronal oxidative phosphorylation (Magistretti and Pellerin, 1999b). Recent studies utilizing techniques with better temporal and spatial resolution (Mangia *et al.*, 2003; Kasischke *et al.*, 2004; Pellerin and Magistretti, 2004) have shed light on the temporal and spatial organization of lactate production and oxidation: Neuronal activity seems to be accompanied by an early increase in neuronal oxidative metabolism and a decrease in lactate (the early dip, duration ~10 s), followed by astrocytic activation of glycolysis and increase in lactate (the overshoot) (Kasischke *et al.*, 2004).

As discussed in previous sections (2.3.2; 5.1.2), the exact origin and role of lactate during stimulation is unclear. The findings of Study II cannot distinguish between the conventional and the new theory as to the role of lactate in brain energy metabolism. However, this study did not involve hypoxic conditions, meaning that the elevation of lactate appears to be a physiologic process normally involved in neuronal activation; this finding supports the new theory.

5.4.2 Effects of aging and sleep deprivation (III)

Previous studies have suggested that unfavorable changes in brain energy metabolism occur during aging and during sleep deprivation (sections 2.3.3 and 2.3.4). Further, a functional impairment of the frontal cortex has been noted in both these conditions. The hypothesis was that aging as well as sleep deprivation alters, probably in a similar manner, the cognitive stimulation-induced brain lactate elevation in the frontal cortex. No prior studies seem to have assessed the effects of aging or of sleep deprivation on the brain lactate response to stimulation.

In Study III, the lactate response to stimulation differed between the older vs. young subjects: unlike in the young subjects, no significant increase in lactate was apparent in the inferior frontal gyrus of the older subjects during the two silent word-generation blocks of 10.5 mins each (lactate 120 ± 70% of the alert baseline levels; Fig. 10). This is compatible with the idea that brain energy metabolism changes during aging.

Also expectedly, the lactate response to stimulation differed between alert vs. sleep-deprived young subjects. Contrary to the alert baseline state, no significant lactate response could be observed during sleep deprivation. The lactate levels of the young at the subsequent time points (h after the first measurement) were: at 12 h, 140 ± 50%, at 24 h, 130 ± 50%, and at 36 h, 120 ± 50% of alert baseline level (time points 12 h and 24 h; unpublished data, Fig. 10). In the older group, similarly to the young, no significant lactate response appeared during sleep deprivation. Lactate levels were 120 ± 67% of the alert baseline value during silent word-generation at time-point 36 h (Fig. 10).

Although the main focus of Study III was brain lactate response to stimulation, the baseline (unstimulated) lactate levels also deserve some attention. The baseline lactate levels of the older subjects did not differ significantly from levels of the younger group. This is in contrast with previous findings (Sijens *et al.*, 2001a), but in accordance with others (Grachev and Apkarian, 2001). In the course of sleep deprivation, no significant change occurred in baseline lactate level of the inferior frontal gyrus (Fig. 10), in line with results from Study I: The baseline lactate increase is site-specific and possibly restricted to the BF area.

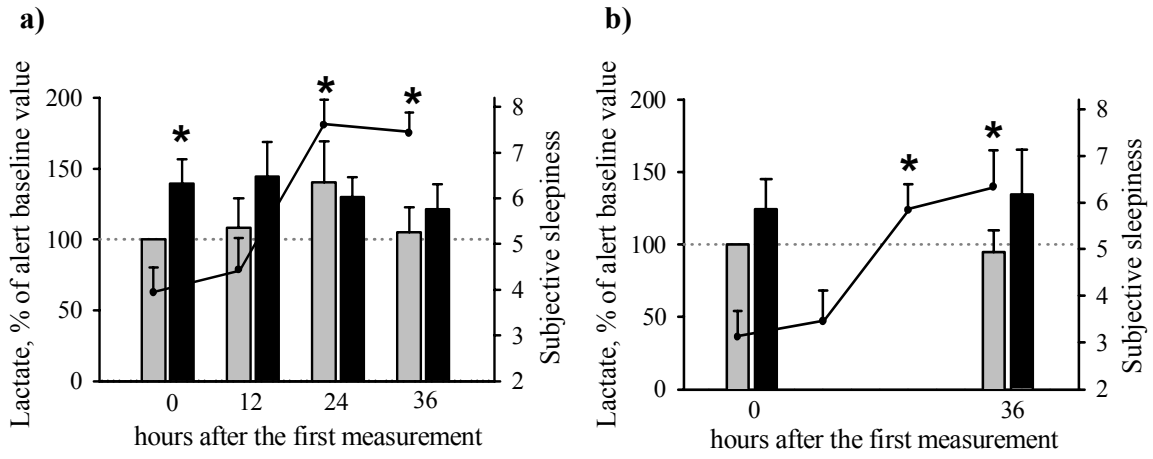

Figure 10. Lactate levels and subjective sleepiness of young (a) and older (b) subjects during the sleep deprivation period. Lactate levels are expressed as percentages of the alert baseline values (mean ± SEM). Gray bars denote baseline values, black bars values during silent word-generation. Subjective sleepiness (scale 1-10; 1=low, 10=high) in data points closest to the ^{1}H MRS lactate measurements are presented as line drawings (mean ± SEM). For lactate measurements, the asterisk denotes a significant difference in lactate level between baseline and silent word-generation (paired t-test; $p<0.05$). For subjective sleepiness ratings, asterisks denote significant differences from the first (time 0) data point (One Way Repeated Measures ANOVA, followed by Dunnett's test; $p<0.05$).

Based on the findings of Study II, the lactate response to stimulation seems to be a physiological response related to the increased energy demand of the neurons upon activation. The diminished lactate response during sleep deprivation and during aging can thus be interpreted as the inability of brain cells to respond normally to a stimulus-induced increase in energy demand. It can be speculated that the dysfunction of brain energy metabolism at least in part gives rise to the functional impairment of the frontal cortex during aging or during sleep deprivation or both. However, it can also be argued conversely that the functional impairment of the frontal cortex accounts for the diminished lactate response to cognitive stimulation during sleep deprivation or aging or both. This study cannot clearly distinguish between these two possibilities, which are tightly interconnected with the conventional and new theories of the role of lactate in brain energy metabolism.

It is also possible that the lactate response is diminished because of more diffuse brain activation in subjects who are older or sleep-deprived or both. In older vs. young subjects, a reduction in hemispheric asymmetry during memory tasks has been noted especially in the frontal lobes (the model of hemispheric asymmetry reduction in older adults, HAROLD) (Cabeza, 2002, 2004). Such hemispheric asymmetry reduction may result from attenuation in the response seen in younger subjects, an inability to activate specific brain areas, or an attempt to compensate for the age-related impaired performance by recruiting additional networks (Cabeza, 2002; Helmuth, 2002). Similarly, it is possible that the sleep-deprived

brain tries to maintain task performance by recruiting new brain areas (Drummond *et al.*, 2000), thus spreading, and locally possibly reducing the lactate response. Consistent with this idea, an increased area of brain lactate response has been noted in dyslexic children before, but not after treatment (Richards *et al.*, 1997, 1999, 2000).

The exact correlations between actual cognitive performance, the fMRI BOLD response, and brain lactate levels remain unknown. Based on our findings, however, the number of words produced in the tape-recorded sessions as well as the fMRI BOLD response (data not shown) was not affected by either aging or sleep deprivation as adversely as was the brain lactate response to stimulation. While fMRI offers an indirect estimation of brain metabolism, the measurement of lactate with ^{1}H MRS addresses the activity-bound neuronal energy metabolism more specifically. It is thus tempting to speculate that a diminished lactate response serves as an early warning sign of soon-to-appear ineffective performance. Accordingly, the decline in older subjects' cognitive performance during sleep deprivation (as shown by the decrease in the number of words produced in the tape-recorded sessions) may be caused by an initially more vulnerable brain energy metabolism (shown by their undetectable brain lactate response in the alert state). Yet, since monitoring task performance simultaneously with the ^{1}H MRS lactate measurements was not possible, further experiments are necessary to test the validity of this interpretation.

The results of Study III provide additional evidence to support the hypothesis that brain energy metabolism changes during sleep deprivation and during aging. The inability of the aged or the sleep-deprived brain to respond normally to cognitive stimulation can be interpreted as an increased vulnerability of the brain energy metabolism in these two conditions. These results also support the idea that sleep deprivation and aging share some common features. Many of the cognitive symptoms related to sleep deprivation are observed also in the course of healthy aging (Zec, 1995; Harrison *et al.*, 2000). Besides cognition, carbohydrate metabolism and endocrine function are also affected similarly during sleep deprivation and aging (Spiegel *et al.*, 1999).

5.5 Effects of sleep deprivation and neuronal activation on the main metabolites of the proton spectrum (III, IV)

Of the main metabolites of the proton spectrum, NAA and tCr are directly linked to brain energy metabolism, while Cho is related to membrane turnover. At the time this study was undertaken, changes in these metabolites had been observed not only in the structurally altered brain, but also in situations in which the structural brain MRI is normal (Lyoo and Renshaw, 2002; Mäkimattila *et al.*, 2004). Factors like gender, age, intelligence, and use-dependent adaptation of the brain affect metabolite levels (Wilkinson *et al.*, 1997; Jung *et al.*, 1999; Grachev and Apkarian, 2001; Aydin *et al.*, 2005). Based on these findings, it was hypothesized that short-term physiological changes as well, such as sleep deprivation or neuronal activation, affect ^{1}H MRS main metabolite levels. These main metabolite levels were the primary focus of Study IV, whereas in Study III their levels were assessed in conjunction with lactate levels. The effects of sleep deprivation on brain ^{1}H MRS main metabolite levels have not as yet been reported.

In Study III, no changes in NAA, tCr, or Cho levels were apparent in the inferior frontal gyrus during sleep deprivation or during silent word-generation.

In Study IV, sleep deprivation caused a 7% decrease in NAA/H_2O, and a 12% decrease in Cho/H_2O in the occipital cortex (see Study IV; Figs. 2 and 3). Sleep deprivation had no effect on tCr/H_2O. In the alert state, photic stimulation caused no changes in the NAA, tCr, or Cho ratios to water. In the sleep-deprived state, no changes in NAA/H_2O or tCr/H_2O were observable during photic stimulation, but Cho/H_2O increased by 10% during the first of the three blocks of photic stimulation (see Study IV; Table 1).

5.5.1 NAA

The decrease in NAA/H_2O in the occipital cortex during sleep deprivation was noted in seven of the eight subjects, and it occurred in successive measurements irrespective of the level of neuronal activity (i.e., baseline or photic stimulation). This decreased NAA/H_2O can be interpreted as a sign of either neuronal loss or neuronal dysfunction (Tsai and Coyle, 1995). Since it is unlikely that neuronal loss would have occurred (Cirelli *et al.*, 1999), the decrease in NAA/H_2O is probably a sign of neuronal dysfunction of the occipital cortical neurons during sleep deprivation.

Synthesis of NAA occurs in mitochondria with a turnover rate of 16.7 h (Baslow, 2002) and is directly coupled with glucose oxidation (Moreno *et al.*, 2001). NAA levels have been observed to decrease in concert with ATP levels, e.g., in the presence of inhibitors of oxidative phosphorylation (Bates *et al.*, 1996; Signoretti *et al.*, 2001). Our decrease in NAA/H_2O in the occipital cortex after 36 h of sleep deprivation agrees with the reported turnover rate of NAA, and supports the hypothesis of disturbed brain energy metabolism during sleep deprivation. More specifically, the decrease in NAA during sleep deprivation may result from impaired mitochondrial energy production of the neurons.

Decrease in NAA during such deprivation agrees with the reported decrease in whole brain cerebral metabolic rate for glucose (CMRglc) (Thomas *et al.*, 2000, 2003). This finding, however, is not entirely consistent with the distribution observed in regional changes in CMRglc, since no change has been observed in absolute regional CMRglc in the occipital cortex during sleep deprivation when compared to the rested baseline. Absolute regional decreases in CMRglc have occurred in the frontal and temporal areas, the thalamus, and the cerebellum (Wu *et al.*, 1991; Thomas *et al.*, 2000, 2003). Thus, based on these PET studies, a decrease in NAA would have been expected to occur in Study III, preferably in the frontal cortex.

Methodological differences may account for some of these contradictory results. CMRglc represents both neuronal and glial metabolism, whereas NAA is more specifically related to the energy metabolism of neuronal mitochondria. Since the glia may consume glucose at an even higher rate than do neurons (Magistretti and Pellerin, 1999b), it can be speculated that in the visual cortex during sleep deprivation, any decrease in neuronal metabolism (reflected in decreased NAA) may be accompanied by an increase in glial glucose consumption, which would render the CMRglc constant. This possibility could find support from the relatively high glial cell/neuron ratio in the visual cortex. The preliminary nature of Study IV should, however, be taken into account, and the findings should be replicated before more extensive interpretation of these data.

Interestingly, decreased NAA/Cho levels have been observed in normal-appearing brain tissue of obstructive sleep apnea patients. In children, this decrease has been noted in the left hippocampus and the right frontal cortex (Halbower *et al.*, 2006), and in adults mainly in the

deep white matter (Kamba *et al.*, 1997, 2001; Alchanatis *et al.*, 2004). Hypoxemia caused by repeated apneic episodes has been hypothesized to be the leading cause for the decrease in NAA in sleep apnea patients, but, based on our findings, fragmented sleep should also be considered as a contributing factor.

5.5.2 Cho

The decreased Cho/H_2O during sleep deprivation that then recovers to alert levels during photic stimulation may point towards a drop in membrane formation, or a diminution in membrane or myelin breakdown that is reversible by neuronal activity. It can be speculated that during sleep deprivation the stability of the cell membranes is altered: The cell membrane becomes more stable and rigid, but also more vulnerable than in the alert state, since the challenge imposed by photic stimulation alters its integrity, and either membrane degradation or remodeling occurs. A generalized decrease in membrane fluidity has been observed in REM-sleep-deprived rats, which supports this hypothesis (Mallick *et al.*, 1995). The interpretation of our finding is, however, complicated, since alterations in Cho may result from either anabolic or catabolic processes. Further, the changes in Cho can result from a combination of changes in many different metabolites contributing to the Cho signal. The major contributors to the Cho peak are phosphocholine and glycerophosphocholine, which cannot be separated with ^{1}H MRS *in vivo* (de Graaf, 1998). Despite difficulties in interpreting the changes in Cho, the significant increase in Cho/H_2O during photic stimulation in the sleep-deprived state supports the observations in Study III of altered brain metabolic response to stimulation during sleep deprivation as compared to the alert state.

5.5.3 tCr

Contrary to expectations, no changes were observed in the levels of tCr or tCr/H_2O during the sleep-deprivation period or during neuronal activation. Since ^{1}H MRS measures the sum of Cr and PCr, it is possible that some specific changes in the Cr/PCr equilibrium occurred, but were not distinguishable with ^{1}H MRS. However, the results from Studies III and IV are in line with previous results from ^{31}P MRS studies, where no changes in Cr or PCr were apparent after sleep deprivation (Murashita *et al.*, 1999b; Dorsey *et al.*, 2003).

Taken together, the effects of sleep deprivation on brain ^{1}H MRS main metabolites appear to vary between the frontal and occipital cortices. The decrease in NAA/H_2O during sleep deprivation in the occipital cortex supports the hypothesis of disturbed brain energy metabolism during sleep deprivation, and the decreased Cho/H_2O that recovers to alert baseline levels during sleep deprivation supports that of altered brain response to stimulation during sleep deprivation.

5.6 General discussion: methodological considerations

5.6.1 Subjects (I-IV)

One of the limitations of this study is its number of subjects (Studies II-IV). Relatively small sample sizes are, however, typical for sleep-deprivation studies requiring burdensome procedures such as PSG or frequent behavioral tests during the sleep-deprivation period. Furthermore, the research protocols in Studies II to IV included MRS measurements, which also are highly labor-intensive and costly, as well as relatively new to the field of sleep

research. Based on these facts, Studies III and IV may be considered as pioneering but preliminary in nature.

The generalizability of the results of Studies II to IV is limited, since only female subjects were studied. On the other hand, a similar limitation applies to Study I, with male animals only. Studies II to IV belonged to a larger EU-funded research project "Sleep in aging women" aimed at increasing knowledge of women's sleep, and at promoting the use of female subjects in sleep research, motivated by the fact that sleep and sleep deprivation have not been extensively studied in women. Further, the detection of minor metabolite differences in Studies II to IV probably benefited from the use of a very homogenous group of volunteers with low inter-individual variation.

Study of women raises the question of the possible effects of fluctuating hormone levels across the menstrual cycle and of women's life span. Preliminary evidence suggests that menstrual cycle phase may indeed influence the neurochemistry of the brain as assessed by ^{1}H MRS (Rasgon *et al.*, 2001). In Studies II to IV, the phase of the menstrual cycle of the young women was always standardized, and the older women were postmenopausal, with hormone therapy forbidden during the study.

Gender differences exist in language skills and in the organization of language-processing brain regions (Shaywitz *et al.*, 1995; Harasty *et al.*, 1997). Further, changes in brain energy metabolism during aging (Sijens *et al.*, 2001a, 2001b, 2003) and during stimulation (Kastrup *et al.*, 1999) may differ between male and female subjects. As a consequence, it is possible that the metabolic responses to silent word-generation as well as photic stimulation would have been different in male subjects.

Age-related cortical atrophy could have influenced the results in Study III. The subjects were, however, carefully screened to be healthy, and their brain MRIs were examined by an experienced neuroradiologist. In the MRIs of the older group of women, mild vascular degeneration was visible in two subjects, and the degree of atrophy was either milder than normal or normal. To avoid the decreased sensitivity caused by the partial volume effect from CSF, voxel size and placement were individually adjusted. Spectral quality in terms of Cr SNR was 15% lower in the older than in the young subjects, but this was partly explained by the minor difference in voxel size between age groups. More importantly, no difference in lactate SNR appeared in the alert baseline condition.

5.6.2 Application of ^{1}H MRS to sleep research (II-IV)

Since Studies III and IV are among the first to apply ^{1}H MRS to sleep research, some general methodological aspects need to be considered.

The reproducibility of ^{1}H MRS has been considered adequate for serial studies of brain metabolism in baseline (non-stimulated) conditions (Brooks *et al.*, 1999). Intra-subject reproducibility of lactate measurement with ^{1}H MRS during stimulation has not been widely tested, but Richards and colleagues (2002) have reported good intersubject reproducibility of the lactate response during a language task in two groups of dyslexic children. Our separate *in vitro* brain phantom measurements also indicated the adequate reproducibility and stability of the MR system (results not shown). It is thus unlikely that the differences observed in metabolic responses were a result of poor reliability.

In none of the ^{1}H MRS studies, absolute quantification was performed, which can be counted as one of the weaknesses of these studies. In all cases, however, the subjects served as their own controls, and thus no absolute quantification of the metabolite levels was necessary. Studies II and III used "raw" metabolite levels, but Study IV metabolite ratios to water. Despite the benefits of the ratio approach (section 2.5.3.), its use limits interpretation of the data, since the changes seen in NAA/H_2O and Cho/H_2O may have at least in part, originated from changes in H_2O content and relaxation. However, a change in brain water content would have probably also resulted in changes in tCr/H_2O.

Although ^{1}H MRS offers a unique possibility to study endogenous metabolites of the human brain *in vivo*, its sensitivity as well as its temporal and spatial resolutions are not optimal. The detection limit for lactate is around 1mmol/l (Prichard *et al.*, 1991), and, as a consequence, a set amount of lactate can be produced in the target area without its being detected with ^{1}H MRS. Further, a number of potentially interesting metabolic compounds—including pyruvate, adenosine, and most of the classical neurotransmitters—cannot be detected with the present ^{1}H MRS methods.

Unlike microdialysis, ^{1}H MRS measures total metabolite levels and does not distinguish between the intra- and extracellular spaces, which limits interpretation of these results. Additionally, many brain areas are unsuitable for ^{1}H MRS measurements either because of their small size (e.g., specific brainstem nuclei), or of their proximity to certain anatomical structures like bone tissue (with different magnetic susceptibility than that of brain tissue), CSF of brain ventricles (causing inhomogeneity in the voxel), or extracranial lipids (contaminating the spectra). In particular, it would have been of great interest to study lactate levels in the human basal forebrain during sleep deprivation, but this was impossible because of the proximity of the skull base bony structures.

Monitoring sleep during ^{1}H MRS is not easy. In principle, it is possible to record EEG during ^{1}H MRS, but in practice it is technically demanding, requires special equipment, and has not been widely tested. Furthermore, EEG recording during fMRI has caused the fMRI SNR to decrease by 20 to 30% compared to fMRI SNR without simultaneous EEG (Lazeyras *et al.*, 2001). Usually, MRS is even more susceptible to interference than is fMRI. Because of these difficulties, in Studies II and IV, wakefulness of the subjects was controlled during ^{1}H MRS by frequent contact via a microphone. As estimated from the absence of responses, the maximum evaluated sleep time inside the magnet was less than 5% of the duration of data collection, which excludes any significant effect of sleep on the results.

5.6.3 Choice of cognitive task (II-III)

The aims of Studies II and III required the use of a prefrontal cortex-oriented cognitive task, which produces a relatively stable and pronounced brain activation pattern. In addition, the task had to be repeatable and easy to perform inside the MR scanner during the entire ^{1}H MRS data-collection period. Choice of the silent word-generation task was based on these requirements.

Silent performance of the word-generation task reduces head motion during the measurement, and produces a greater activation than when the words are vocalized (Yetkin *et al.*, 1995). However, silent word-generation does not allow objective monitoring of task performance. Loss of motivation or the learning effect or both are possible explanations for the lack of lactate response during sleep deprivation (III). Efforts were made to minimize both of these

phenomena. The tasks were rehearsed prior to sleep deprivation in order to attain stable performance during the ^{1}H MRS measurements, and the letter and category tasks were used alternately to subtly modulate the task and thus avoid boredom. The ability of the subjects to perform the word-generation task was controlled with a tape-recorded task immediately after ^{1}H MRS.

5.6.3 Timing of the measurements and the circadian component (III-IV)

The ^{1}H MRS measurements were performed at 12-h intervals for the young in Study III. Since the data from the young subjects showed the clearest difference between the first (morning) and the last (evening) ^{1}H MRS session, we chose to perform the strenuous and labor-intensive MR measurements for the older subjects and the subjects in Study IV only at those times of the sleep-deprivation period.

Hypothetically, the metabolite changes in Studies III and IV may reveal some effect of physiological morning-evening differences in metabolite levels. In particular, decreased NAA/H_2O levels (IV) after sleep deprivation may in part arise from a circadian change in occipital lobe glucose metabolism, which has indeed been observed in a preliminary study (Buysse *et al.*, 2004). Further research is required to clarify the contribution of circadian time to brain metabolite changes assessed by ^{1}H MRS. In addition, it will be important to assess the effects of recovery sleep on ^{1}H MRS metabolite levels.

5.6.4 Future perspectives

The findings presented in this thesis can not provide a comprehensive answer to the multifaceted question of the relationship between sleep deprivation and brain energy metabolism. Many interesting aspects of this relationship still remain to be clarified.

Naturally, in future studies, the preliminary findings reported in this thesis need to be replicated. Further, ^{1}H MRS can be applied during sleep deprivation to study different cognitive tasks, different types of sensory stimulation, and different brain areas—as well as rats or other animal species. Brain samples from sleep-deprived animals might also be studied with *in vitro* applications of ^{1}H MRS.

After the publication of Study I, several researchers have pursued work in clarifying biochemical events occurring in the BF during sleep deprivation. Notably, the changes in energy metabolism of the BF reported in Study I have been recently confirmed (Kalinchuk *et al.*, 2006a), with a mechanism potentially accounting for the local energy depletion proposed. Inducible nitric oxide synthase is induced in the BF during sleep deprivation (Kalinchuk *et al.*, 2006b), leading to an increase in the level of nitric oxide (Kalinchuk *et al.*, 2006a). Nitric oxide acts as an inhibitor of the mitochondrial electron transfer chain (Clementi *et al.*, 1998), and its increase may thus be linked to the local accumulation of adenosine, lactate, and pyruvate in the BF during sleep deprivation.

In human studies, one of the major aims of future studies will be to correlate more precisely the changes occurring in brain energy metabolism with the behavioral deficits observed during sleep deprivation, and to assess the extent to which inter-individual differences in brain energy metabolism may explain inter-individual differences in vulnerability to sleep deprivation. Recent evidence suggests that inter-individual differences in brain activation correlate with the decline in working-memory performance accuracy during sleep deprivation

(Chee *et al.*, 2006). A similar analysis of the correlation between changes in ^{1}H MRS metabolites and decrements in cognitive performance or in visual vigilance requires a larger and more heterogenous group of volunteers. Finally, although of major interest, knowledge of the interrelationship between brain and whole body energy metabolism during sleep deprivation is still incomplete, and should be studied perhaps by combining brain imaging and blood sampling procedures.

6. CONCLUSIONS

The main results and conclusions of this study can be summarized as follows:

I. Local energy depletion in the basal forebrain mimicked the effects of sleep deprivation leading to increased extracellular concentrations of adenosine, lactate, and pyruvate, and by elevating subsequent sleep. During sleep deprivation, the waking-promoting cells in the basal forebrain can be hypothesized to be continuously active and thus beginning to suffer from energy depletion, resulting in the increase of these metabolites. The increase in adenosine may contribute to the subsequent increase in sleep by acting as a sleep-inducing agent.

II. Silent word-generation induced an increase in lactate levels in the inferior frontal gyrus, showing that metabolic imaging of neuronal activity related to healthy adult cognition is possible with ^{1}H MRS. This finding supports the theory that lactate elevation is a physiologic process normally involved in neuronal activation.

III. During sleep deprivation and in alert older subjects, no cognitive stimulation-induced increase in lactate appeared. This absence of lactate response in the sleep-deprived and in the aging subjects can be interpreted hypothetically as a sign of malfunctioning brain energy metabolism leading to functional impairment of the frontal cortex.

IV. NAA/H_2O decreased during sleep deprivation in the occipital cortex, supporting the hypothesis of disturbed brain energy metabolism during sleep deprivation. Cho/H_2O decreased during sleep deprivation but recovered to alert levels during photic stimulation. The latter result supports earlier observations of altered brain response to stimulation during sleep deprivation.

Taken together, site-specific- and neuronal activity-dependent effects of sleep deprivation on brain energy metabolism were evident. Although effects of sleep deprivation were not detectable in all cases in the non-stimulated baseline state, the challenge imposed by cognitive or photic stimulation revealed significant changes. This suggests that brain energy metabolism is more vulnerable during sleep deprivation than after a night of normal sleep.

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