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INTENSE EXERCISE INCREASES ADENOSINE CONCENTRATIONS IN RAT BRAIN: IMPLICATIONS FOR A HOMEOSTATIC SLEEP DRIVE

M. DWORAK,[a]* P. DIEL,[b] S. VOSS,[c] W. HOLLMANN[d] AND H. K. STRÜDER[a]

[a]*Institute of Motor Control and Movement Technique, German Sport University Cologne, Carl-Diem-Weg 6, 50933 Cologne, Germany*

[b]*Institute of Molecular and Cellular Sports Medicine, German Sport University Cologne, Carl-Diem-Weg 6, 50933 Cologne, Germany*

[c]*Institute of Biochemistry, German Sport University Cologne, Carl-Diem-Weg 6, 50933 Cologne, Germany*

[d]*Institute of Cardiology and Sport Medicine, German Sport University Cologne, Carl-Diem-Weg 6, 50933 Cologne, Germany*

Abstract—Intense exercise and sleep deprivation affect the amount of homeostatically regulated slow wave sleep in the subsequent sleep period. Since brain energy metabolism plays a decisive role in the regulation of behavioral states, we determined the concentrations of nucleotides and nucleosides: phosphocreatine, creatine, ATP, ADP, AMP, adenosine, and inosine after moderate and exhaustive treadmill exercise as well as 3 and 5 h of sleep deprivation and sleep in the rat brain using the freeze-clamp technique.

High intensity exercise resulted in a significant increase of the sleep-promoting substance adenosine. In contrast, following sleep, inosine and adenosine levels declined considerably, with an accompanied increase of ADP after 3 h and ATP after 5 h. Following 3 h and 5 h sleep deprivation, ADP and ATP did not differ significantly, whereas inosine increased during the 3 and 5-h period. The concentrations of AMP, creatine and phosphocreatine remained unchanged between experimental conditions.

The present results are in agreement with findings from other authors and suggest that depletion of cerebral energy stores and accumulation of the sleep promoting substance adenosine after high intensity exercise may play a key role in homeostatic sleep regulation, and that sleep may play an essential role in replenishment of high-energy compounds.

Key words: physical activity, sleep deprivation, nucleotides, nucleosides, adenosine, homeostatic regulation.

Physical exercise is known to impact on nearly every system of the body, including the brain (Cotman and Engesser-Cesar, 2002; Cotman and Berchtold, 2002). Abundant experimental evidence strongly suggests that dynamic physical exercise produces elevated regional cerebral blood flow (CBF), alterations in endogenous peptides, increased amino acid transport through the blood–brain-barrier and neurotransmitter alterations (Herholz et al., 1987; Hollmann et al., 1994; Ide et al., 1999). Dynamic exercise stimulates the formation of synapses and neuronal spines, promotes neurogenesis and improves cognitive brain functions and age-related degeneration processes (Cotman and Berchtold, 2002). However, the relationship between physical exercise and sleep in humans is not completely understood. Only high intensity exercise affects the amount of homeostatic-regulated slow wave sleep in the subsequent sleep period (Shapiro et al., 1981; Dworak et al., in press). The reason for this phenomenon is still unclear. Modern neuroscientific theories support the hypothesis that the brain energy metabolism and specific neurotransmitter systems play a decisive role in mammalian sleep regulation (Benington and Heller, 1995; Maquet, 1995).

Recent evidence showed that prolonged sleep deprivation (SD) results in spatial and temporal alterations in brain glycogen levels (Kong et al., 2002; Gip et al., 2002; Franken et al., 2003, 2006). Slow-wave sleep is thought to be essential for glycogen replenishment, since brain energy metabolism is dramatically decreased in this state (Nofzinger et al., 2000; Arrigoni et al., 2006). Discrepancies between energy demand and energy supply result in increased extracellular adenosine concentrations and reflect an energy deficit during prolonged wakefulness (recently reviewed in Basheer et al., 2004). Adenosine plays an important role in regulation of blood flow, synaptic transmission and neuronal excitability (Latini and Pedata, 2001). Stimulation of neuronal adenosine receptors mediates presynaptic inhibition in the transmitter release of neuronal networks involved in the regulation of wakefulness (Rainnie et al., 1994; Arrigoni et al., 2006) as well as post-synaptic hyperpolarization that regulates behavioral states (Basheer et al., 2004; Latini and Pedata, 2001). Thus, this nucleotide is a product of cerebral energy consumption and a potential sleep-promoting factor in the CNS (Porkka-Heiskanen et al., 2000).

Since physical exercise is narrowly linked with neuronal metabolic activity, CBF, neurotransmitter alterations and increased brain temperature (Nybo and Secher, 2004), it can be assumed that intense physical exercise is a potential stimulus to reduce cerebral energy compounds, increase brain adenosine levels and promote subsequent sleep. SD is also known to increase the homeostatic drive to sleep (Tobler and Borbely, 1990) and raise local extracellular adenosine level (Basheer et al., 2004; Latini and Pedata, 2001). Therefore we used both, physical exercise and SD to examine the effects on brain energy metabolism.

*Correspondence to: M. Dworak, Institute of Motor Control and Movement Technique, German Sport University Cologne, Carl-Diem-Weg 6, 50933 Cologne, Germany. Tel: +49-0-221-4982 4200.
E-mail address: dworak@dshs-koeln.de (M. Dworak).

Abbreviations: ANOVA, analysis of variance; CBF, cerebral blood flow; Cr, creatine; EEG, electroencephalography; EX, exercise group; iEX, intense exercise group; mEX, moderate exercise group; PCr, phosphocreatine; SD, sleep deprivation.

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In the present study we tested the hypotheses that physical exercise and SD decrease phosphocreatine (PCr) and ATP levels in the rat brain, that physical exercise and SD increase adenosine levels in the rat brain, and that duration and intensity of the exercise, sleep and SD sessions are decisive for the extent of these alterations.

EXPERIMENTAL PROCEDURES

Animals

All procedures were conducted in accordance with the European Union Guidelines for the Care and Use of Laboratory Animals and undertaken with the approval of the regional administration of the governmental body. All experiments conformed to named local guidelines on the ethical use of laboratory animals and were conducted to minimize the number of laboratory animals and their suffering. Male adult Wistar rats (70 days old), weighing 376.7±72.3 (sd) g, were housed under constant temperature (22 °C) on a 12-h light/dark cycle (lights on from 07:00 h to 19:00 h). Food and water were provided *ad libitum*. After a 1-week acclimation period, rats were randomly divided into the following groups: Control (C, $n=18$), SD ($n=12$) and exercised (EX, $n=12$). Furthermore EX rats were randomly assigned to one of the following two subgroups: moderate exercise (mEX) and intense exercise (iEX). Considering that the brain is one of the most active tissues in terms of nucleoside and nucleotide synthesis, and that after removal metabolites are very unstable during ischemia, we used freeze-clamp technique to freeze brain tissue immediately and prevent enzymatic activity (Palladino et al., 1980; Helzberg et al., 1987). The tissue was rapidly clamped between aluminum blocks, pre-cooled in liquid nitrogen, and frozen immediately to a temperature approximating that of liquid nitrogen. Studies showed that freeze-clamp measurements correlated well with focused microwave irradiation (Beal et al., 1993) and nuclear magnetic resonance (NMR) data (Camacho et al., 1988).

Exercise session

Animals were familiarized with a motor-driven treadmill for 3 days, 5 min/day, on a 10% grade. On experimental days, both EX-groups performed an acute bout of treadmill running. For the mEX group the running speed was initially 15 m/min and was gradually increased to 20 m/min at 10% grade for 30 min. Intense treadmill exercise was initially performed at 15 m/min and increased to 25 m/min for a maximum of 60 min to exhaustion.

SD

SD was achieved by the gentle handling method, as described earlier (Franken et al., 1991). Rats were inspected continuously and kept awake by introducing and removing objects from the cage, and after a prolonged period of wakefulness by tapping lightly the cage or touching the animal with a paintbrush. SD of 3 h ($n=6$) or 5 h ($n=6$) durations were started at 7:00 AM and were performed under lights-on conditions. Both SD groups had their own control group ($n=2\times6$) consisting of animals kept undisrupted under the same conditions. Electroencephalography (EEG) was not monitored but the criteria for sleep behavior were identical with other studies (Tobler and Borbely, 1990). Control animals were allowed to sleep *ad libitum* and spared from any physical activity. The unanesthetized animals were decapitated at three different times across a 24-h day. The sampling time points were: 7:00 AM, 10:00 AM and 12:00 AM. The brains were rapidly removed, frozen by freeze-clamp-technique, placed in liquid nitrogen and stored at −85 °C.

Biochemical analyses

All reagents were of the highest purity available. Adenosine, creatine (Cr), PCr, and ADP were purchased from Fluka (Sigma-Aldrich, Taufkirchen, Germany). Inosine, AMP and ATP were purchased from Sigma-Aldrich. The frozen brain was weighed and homogenized in 1.0 M $HClO_4$/50 mg. The protein precipitate was spun off, and the supernatant was neutralized with 2.0 M K_2HPO_4/50 mg. The supernatant was utilized for the determination of inosine, adenosine, AMP, ADP, ATP, Cr and PCr levels by HPLC/UV detection with comparison to known standards as described earlier (Helzberg et al., 1987).

Data analysis

Reported values are means±sd from at least six rats per group. Statistical analysis of the nucleotide and nucleoside data was performed with one-way analysis of variance (ANOVA), followed by the Bonferroni test as a post hoc test to compare interactions between the groups using SPSS 12.0 (SigmaStat Statistical Software, Chicago, IL, USA) and STATISTICA 7.1. software (StatSoft, Tulsa, OK, USA) for MS Windows. All statistical tests were considered significant if $P<0.05$.

RESULTS

Metabolite changes after exercise

Nucleotide and nucleoside concentrations in the rat brain were measured after “lights-on” (baseline), 3 h and 5 h (control) of sleep, moderate and intense exercise as well as after 3 h and 5 h SD. Resting values (control) were defined after 5 h of undisrupted sleep.

After high intensity exercise adenosine and inosine levels were significantly elevated (ANOVA $F=5.67$; $P=0.0003$ and

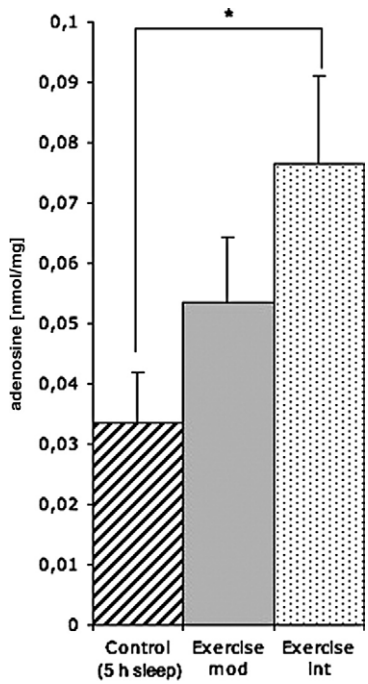

Fig. 1. Adenosine concentrations measured after control conditions (5 h sleep), moderate (mEX) and high intensity (iEX) exercise. Intense exercise increase adenosine concentrations significantly (* $P<0.05$) related to sleeping controls.

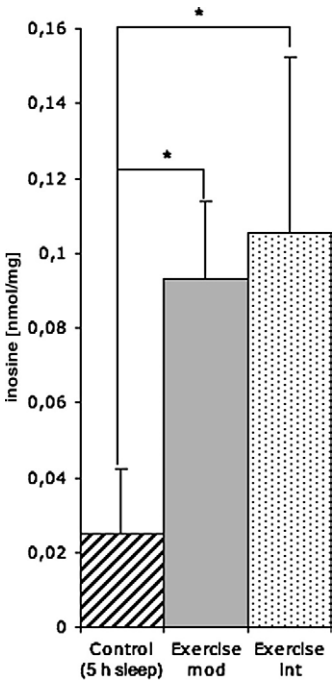

Fig. 2. Whole brain inosine concentrations measured after control conditions (5 h sleep), moderate (mEX) and high intensity (iEX) exercise. Inosine concentrations were significantly elevated (* $P<0.05$) after the moderate and high intensity exercise session.

$F=8.43$; $P=0.00001$) and reached 229.04% and 425.21% of the control levels respectively. Only the intense, not moderate exercise session resulted in a significant (ANOVA $F=5.67$; $P=0.0003$; post hoc $P=0.0002$) increase of adenosine when compared with resting controls (5 h sleep). In the iEX group, ADP and ATP concentrations were 0.05 ± 0.01 nmol/mg and 0.03 ± 0.01 nmol/mg without any statistical significance. Figs. 1 and 2 depict the level of adenosine and inosine for the mEX and iEX groups. A significant ($F=31.30$; $P=0.0002$; post hoc $P=0.0036$) increase of inosine was also observed after the mEX session, when compared with resting controls (5 h sleep). The respective concentrations for ATP and adenosine in the moderate exercised group were 0.09 ± 0.04 nmol/mg and 0.03 ± 0.01 nmol/mg, and were not statistically significant relative to control conditions. The concentrations of AMP, Cr and CrP did not differ significantly between the experiments.

Metabolite changes after SD and resting conditions

Total brain adenosine concentrations did not change after 3 and 5 h of SD. Also, comparisons of total brain AMP, Cr and CrP showed no significant changes between the groups and time points of measurement.

Figs. 3 and 4 depict the changes of total ATP and ADP concentrations after 3 and 5 h of sleep. Significant increases in brain ADP concentrations after 3 h (ANOVA $F=3.71$; $P=0.060$; post hoc $P=0.042$) and ATP concentrations after 5 h (ANOVA $F=5.17$; $P=0.0006$; post hoc $P=0.0039$) of sleep were observed. In addition, significant declines in inosine and adenosine concentrations were observed in a duration-dependent manner. Inosine concentrations showed a significant (ANOVA $F=8.43$; $P=0.00001$) progressive decline to 57.42% and to 29.22% of the baseline values after 3 h and 5 h of sleep (Fig. 5). Brain adenosine concentrations were also significantly reduced when compared with the intense exercised rats (ANOVA $F=5.67$; $P=0.0003$; post hoc $P=0.0002$). Respective values for Cr and PCr were 9.60 ± 1.10 and 0.39 ± 0.10 nmol/mg under baseline conditions and 5.93 ± 2.42 and 0.61 ± 0.27 nmol/mg after 5 h sleep, without any statistic significance when compared with control conditions.

DISCUSSION

The present study demonstrates that, sleep and SD conditions, in contrast to intense physical exercise increase total brain adenosine and inosine concentrations. Undisrupted sleep resulted in significantly elevated ADP and ATP concentrations as well as a progressive decline of inosine and adenosine in a duration-dependent manner. SD (3 h and 5 h) did not affect brain ATP and adenosine concentrations significantly. However a great increase of inosine between the 3- and 5-h deprivation period was observed. The present results are in congruence with other studies concerning the effects of exercise and sleep on the brain energy metabolism, providing supplementary evidence for a homeostatic regulation of brain energy stores and the role of sleep in replenishment of brain energy compounds.

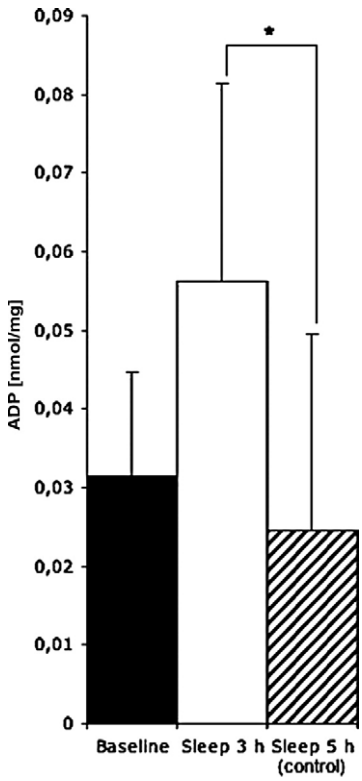

Fig. 3. ADP concentrations measured after Baseline conditions, 3 h and 5 h (control) of sleep. Baseline measurements were performed at beginning of "lights-on"-conditions, i.e. after the active period. ADP concentrations increase significantly (* $P<0.05$) after 3 h of sleep when compared with the 5-h sleep period.

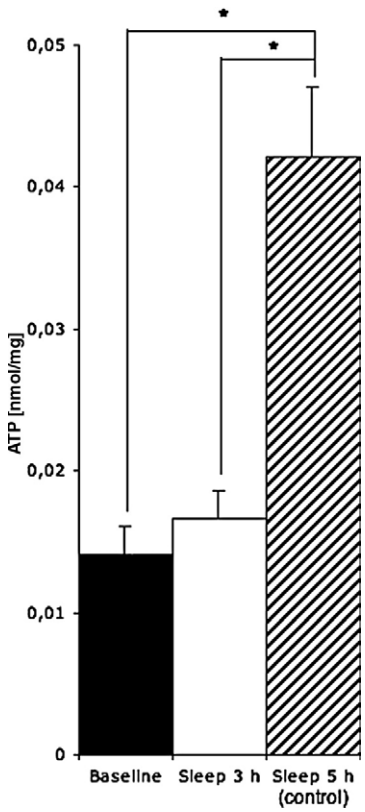

Fig. 4. ATP concentrations measured after baseline conditions, 3 h and 5 h (control) of sleep. After 5 h of sleep ATP concentrations were significantly higher (* $P<0.05$) related to basal values and 3 h of sleep.

Brain energy metabolism during exercise

Physical exercise is known to impact nearly every system of the body, including the brain (Hollmann et al., 1994; Cotman and Engesser-Cesar, 2002). Dynamic physical exercise produces an elevated regional CBF (Herholz et al., 1987; Ide and Secher, 2000), alterations in endogenous peptides, amino acid transport through the blood–brain-barrier and neurotransmitter alterations (Hollmann et al., 1994). Furthermore it stimulates the formation of synapses and spines as well as neurogenesis, improves cognitive brain functions and age-related degeneration processes (Cotman and Engesser-Cesar, 2002; Cotman and Berchtold, 2002). Physical exercise is narrowly linked with neuronal activity (Ide and Secher, 2000). Previous studies have shown that dynamic movements are associated with cortical activation and increases in blood flow to the primary sensorimotor area and supplementary motor area (Orgogozo and Larsen, 1979). The cerebral metabolic rate for glucose determined after running indicates an involvement of the hypothalamus, the posterior parietal, the temporoparietal, the prefrontal, the premotor and the primary motor cortex (Orgogozo and Larsen, 1979). The magnitude of brain activation increases with the intensity of exercise (Williamson et al., 1999) and the brain may become maximally stimulated when exercise is performed at a level near to exhaustion (Kayser, 2003). Additionally, whole brain metabolic activity increases (Ide and Secher, 1999; Williamson et al., 1999) since the increased motor command results in elevated metabolic rates in the activated brain structures associated with exercise execution (Kayser, 2003). During intense brain activation neurons prefer lactate to glucose as their primary energy substrate, which raises the production of ATP (Schurr, 2006). High-intensity exercise increases the ratio of metabolite demand to metabolite availability with an accompanied production of adenosine from AMP. We found significantly elevated adenosine and inosine concentrations after intense exercise, relative to moderate exercise, SD and resting conditions. Also, previous studies showed significantly higher adenosine concentrations in the rat neostriatum and in the hippocampus during the dark period (active period of rats) than during the light period, a finding that was interpreted as association of adenosine concentrations with motor activity (Huston et al., 1996). The observed findings of increased adenosine concentrations might reflect a state of bioenergetic stress, possibly as a result of an increased breakdown of high-energy phosphates.

Brain energy metabolism during SD

SD is assumed to affect the brain proportionally more than any other organ in the body. In the rat, SD resulted in a unique appearance of peripheral symptoms, including increases in whole body energy expenditure and immunodeficiency that develops progressively and could be lethal after about 19 days (Rechtschaffen et al., 1983; Rechtschaffen and Bergmann, 1995). In the present study SD was induced under light-on conditions, directly after the dark period, for 3 h and 5 h to avoid stressing the animals by prolonging the SD, because acute and chronic stress might affect brain energy metabolism. Additionally, previ-

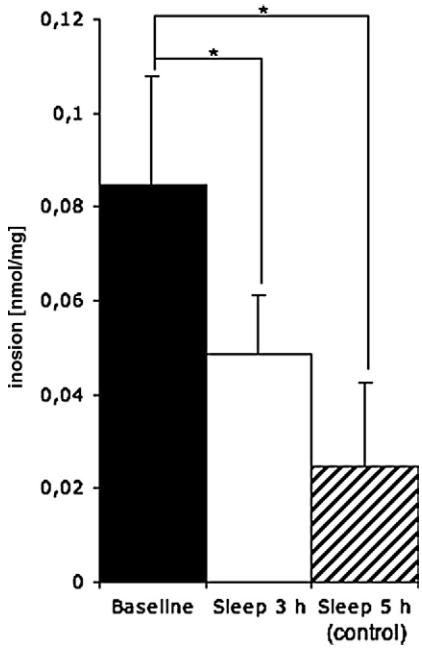

Fig. 5. Whole brain inosine concentrations measured after baseline, 3 h and 5 h (control) of sleep. Inosine concentrations were significantly reduced (* $P<0.05$) after 3 and 5 h of sleep.

ous experiments showed that during the dark phase rats normally sleep 25–35% of the time, whereas during the first half of the light phase rats normally sleep 60–80% of the time (Tobler and Borbely, 1990). Thus, it can be estimated that 3 h of SD during light-on conditions caused on average 2.1 h lack of sleep whereas 5 h SD caused in average 3.5 h lack of sleep.

Whole adenosine concentrations did not differ significantly after 3 h and 5 h of SD. This may very well be due to whole brain measurements of nucleotides and nucleosides using the freeze-clamp technique. It is well known that the SD-induced increase in adenosine in the first few hours is limited to the basal forebrain area (reviewed in Basheer et al., 2004). Porkka-Heiskanen et al. (2000) showed accumulation of extracellular adenosine in a site-specific manner after prolonged wakefulness, selectively in the basal forebrain and to a lesser extent in the cortex, while in other subcortical structures the concentrations tended to decline. Since total adenosine concentrations (extra- and intracellular) in the rat brain were examined in the present study, our data provide a net effect for adenosine in the whole brain and may dilute the effects that occur in localized areas. Thus, the present results are in accordance with the observation that changes in extracellular adenosine during SD may be a regionally specific phenomenon. However, during intense exercise it seems that rise in adenosine levels is rather global, since whole brain measurements show a significant increase in brain adenosine concentrations. Also, it could be assumed that the duration of SD was not long enough to increase metabolic demand and total adenosine concentrations. In a recent PET study it was shown that after 24 h SD in humans cortical A1 receptor binding is increased, presumptively because of adenosine increases in the cortex at this time point (Elmenhorst et al., 2007). Possibly longer periods of SD might show evidence for adenosine changes in other brain areas.

In addition, after SD no changes in total brain ADP and ATP concentrations were observed. Previous studies have produced contrasting results with respect to the regulation of glucose and glycogen metabolism during SD. It was shown that the mean rate of glucose utilization in the brain remained unchanged between sleep-deprived rats and yoked controls (Everson et al., 1994). However, significant reductions in the hypothalamus, thalamus, and to a lesser extent in the limbic system were observed after SD (Everson et al., 1994). SD studies with longer durations (12 or 24 h) resulted in significantly decreased brain glycogen levels in white and gray matter that reversed to basal conditions during recovery sleep (Kong et al., 2002). Spatial differences were also observed in other studies. After 6 h SD, reductions in glycogen concentrations were only detectable in the cerebellum but not in the cortex of young rats (Gip et al., 2002). These results were confirmed in a following study with significant declines in glycogen content in the cerebellum and hippocampus, but not in the cortex and brain stem (Gip et al., 2002). In AKR/J and DBA/3J mice, glycogen content significantly decreased in the cerebellum and brain stem but did not change in the cortex after 6 h SD whereas significant increases were observed in C57BL/6J (B6) mice (Franken et al., 2003, 2006).

These contrary findings support the notion that metabolic changes in the brain occur in a spatial and temporal manner and are in accordance with our results, since we found no evidence for changes in total brain adenosine concentrations following SD (3 h and 5 h). However it could be assumed that the dynamics of the changes in brain energy metabolism in relation to sleep and wakefulness are complex and affected by age, genotype and brain region (Franken et al., 2003).

Brain energy metabolism and sleep regulation

Sleep is homeostatically regulated and strongly correlated with adenosine metabolism (Basheer et al., 2004; Porkka-Heiskanen et al., 2002). The primary effect of the increase in adenosine in cholinergic basal forebrain is on the ensuing sleep as indicated by an increase in delta power during slow wave sleep (Basheer et al., 2000; Porkka-Heiskanen et al., 1997; Alam et al., 1999). The increased levels of EEG delta frequency are suggested to predict the intensity of sleepiness based on the duration of prior wakefulness (Borbely and Achermann, 2000). Neuronal downscaling during sleep reflects reductions in metabolic rate indicating that during slow-wave sleep brain energy expenditure decreases, (Kennedy et al., 1981; Netchiporouk et al., 2001) CBF (Braun et al., 1997) and cerebral metabolic rate decrease, while glucose (Nofzinger et al., 2000) and ATP concentrations increase (Van den Noort and Brine, 1970). The great increase of brain ADP levels after 3 h, and brain ATP levels after 5 h sleep in the present study might reflect an assembly of high-energy compounds. Since wakefulness results in a greater metabolic activity than sleep, our results are in accordance with the hypothesis that brain energy stores are replenished during sleep (Benington and Heller, 1995).

Additionally, it is assumed that physical exercise has a sleep-promoting effect (Shapiro et al., 1981; O'Connor and Youngstedt, 1995). Previous studies showed that only high-intensity exercise results in higher sleep efficiency, shorter sleep onset latency and proportional elevated levels of slow-wave sleep, while no effects after moderate exercise could be shown (Shapiro et al., 1981; Dworak et al., in press). If the role of adenosine as a sleep-promoting factor is taken into account, the results of the present study could provide a simple explanation for the sleep-promoting role of high intensity exercise. Our results suggest that only high intense exercise resulted in significant elevated adenosine and inosine concentrations, whereas no changes in these nucleosides were found after moderate exercise. These observations were supported by a recent *in vivo* microdialysis study, showing that minimal exercise did not affect adenosine concentrations in the basal forebrain (McKenna et al., 2007).

The relationship between energy depletion and sleep induction has been addressed in previous studies (Kalinchuk et al., 2003; Shepel et al., 2005). Elevation of extracellular adenosine concentrations is essential for the subsequent induction of non–rapid eye movement sleep (Kalinchuk et al., 2003). Adenosine may affect sleep and waking behavior through several mechanisms (reviewed in

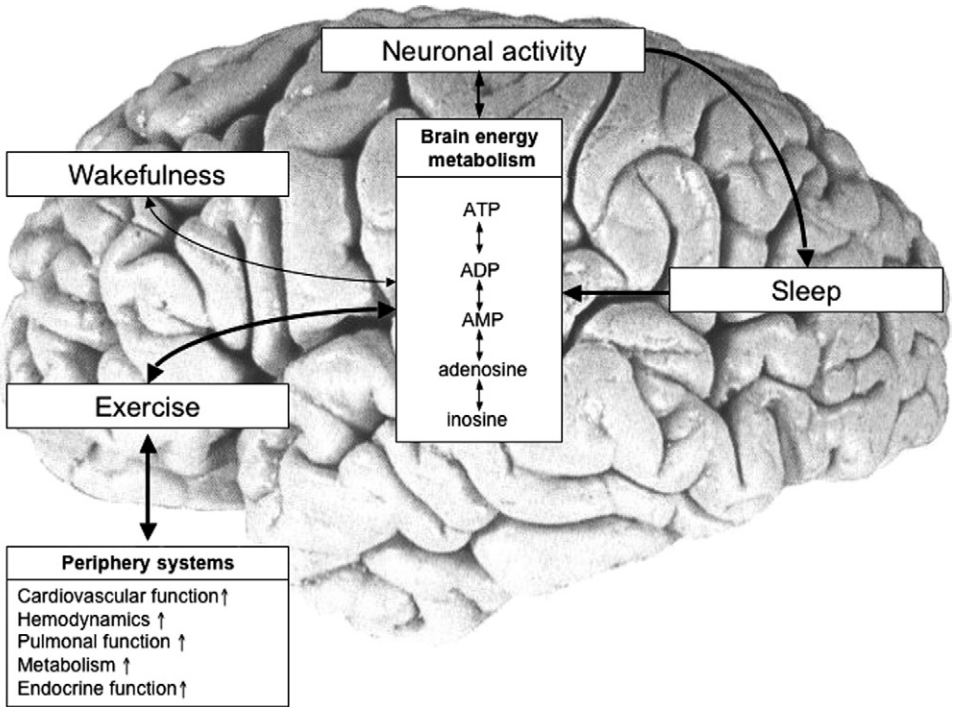

Fig. 6. Relationships between physical exercise, brain energy metabolism and sleep. Exercise affects both, periphery and brain metabolic systems. During intense exercise when metabolic demand exceeds metabolic supply, the formation of adenosine from AMP increases. Adenosine depresses neuronal activity, by pre- and postsynaptic actions and facilitates sleep. During sleep, brain energy expenditure decreases while ADP and ATP concentrations increase.

Basheer et al., 2004). Thus, it could be hypothesized that increased adenosine concentrations after intense exercise in the whole brain, including the cerebral cortex, hypothalamus, and basal forebrain structures result in a depression of neuronal activity due to the inhibitory actions of this nucleotide. A significant accumulation of adenosine due to a depletion of brain energy stores may play a central role in homeostatic sleep regulation, and consequential increases in slow-wave sleep in subsequent sleep may serve a compensatory function to alleviate this deficit. In conclusion, our data indicate that intense exercise affects brain energy metabolism by accumulation of adenosine and inosine. In contrast, sleep leads to a progressive decline of inosine and adenosine as well as significantly elevated ADP and ATP concentrations in a duration-dependent manner. Thus, the present study and findings from other authors suggest that the accumulation of the sleep-promoting substance adenosine after high intensity exercise may play a key role in homeostatic sleep regulation and that sleep could play an essential role in replenishment of high energy compounds (Fig. 6).

Limitations of the study

Only nucleosides and nucleotides were measured in the present study. Therefore we cannot assess the influence of other energy compounds such as glycogen, glucose, and lactate on energy requirements. Furthermore, we measured concentrations of nucleotides and nucleosides in whole rat brain using freeze clamp technique (Palladino et al., 1980; Helzberg et al., 1987). Thus, it is not possible to get any information about spatial changes of nucleotide and nucleoside concentrations in the rat brain. Since the tissue was rapidly clamped, we were not able to detect metabolite changes in specific brain areas such as the basal forebrain, hypothalamus, and cortex. Especially adenosine and other metabolites may accumulate during different behavioral states in a spatial and temporal manner and further research is necessary to examine the effects of physical exercise on brain energy metabolites in specific brain structures.

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