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Sleep deprivation increases A₁ adenosine receptor density in the rat brain

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Abstract

Adenosine, increasing after sleep deprivation and acting via the A₁ adenosine receptor (A₁AR), is likely a key factor in the homeostatic control of sleep. This study examines the impact of sleep deprivation on A₁AR density in different parts of the rat brain with [³H]CPFPX autoradiography. Binding of [³H]CPFPX was significantly increased in parietal cortex (PAR) (7%), thalamus (11%) and caudate-putamen (9%) after 24 h of sleep deprivation compared to a control group with an undisturbed circadian sleep-wake rhythm. Sleep deprivation of 12 h changed receptor density regionally between –5% and +9% (motor cortex (M1), statistically significant) compared to the circadian control group. These results suggest cerebral A₁ARs are involved in effects of sleep deprivation and the regulation of sleep. The increase of A₁AR density could serve the purpose of not only maintaining the responsiveness to increased adenosine levels but also amplifying the effect of sleep deprivation and is in line with a sleep-induced homeostatic reorganization at the synaptic level.

Keywords

Adenosine A₁ receptor; Receptor autoradiography; [³H]CPFPX; Sleep deprivation; Sleep homeostasis; Rat

1. Introduction

Endogenous adenosine is considered a candidate molecule for the homeostatic sleep drive, inducing sleep after prolonged wakefulness (for review see Basheer et al., 2004).

In freely moving cats, microdialysis showed accumulating adenosine levels in the cortex during prolonged wakefulness (6 h) (Porkka-Heiskanen et al., 1997). Subsequent recovery-sleep restored the adenosine concentration to baseline levels. Similar experiments have been repeated in rat with comparable results in the basal forebrain (Basheer et al., 1999; Murillo-Rodriguez et al., 2004). An artificial increase of the adenosine concentration in the basal forebrain by infusion of adenosine in cats (Portas et al., 1997) or rats (Basheer et al., 1999)

increased sleep. Inhibition of equilibrative nucleoside transporters (Porkka-Heiskanen et al., 2000) resulted in electroencephalographic and behavioral effects comparable to those under sleep deprivation.

It is a matter of debate which adenosine receptor subtype mediates the sleep inducing action of adenosine. The A₁ receptor subtype (A₁AR) has the widest distribution in the central nervous system with particularly high concentrations in the cortex, cerebellum, hippocampus, striatum and thalamus (Fredholm, 1995). Selective A₁AR agonists increased the sleep drive in rats after either systemic or cerebroventricular administration (Benington et al., 1995; Schwierin et al., 1996). Inhibition of equilibrium nucleoside transporters (which in turn increased adenosine levels) had the same effect in cats, and correspondingly a selective A₁AR antagonist infused into the basal forebrain decreased sleep propensity in cats (Strecker et al., 2000). Microdialysis perfusion of A₁AR antisense oligonucleotides inhibiting the translation of A₁AR mRNA, significantly decreased nonREM sleep and increased wakefulness in rats (Thakkar et al., 2003). However, A₁AR knockout mice reacted similarly to sleep deprivation as their wild type littermates (Stenberg et al., 2003) and it has been reported that subarachnoidal infusion of the selective A_{2A} adenosine receptor (A_{2A}AR) agonist CGS21680 (Sato et al., 1999) promoted deep sleep.

Intraperitoneal injections of the A₁AR agonist CPA over 5 days significantly decreased A₁AR density in the rat cortex, suggesting desensitization (Roman et al., 2008). Since sleep deprivation increased cerebral adenosine levels it was initially hypothesized that A₁AR density might similarly decrease with deprivation. However, 3 and 6 h of total sleep deprivation increased A₁AR mRNA in the basal forebrain but not $B_{\max}$ or K_D values determined with [³H]DPCPX autoradiography (with and without GTP in the incubation medium) (Basheer et al., 2001). Moreover, A₁AR density in the basal forebrain increased after sleep deprivations of 12 h (10%) and 24 h (14%) (Basheer et al., 2007). In addition, rapid eye movement sleep deprivation for 48 and 96 h was reported to increase A₁AR density in cortical (15 and 20%) and subcortical (23 and 48%) areas of rat brains, as determined by [³H]L-PIA saturation binding experiments in membrane preparations. The K_D value did not change significantly (Yanik and Radulovacki, 1987).

In humans, in vivo imaging of the A₁AR with positron emission tomography and CPFPX showed an increase of A₁AR availability in cortical and subcortical regions after one night of sleep deprivation (Elmenhorst et al., 2007). These findings also did not support the hypothesis that the increase in adenosine concentration during sleep deprivation will induce receptor desensitization due to a high agonist load. Instead, they were in accord with the results of Basheer et al. (2007) who found an increase in both A₁AR mRNA and density after 12 and 24 h in the basal forebrain of the rat. The rationale of the present study was therefore to evaluate whether the widespread, particularly neocortical increase of A₁ARs observed in humans could be replicated in rats. Such a finding would extend the role of adenosine from a focal sleep modulator in the basal forebrain to, with longer durations of sleep deprivation, a ubiquitous player in sleep regulation in many parts of the brain.

2. Results

The results of the effect of sleep deprivation on A₁AR binding of [³H]CPFPX are given in Table 1, and relative regional changes are depicted in Fig. 1. A representative autoradiogram of [³H]CPFPX binding of a rat of the 12 h control group is shown in Fig. 2. The analysis of the data by two-way ANOVA showed that the means of the factors 'group' and 'region' are significantly different ($F_{(3,276)}=267.12$; $p<0.0001$ and $F_{(9,276)}=12.95$; $p<0.0001$, respectively) which is specified by the results from the *t*-test. The interaction 'group×region' was not significant ($F_{(27,176)}=0.49$; $p=0.98$) showing that the changes in the different regions

are congruent. Sleep deprivation of 12 h changed receptor density regionally in a range of -5% and $+9\%$ compared to the circadian control group with a statistically significant increase in the cortical region M1 (9%). 24 h of sleep deprivation increased the binding in all observed regions between 3% and 11% . Differences were significant for the parietal cortex, PAR (7%), thalamus (11%) and caudate-putamen (9%). In most of the regions $B_{\max}$ values were higher in the 24 h control animals than in the 12 h control animals in a range between 4% and 11% increase without reaching statistical significance. Cerebellum, hippocampus and caudate-putamen showed no circadian variation (relative differences: -1.9 , -0.5 and -0.5% respectively).

The ratio between [^3H]CPFPX binding with and without Gpp (NH)p in the buffer reflecting the uncoupled and coupled states of A_1 ARs was about 1.9 in the 12 h and about 2.7 in the 24 h control group. Two-way ANOVA of the coupling state showed that the factors 'group' and 'region' are significantly different ($F_{(3,182)}=31.01$; $p<0.0001$ and $F_{(9,182)}=21.24$; $p<0.0001$, respectively). The interaction 'group $\times$ region' was not significant ($F_{(27,182)}=0.76$; $p=0.8$). After sleep deprivation, the ratio was significantly higher after 12 h of deprivation (2.5) but unchanged after 24 h (2.6). Regional results and statistics are reported in Table 2.

3. Discussion

The results of the present study suggest that sleep deprivation for 24 h produces an increase of A_1 AR density in cortical and subcortical regions of the rat brain.

This finding is consistent with an increase of A_1 ARs in the basal forebrain (12 h, 10% ; 24 h, 14%) established in a subset analysis in some of the animals reported here (Basheer et al., 2007). The results of the present study enlarge and extend these previous findings significantly, because 12 and 24 h of total sleep deprivation changed the A_1 AR density not only in the basal forebrain but in numerous neocortical and subcortical areas. Our findings are likely to lead to a shift in the perception of adenosine. It is not only a local regulator of the induction of sleep in the cholinergic basal forebrain but is additionally participating in the neo- and subcortical modulation during sleep deprivation, consistent with our hypothesis that over longer periods of sleep loss, there is a second mechanism of homeostatic control through transcriptional modification and A_1 AR receptor upregulation that extends to many brain regions (McCarley, 2007).

The underlying mechanism of the observed increase in A_1 ARs warrants further investigation. Although likely, it still must be proven that sleep deprivation in the rat model leads to a wide-spread increase in the cerebral adenosine concentration comparable to that observed in cats (Porkka-Heiskanen et al., 1997). This elevated agonist load would most likely induce a desensitization of A_1 ARs. However, we found an increase of [^3H]CPFPX binding after 12 and 24 h of sleep deprivation. The observed increase was lower than the changes in binding reported for an A_1 AR agonist after 48 h ($15\text{--}23\%$) and 96 h ($20\text{--}48\%$) of REM sleep deprivation but nevertheless in a comparable range considering the longer deprivation period (Yanik and Radulovacki, 1987).

It has been reported that A_1 AR densities undergo circadian variation in mice, characterized by a decrease of $B_{\max}$ during the sleeping period and an increase during the active period (Florio et al., 1991). The relative difference between nadir and peak was 33% but without a change in K_D . That the average density in the 12 h control group of the present study was lower than the density of the 24 h control group could be explained by this circadian variation.

Gpp(NH)p uncouples the A_1 AR from the G-protein resulting in a predominantly high affinity state for [^3H]CPFPX which acts as an inverse agonist at A_1 ARs. In contrast, the

presence of MgCl_2 in the incubation medium decreases the affinity. The ratio of B_{max} of the uncoupled to the coupled state was only different for the 12 h control group compared to the other conditions. The difference in the ration between the 12 and 24 h control group could reflect a mechanism that is active during normal circadian variations of the receptor density. As these physiological changes occurred after the active period, the same mechanisms could be active during sleep deprivation.

We note that the reported B_{max} values are based on a standard K_D value. Saturation binding experiments have not been performed, because it was not likely that the K_D value changes due to sleep deprivation. In particular, the K_D value is reported to be constant after 3 and 6 h (Basheer et al., 2001) and 24, 48 and 96 h of sleep deprivation (Yanik and Radulovacki, 1987). As all groups were processed in the same experiment, the relative changes would be identical.

Most studies which investigated the impact of $A_1\text{AR}$ changes on sleep regulation have focused on cholinergic neurons in the basal forebrain. However, the widespread changes of $A_1\text{AR}$ density after sleep deprivation are not restricted to the basal forebrain but seem to affect the whole brain. This implies that sleep deprivation induced regulatory effects are taking place ubiquitously, albeit not homogeneously or perhaps with the same temporal sequence in the brain. The present data confirm data of a previous positron emission tomography study in healthy human subjects, in which we found an increase of $A_1\text{AR}$ availability in cortical and subcortical regions after one night of sleep deprivation (Elmenhorst et al., 2007).

Interestingly, our findings are also in line with a recent hypothesis of the function of sleep (Tononi and Cirelli, 2006). It links the plastic processes at the cortical synaptic level during wakefulness, which result in a net build up of synaptic structures, to the need to downscale synapses to baseline levels, which is proposed to happen during sleep. Synaptic receptors are likely to be involved in this regulation and to be increased by extending the wake period by sleep deprivation. The rather ubiquitous character of the cortical increase of brain $A_1\text{AR}$ s reported in this article fits well into that concept.

The results of our study suggest that not only adenosine but also its $A_1\text{AR}$ are involved in the homeostatic regulation of sleep. Adenosine and its $A_1\text{AR}$ are therefore not only factors of sleep regulation in local basal forebrain networks but also players in the modulation of neo- and subcortical regions during sleep deprivation. The observed increase of $A_1\text{AR}$ density could serve the purpose of not only maintaining the responsiveness to increased adenosine levels but also amplifying the effect of sleep deprivation and is in line with a sleep-induced homeostatic reorganization at the synaptic level.

4. Experimental procedures

4.1. Animals

Thirty-two male Sprague Dawley rats (250–350 g) were used. Rats were housed in a 12 h light/dark cycle (light on 7:00 AM to 7:00 PM), at 23 ± 1 °C with access to food and water *ad libitum*; treatment was according to the Association for Accreditation of Laboratory Animal Care and Use Committee as Boston VA Healthcare system, Harvard University and US National Institute of Health. In a subset of these animals ($n=24$) an analysis of $A_1\text{AR}$ density changes in the basal forebrain has been performed and published by Basheer et al., (2007).

4.2. Radioligands and chemicals

8-Cyclopentyl-3-(3-fluoropropyl)-1-propylxanthine (CPFPX) and [^3H]8-cyclopentyl-3-(3-fluoropropyl)-1-propylxanthine ([^3H]CPFPX; specific activity 2179.3 GBq/mmol) were

synthesized as previously described (Holschbach et al., 2002). Adenosine deaminase, 5'-guanylyl-imidodiphosphate (Gpp(NH)p) and R(-)-N⁶-(2-phenylisopropyl)adenosine (R(-)-PIA) were purchased from Sigma-Aldrich Co. (St. Louis, MO). All other chemicals were of reagent grade and obtained commercially.

4.3. Surgery, sleep recording and sleep deprivation

Under general anesthesia [i.m. injection of a cocktail of ketamine (7.5 mg/100 g body wt.), xylazine (0.38 mg/100 g) and acepromazine (0.075 mg/100 g)], rats were stereotactically implanted with bilateral EEG electrodes 2 mm anterior to the Bregma and 3.5 mm lateral to the mid-sagittal suture, with flexible wire loops into the external canthus for EOG; and into nuchal muscles for EMG. After week-long recovery and 3 days adaptation to cables, the EEG, EMG and EOG recordings were performed (data not reported).

Sleep deprivation was performed by gentle handling, with minimal stress to the animals, as described in previous reports (Basheer et al., 1999). Deprivation was started at 8 AM and continued for 12 or 24 h. Controls were undisturbed sleeping animals sacrificed at the same circadian time.

4.4. Receptor protein density by autoradiography

Rats with 12 and 24 h sleep deprivation and their undisturbed controls ($n=8$ /group) were decapitated, their brains rapidly removed, blotted free of excess blood, frozen in 2-methylbutane (-50°C) and stored at -80°C . Brain sections ($20\text{ }\mu\text{m}$ thick) were thaw-mounted onto silica-coated slides and stored at -80°C . Autoradiographic procedures were as described previously (Bauer et al., 2003). In brief, after preincubation in Tris-HCl buffer (170 mmol/l; pH 7.4) for 15 min (4°C), sections were incubated (120 min; 22°C) in buffer containing [³H]CPFPX ($n=6$; 4.04 nmol/l and $n=2$; 4.2 nmol/l) and 2 U/l adenosine deaminase to remove endogenous adenosine. To assess G-protein coupled and uncoupled states of the A₁AR, buffer contained 1 mmol/l MgCl₂ or 100 mmol/l Gpp(NH)p, respectively. For the assessment of non-specific binding, selected slices were incubated with R(-)-PIA (100 nmol/l) in the medium. After two washes in incubation buffer (4°C) and a rapid rinse in ice-cold water the sections were dried. Specific binding was calculated as the difference of total and nonspecific binding. Adjacent sections were Nissl stained for cytoarchitectonic identification of brain nuclei. Labeled sections were placed on phosphor-imaging plates (BAS-TR 2025, Raytest-Fuji, Straubenhardt, Germany) with industrial tritium activity standards (Microscales; Amersham Biosciences, Freiburg, Germany). Upon exposure, the plates were scanned with a high-performance plate reader (spatial resolution of $50\text{ }\mu\text{m}$; BAS5000 BioImage Analyzer, Raytest-Fuji, Straubenhardt, Germany). The digital receptor autoradiography was processed using image analysis software (AIDA 2.31, Raytest-Fuji, Straubenhardt, Germany) by an investigator who was blinded to the group assignment and defining regions of interest according to a standard rat brain atlas (Paxinos and Watson, 1998). The average number of regions of interest placed and the average area covered were: cerebellum 5.5 ± 1.2 , $22.7\pm 2.5\text{ mm}^2$; motor cortex M1 35.0 ± 2.0 , $1.8\pm 0.1\text{ mm}^2$; parietal cortex PAR 28.0 ± 1.2 , $2.2\pm 0.2\text{ mm}^2$; cingulate cortex 61.0 ± 2.0 , $0.8\pm 0.3\text{ mm}^2$; hippocampus 4.8 ± 1.2 , $3.1\pm 0.3\text{ mm}^2$; dental gyrus 5.8 ± 1.2 , $1.3\pm 0.5\text{ mm}^2$; thalamus 5.0 ± 1.2 , $5.7\pm 0.4\text{ mm}^2$; hypothalamus 6.3 ± 1.5 , $3.0\pm 0.5\text{ mm}^2$; caudate-putamen 44.8 ± 1.2 , $8.3\pm 1.0\text{ mm}^2$; nucleus accumbens 12.8 ± 1.2 , $0.2\pm 0.1\text{ mm}^2$. As determined in previous experiments, an assumed K_D of 4.4 nmol/l (6.4 nmol/l for the uncoupled state) and a factor of 18.4 for the conversion of mol/g wet weight to mol/g protein were used for the calculation of receptor density (B_{max}) in mol/g protein.

To test for the factors 'group' and 'region' and the interaction 'group $\times$ region' a mixed model two-way ANOVA was performed on the binding data and the uncoupled-to-coupled

receptor state ratio. Student's *t*-test was used to compare groups, all reported average values are mean $\pm$ standard deviation.

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Abbreviations

A₁AR	A ₁ adenosine receptor
CPFPX	8-cyclopentyl-3-(3-fluoropropyl)-1-propylxanthine
PAR	parietal cortex
M1	motor cortex
A_{2A}AR	A _{2A} adenosine receptor
CPA	N6-Cyclopentyladenosine
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
PIA	N ⁶ -(2-phenylisopropyl)adenosine

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Fig. 1.

A₁AR density: relative differences of the 12 and 24 h sleep deprivation condition to the circadian control group [(sleep deprivation-control)/ control] n=8. Statistical analysis by Students *t*-test (*p<0.05).

Fig. 2. Representative example of [^3H]CPFPX binding to A_1AR in one rat of the 12 h control group. Upper row shows the binding in the presence of Gpp(NH)p (G-protein uncoupled state) and addition of the competitor R(-)-PIA (right part, unspecific binding). Lower row shows binding in the presence of MgCl_2 (G-protein coupled state) and competitor. Delineation show the regions of interest used for analysis.

Table 1

	A ₁ AR density fmol/mg protein				t-test	
	ct12	sd12	ct24	sd24	sd12 vs ct12	sd24 vs ct24
Cerebellum	4188 ± 625	3979 ± 600	4107 ± 862	4346 ± 437	0.51	0.50
Cortex M1	2824 ± 276	3081 ± 165	3017 ± 138	3105 ± 159	0.04	0.26
Cortex Par	3474 ± 383	3760 ± 388	3787 ± 188	4056 ± 237	0.16	0.02
Cingulate ctx.	3113 ± 304	3106 ± 184	3227 ± 169	3311 ± 147	0.96	0.31
Hippocampus	5003 ± 498	4910 ± 507	4977 ± 471	5292 ± 325	0.73	0.14
Dentate gyrus	4510 ± 641	4692 ± 463	4710 ± 363	4879 ± 267	0.54	0.31
Thalamus	3756 ± 456	3971 ± 461	3951 ± 378	4380 ± 288	0.38	0.02
Hypothalamus	1131 ± 153	1205 ± 196	1253 ± 159	1375 ± 237	0.43	0.24
Caudate-putamen	2556 ± 459	2455 ± 247	2542 ± 118	2770 ± 219	0.59	0.02
Ncl. accumbens	2341 ± 607	2321 ± 158	2574 ± 404	2699 ± 468	0.93	0.57

A₁AR density in various rat brain regions of the 12 and 24 h sleep deprivation (sd) group and of the circadian control (ct) group. Values are means ± standard deviation; results of statistical testing with Student's *t*-test are reported; *p*<0.05 values are highlighted in bold numbers.

Table 2

	Ratio of uncoupled to coupled A ₁ AR				t-test	
	ct12	sd12	ct24	sd24	sd12 vs ct12	sd24 vs ct24
Cerebellum	2.2 ± 0.3	2.6 ± 0.4	2.6 ± 0.3	2.4 ± 0.4	0.09	0.36
Cortex M1	1.6 ± 0.2	2.1 ± 0.2	2.3 ± 0.4	2.2 ± 0.3	0.01	0.53
Cortex Par	1.7 ± 0.2	2 ± 0.2	2.5 ± 0.2	2.3 ± 0.1	0.04	0.08
Cingulate ctx.	2.1 ± 0.3	2.7 ± 0.3	2.8 ± 0.4	2.9 ± 0.2	0.01	0.74
Hippocampus	1.6 ± 0.2	2 ± 0.4	2.3 ± 0.3	2.1 ± 0.2	0.16	0.36
Dentate gyrus	1.6 ± 0.2	2.1 ± 0.5	2.3 ± 0.4	2.3 ± 0.2	0.09	0.91
Thalamus	1.7 ± 0.3	2.2 ± 0.5	2.3 ± 0.3	2.3 ± 0.2	0.11	0.95
Hypothalamus	2.4 ± 0.8	3.1 ± 0.9	3.3 ± 0.9	3.1 ± 0.6	0.32	0.64
Caudate-putamen	1.8 ± 0.3	2.5 ± 0.2	2.8 ± 0.5	2.7 ± 0.2	0.002	0.64
Ncl. accumbens	2.3 ± 0.6	3.8 ± 0.5	3.8 ± 1.1	3.3 ± 0.6	0.002	0.40

Ratios of A₁ adenosine receptors uncoupled (addition of Gpp(NH)p to buffer) and coupled (addition of MgCl₂ to the buffer) to the G-protein in various rat brain regions of the 12 and 24 h sleep deprivation (sd) group and of the circadian control (ct) group. Values are means ± standard deviation; results of statistical testing with Student's *t*-test are reported; *p*<0.05 values are highlighted in bold numbers.