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Dominant localization of adenosine deaminase in leptomeninges and involvement of the enzyme in sleep☆

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Abstract

Adenosine is an endogenous hypnotic molecule. However, the mechanism by which the level of extracellular adenosine is regulated remains to be elucidated. We found by Northern hybridization and enzyme assay that ecto-5′-nucleotidase and adenosine deaminase (ADA), major enzymes responsible for the production and degradation of adenosine, respectively, were localized most abundantly in the leptomeninges within the rat brain. Immunohistochemical study showed that ADA was dominantly localized in arachnoid barrier and trabecular cells of the leptomeninges. In vivo microdialysis demonstrated that externally applied adenosine was rapidly metabolized by ADA to inosine in the subarachnoid space. Perfusion of an ADA inhibitor, coformycin, increased the extracellular adenosine level in the subarachnoid space under the rostral basal forebrain. When coformycin was continuously infused into the subarachnoid space, non-rapid eye movement sleep was increased with prolonged duration of the sleep episode. These results demonstrate that the leptomeninges control the extracellular level of adenosine in the subarachnoid space by their high 5′-nucleotidase and ADA activities and regulate non-rapid eye movement sleep.
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Adenosine is proposed to be an endogenous sleep-inducing molecule, as concluded from a variety of pharmacological and behavioral experiments [1,2]. For example, sleep is induced in rats after administration of metabolically stable adenosine analogs, such as N^6-L-(phenylisopropyl)-adenosine, adenosine-5′-*N*-ethylcarboxamide, and cyclohexyladenosine, which are agonists for adenosine A_1 and/or A_{2A} receptors [3,4]. The adenosine content is increased in the basal forebrain, one of the sleep centers, after sleep deprivation [5]. CGS-21680, an adenosine A_{2A} receptor agonist, induces sleep after administration to the subarachnoid space of the rostral basal forebrain [6,7]. However, the mechanism regulating the extracellular adenosine concentration remains to be elucidated.

The extracellular adenosine concentration is theoretically controlled by three processes: the biosynthesis of adenosine from AMP by 5′-nucleotidase (5′-NT) or from *S*-adenosylhomocysteine by *S*-adenosylhomocysteine hydrolase (SAHH), adenosine uptake by purine nucleoside transporters, and the metabolism of adenosine to AMP by adenosine kinase (AK) or to inosine by adenosine deaminase (ADA) [8]. Among these processes, the transport of adenosine into cells has been proposed to be primarily responsible for removing adenosine from the extracellular space in the brain [9]. However, in this study, we found that 5′-NT and ADA were remarkably abundant in the leptomeninges rather

☆ *Abbreviations used:* ADA, adenosine deaminase; ecto-5′-NT, ecto-5′-nucleotidase; SAHH, *S*-adenosylhomocysteine hydrolase; AK, adenosine kinase; non-REM, non-rapid eye movement; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPGDS, lipocalin-type prostaglandin D synthase; EEG, electroencephalogram; EMG, electromyogram; PG, prostaglandin; TMN, tuberomammillary nucleus.

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than the brain parenchyma and that administration of the ADA inhibitor coformycin to the subarachnoid space of the rostral basal forebrain increased the extracellular adenosine concentration and induced non-rapid eye movement (non-REM) sleep.

Materials and methods

Male Sprague Dawley rats, weighing 250–350 g (8–12 weeks old), were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). They were housed at a constant temperature (24 ± 0.5 °C) with a relative humidity (60 ± 2%) on an automatically controlled 12:12 light/dark cycle (light on at 8:00 a.m.). Adenosine was purchased from Wako Pure Chemical Industries (Osaka, Japan); and coformycin, from Calbiochem (San Diego, CA).

RNA extraction and Northern hybridization. Rats were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused with saline transcardially. After isolation of the leptomeninges, the brain was dissected as described in Fig. 1. RNA extraction and Northern hybridization were performed as described [10]. The cDNA fragments of rat ADA, ecto-5′-NT, AK, SAHH, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as probes. The signals were visualized with an FLA2000 fluorescence imaging analyzer (Fuji Photo Film, Tokyo, Japan).

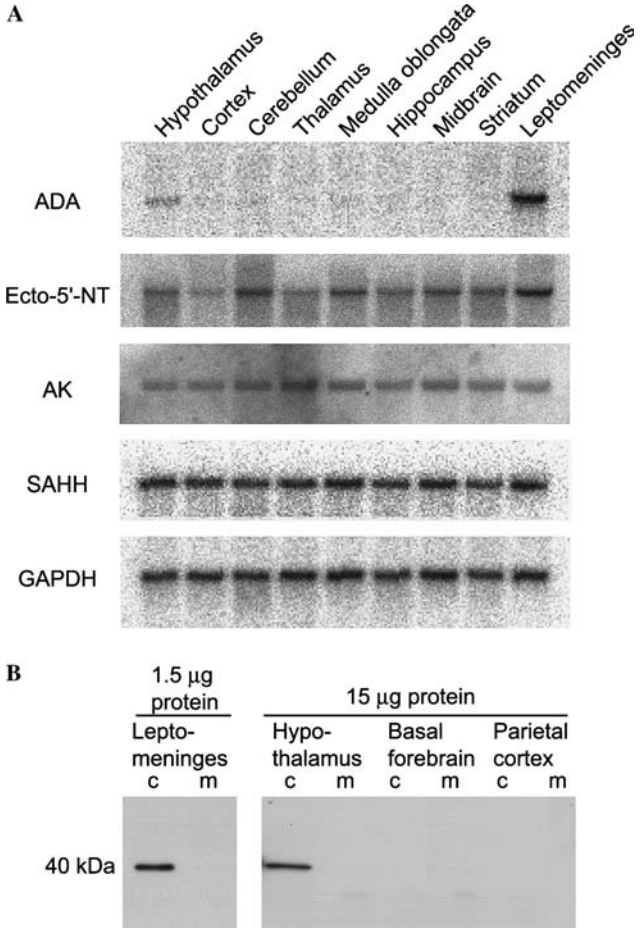

Fig. 1. Distribution of the metabolic enzymes of adenosine in the rat brain. (A) Northern hybridization for ADA, ecto-5′-NT, AK, SAHH, and GAPDH mRNAs. Each lane contained 10 μg of total RNA. After removal of the first probe, the membrane was hybridized again sequentially with the probes for the other adenosine metabolic enzymes. (B) Western blot analysis of ADA in the cytosolic (c) and membrane fractions (m) of various brain tissues.

Western blotting. Tissues were homogenized in 50 mM Tris–Cl (pH 7.3) on ice. The homogenates were centrifuged at 100,000*g* and 4 °C for 1 h to separate the cytosolic and membrane fractions. After SDS–polyacrylamide gel electrophoresis, the proteins were transferred onto an Immovilon PVDF membrane (Millipore, Bedford, MA). The membrane was incubated at 4 °C overnight with sheep antiserum against rat ADA (1:250,000 dilution) [11], and then at room temperature for 1 h with horseradish peroxidase-conjugated anti-sheep IgG antibody (1:1000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). The immunoreactive band was detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Enzyme assay. Tissue homogenates were incubated with 0.5 mM adenosine for the ADA assay or with AMP for the 5′-NT assay in a total volume of 50 μl of 50 mM Tris–Cl (pH 7.3) containing 10 mM $MgCl_2$. The reaction was stopped by the addition of 5 μl of 25% (v/v) perchloric acid. After centrifugation at 14,000*g* and 4 °C for 5 min, the supernatant was used to measure adenosine and its metabolites by HPLC. The mobile phase, consisting of 10 mM ammonium phosphate (pH 6.0) and 14% (v/v) methanol, was pumped at 1 ml/min through a reverse-phase C_{18} column (Symmetry, 4.6 × 150 mm; Waters, Milford, MA) at 30 °C. The chromatograms were recorded at 254 nm and analyzed with a computer using Millennium[32] Chromatography Manager (Waters). ADA activity was determined by measuring the conversion of adenosine to inosine and hypoxanthine [12]. Total 5′-NT activity was observed by monitoring the conversion of AMP to adenosine, inosine, and hypoxanthine.

Immunohistochemistry. Under pentobarbital anesthesia, rats were transcardially perfused with saline followed by phosphate-buffered saline (pH 7.3) containing 4% (w/v) formaldehyde. The brain was placed in the same fixative solution for 4 h at 4 °C. Cryosections (20-μm thickness) were incubated with the sheep anti-ADA antiserum (1:50,000 dilution) overnight at 4 °C. The antibody was detected with biotin-conjugated anti-sheep IgG antibody (1:2000, Jackson ImmunoResearch Laboratories) and horseradish peroxidase-conjugated avidin (Vector Laboratory, Burlingame, CA). Finally, the sections were visualized with 3,3′-diaminobenzidine.

Immunofluorescence staining. Brain sections (10 μm) were prepared as described above and incubated with sheep anti-ADA antiserum (1:5000) and rabbit anti-lipocalin-type prostaglandin D synthase (LPGDS) antiserum (1:500) [13], followed by fluorescein-conjugated anti-sheep (1:200, Jackson ImmunoResearch Laboratories) and rhodamine-conjugated anti-rabbit IgG antibodies (1:10, Boehringer, Mannheim, Germany), respectively. Immunofluorescence was observed with an Axiovert 100M microscope (Carl Zeiss, Oberkochen, Germany) connected to a Zeiss laser-scanning microscope 510 (Carl Zeiss).

Microdialysis. Rats were anesthetized with urethane (1.2 g/kg, i.p.). A microdialysis probe (membrane length, 1 mm; CMA/Microdialysis AB, Stockholm) was stereotaxically inserted into the subarachnoid space of the rostral basal forebrain. The coordinates were the following: anteroposterior, 0.9 mm from the bregma; dorsoventral, −8.2 mm from the brain surface according to the atlas of Paxinos and Watson [14]. Perfusion with artificial cerebrospinal fluid [composition (mM): 140 NaCl, 3 KCl, 1.0 $MgCl_2$, 1.3 $CaCl_2$, 2 Na_2HPO_4, and 0.2 NaH_2PO_4; pH 7.4] was started immediately after insertion of the probe at a flow rate of 2 μl/min. Three hours after insertion of the microdialysis probe, the dialysate was continuously collected at 20-min intervals. Adenosine and coformycin were dissolved in artificial cerebrospinal fluid, and administered through the microdialysis membrane 2 h after starting the sample collection. When the combination of these two chemicals was used, perfusion with coformycin was started 20 min prior to that with adenosine. After treatment of the

dialysate with 2.5% (v/v) perchloric acid, the levels of adenosine and inosine were measured by HPLC as described above. Based on our in vitro study, the permeation efficiency of the dialysis membrane for adenosine and inosine was 15%.

Electroencephalogram (EEG) and electromyogram (EMG) recordings during microinfusion. Under pentobarbital anesthesia (50 mg/kg, i.p.), rats were implanted with electrodes for EEG and EMG recordings, and with a cannula for microinfusion as described [15,16]. The EEG/EMG signals were recorded and analyzed by the *SleepSign* program (Kissei Comtec Co. Ltd., Nagano, Japan). The recordings were taken in each rat for 2 consecutive 24-h periods, and the first 24 h (day 1) served as the baseline for the same animal. On the experimental day (day 2), coformycin was infused for 5 h starting at 23:00 at a rate of 400 pmol/0.4 μl/min. The vigilance states were automatically classified by 10-s epochs into wakefulness, REM sleep, and non-REM sleep, according to the criteria previously described [17]. As a final step, defined sleep–wake stages were examined visually, and corrected, if necessary. Episode number, average duration, and EEG power distribution of non-REM sleep were analyzed by the *SleepSign* program.

Results

Abundance of metabolic enzymes of adenosine in the leptomeninges of the rat brain

We examined the mRNA expression of metabolic enzymes for adenosine in distinct regions of the brain parenchyma and the leptomeninges of adult rats by Northern hybridization (Fig. 1A). ADA mRNA was abundantly expressed in the leptomeninges but was almost under the detection limit in all regions of the parenchyma except the hypothalamus. The mRNA expression ratio of ADA to GAPDH in the leptomeninges was 5.5-fold higher than that in the hypothalamus. Alternatively, the leptomeninges was also enriched in ecto-5′-NT mRNA, which was widely distributed in various brain regions, thus differing from the ADA mRNA. The mRNA expression ratio of ecto-5′-NT to GAPDH was 1.3- to 2.8-fold higher in the leptomeninges than in the other regions of the brain parenchyma. In contrast, the mRNAs for AK and SAHH were almost ubiquitously distributed throughout the brain parenchyma and leptomeninges.

The abundance of ADA and 5′-NT in the leptomeninges was confirmed by the results of enzyme assays. The ADA activity in the leptomeninges (152.8 ± 19.3 nmol/min/mg protein, mean ± SEM, $n = 6$) was 12-, 48-, and 109-fold higher than that in the hypothalamus (12.5 ± 4.7), basal forebrain (3.2 ± 0.7), and parietal cortex (1.4 ± 0.6), respectively. The 5′-NT activity in the leptomeninges (67.1 ± 2.8 nmol/min/mg protein, $n = 6$) was 1.9-, 2.8-, and 3.4-fold higher than that in the basal forebrain (35.5 ± 16.0), hypothalamus (24.3 ± 9.3), and parietal cortex (19.9 ± 7.7), respectively.

We then investigated the subcellular localization of ADA by Western blot analysis (Fig. 1B). Anti-ADA antibody detected a single immunoreactive band at a position of about 40 kDa in the cytosol, but not in the membrane, fraction of the leptomeninges (1.5 μg of protein) and the hypothalamus (15 μg of protein). The immunoreactive protein was not detected in the basal forebrain or parietal cortex, being consistent with the results of Northern hybridization and enzyme assay.

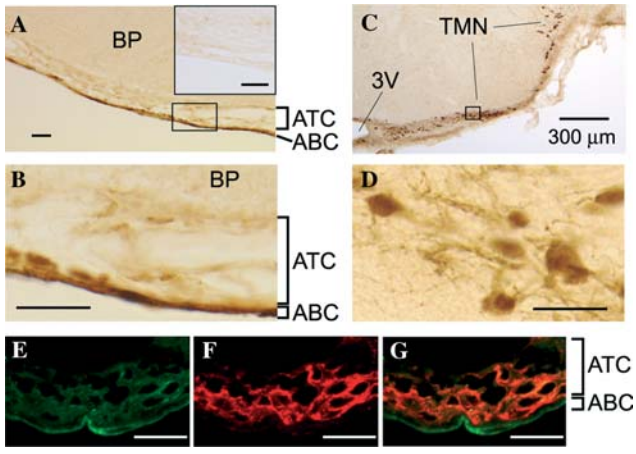

Fig. 2. Histological determination of ADA localization in the leptomeninges. (A,B) Low- (A) and high- (B) magnification views of the leptomeninges of the rostral basal forebrain after immunostaining. The inset to A shows the staining with serum from normal sheep. (C,D) Low- (C) and high- (D) magnification views of the TMN neurons of the posterior hypothalamus. (E–G) Immunofluorescence staining for ADA (E; green) and LPGDS (F; red) in the leptomeninges of the rostral basal forebrain. (G) Merged view of E and F. ATC, arachnoid trabecular cells; ABC, arachnoid barrier cells; BP, brain parenchyma; 3V, third ventricle. Scale bar: 30 μm otherwise indicated.

Immunohistochemical staining with anti-ADA antibody revealed that ADA was predominantly localized in the leptomeninges of the rostral basal forebrain (Figs. 2A and B) and in the neurons of the tuberomammillary nucleus (TMN) of the posterior hypothalamus (Figs. 2C and D), where the histaminergic neurons are located. The localization of ADA in the TMN neurons is consistent with the results of previous immunohistochemical studies [18,19]. The ADA immunoreactivity was also found in the leptomeninges of the cortex, cerebellum, medulla oblongata, and so on (data not shown). No positive staining was observed with normal sheep serum used as the primary antibody (inset to Fig. 2A). Double immunofluorescence staining for ADA and LPGDS, the latter being a marker protein for the arachnoid barrier and trabecular cells [13], demonstrated that ADA was colocalized with LPGDS in the leptomeninges and that a thin layer of arachnoid barrier cells had the intense green ADA immunoreactivity, whereas the arachnoid trabecular cells showed moderate ADA immunoreactivity (Figs. 2E–G).

ADA inhibitor coformycin attenuated the degradation of adenosine and increased the extracellular adenosine concentration in the subarachnoid space

Due to the restricted localization of ADA in the leptomeninges, we hypothesized that the leptomeningeal

ADA plays an important role in the regulation of the adenosine concentration in the subarachnoid space. To explore this hypothesis we monitored the metabolism of adenosine in the subarachnoid space by using in vivo microdialysis. When we perfused 10 μM adenosine for 1 h into the subarachnoid space under the rostral basal forebrain through the microdialysis membrane, the amount of inosine in the dialysate increased from the basal level (17.8 ± 3.2 nM) to 238.7 ± 46.7 nM (Fig. 3A), whereas the AMP concentration remained unchanged (data not shown). Co-administration of adenosine with the ADA inhibitor coformycin decreased the inosine concentration by 53% (112.7 ± 14.6 nM) at a dose of 1 μM, and by 81% (45.7 ± 17.9 nM) at 10 μM, indicating that adenosine was converted to inosine by ADA.

We then monitored the changes in the extracellular adenosine concentration in the subarachnoid space of the rostral basal forebrain during the local perfusion with coformycin. Compared with the vehicle administration (6.0 ± 1.7 nM), continuous application of 1 μM coformycin for 2 h increased the adenosine level in the subarachnoid space by 2.0-fold (11.9 ± 1.3 nM); and 10 μM coformycin caused a further increase, 2.3-fold (13.6 ± 1.9 nM, Fig. 3B), and decreased the inosine concentration to an undetectable level (<0.5 nM, Fig. 3A). These data indicate that inhibition of the leptomeningeal ADA caused elevation of the extracellular adenosine concentration in the subarachnoid space.

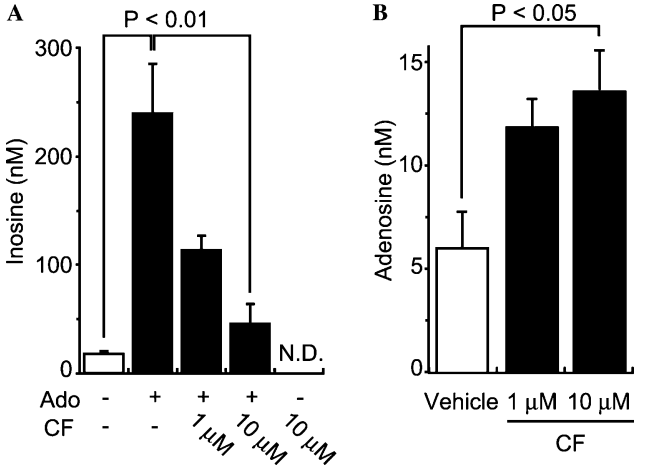

Fig. 3. Microdialysis studies for adenosine metabolism in the subarachnoid space. (A) Concentration of inosine in the dialysate 60 min after the continuous administration of vehicle (open column), 10 μM adenosine (Ado) or 10 μM adenosine with coformycin (CF). (B) Concentration of adenosine in the dialysate 2 h after the continuous administration of vehicle or coformycin. N.D. means not detectable. Values are expressed as the mean ± SEM ($n = 4$–6). One-way ANOVA followed by the Fisher's PLSD test was used for statistical analysis.

Administration of coformycin into the subarachnoid space of the rostral basal forebrain induced non-REM sleep

Application of adenosine A_{2A} receptor agonist into the subarachnoid space under the rostral basal forebrain has been demonstrated to induce sleep [6]. We therefore monitored the changes in the amount of non-REM and REM sleep after causing an increase in the extracellular level of adenosine by continuously infusing coformycin into the subarachnoid space under the rostral basal forebrain in the dark (active) period (Fig. 4A). At a dose of 400 pmol/0.4 μl/min, the accumulative amount of non-REM sleep increased 30%, at 2 h compared with

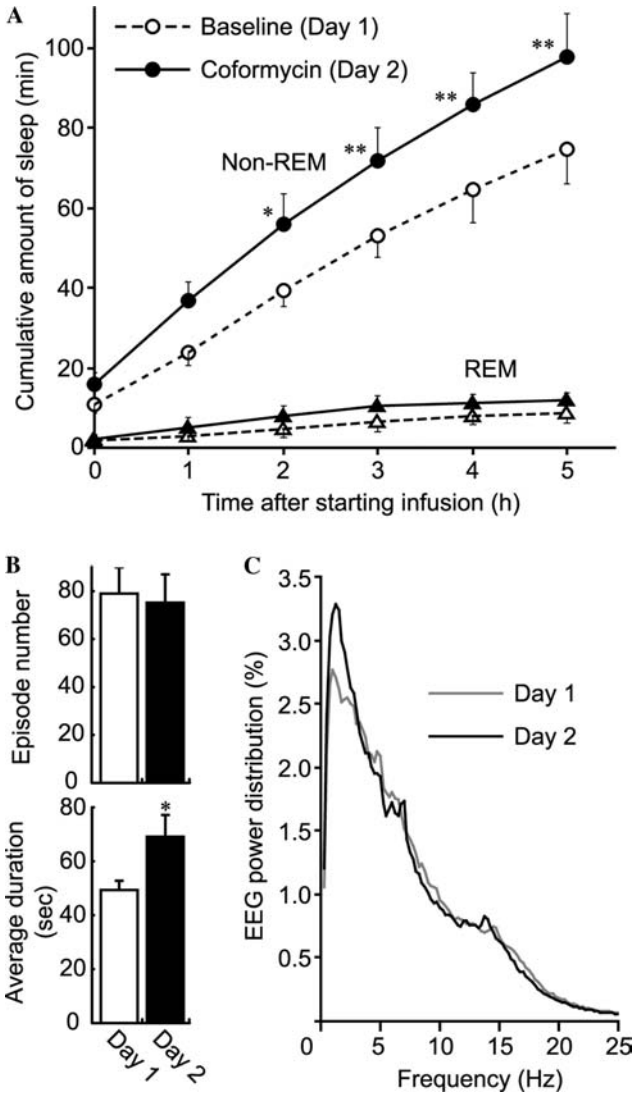

Fig. 4. Effect of subarachnoid administration of coformycin on sleep profiles. (A) Cumulative amount of non-REM (circles) and REM (triangles) sleep on baseline (day 1) and experimental day (day 2). (B) Episode numbers (upper) and average duration (lower) of non-REM sleep during the 5-h infusion period of days 1 and 2. (C) EEG power distribution of non-REM sleep during the same 5-h period of days 1 (dotted line) and 2 (solid line). Values are expressed as the mean ± SEM ($n = 5$). * $P < 0.05$, ** $P < 0.01$ vs. day 1 (paired t test).

that of the baseline day (day 1) and this increased level lasted until the end of the drug administration. In contrast, the cumulative amount of REM sleep was not increased by the coformycin administration.

The number of non-REM sleep episodes remained unchanged during 6-h coformycin administration (day 2) compared with that of baseline day (day 1). However, the average duration was prolonged by 40% during coformycin administration (Fig. 4B). The EEG spectrum analysis of non-REM sleep revealed that the EEG power distribution on day 2 was essentially identical to that on day 1, with a slight increase in the relative power of the delta wave (around 2 Hz), a characteristic of deep sleep (Fig. 4C). These results indicate that the inhibition of leptomeningeal ADA induced non-REM sleep, probably due to the increase in the subarachnoid adenosine level, and suggest that leptomeningeal ADA in the rostral basal forebrain plays a crucial role in the regulation of non-REM sleep.

Discussion

Active metabolism of adenosine in leptomeninges

We demonstrated for the first time that ADA is predominantly localized in the leptomeninges and regulates the adenosine concentration in the subarachnoid space of rats. Enzyme assay and Northern analyses revealed that ADA was present almost completely in the soluble fraction, although ADA has reportedly been detected in the membrane fraction of the brain [20]. Since purine nucleoside transporters were widely distributed in the leptomeninges and brain parenchyma as judged from RT-PCR data (Okada et al., unpublished data), adenosine is probably taken up by leptomeningeal cells from the subarachnoid space through purine nucleoside transporters and metabolized to inosine by ADA within these cells.

We also found that the leptomeninges had the highest 5′-NT activity in the brain. Earlier studies showed that this activity was detectable throughout the brain in a range of 15 to 46 nmol/min/mg protein [21–23]. The much greater abundance of ADA and 5′-NT in the leptomeninges indicates that adenosine metabolism in the leptomeninges is more active than that in the brain parenchyma. On the other hand, the AK activity was widely distributed throughout the brain parenchyma having negligible ADA activity, suggesting that adenosine is metabolized to AMP, but not to inosine, in the parenchyma. Since ADA is also localized in limited populations of neurons in the brain parenchyma, such as histaminergic neurons of the TMN [19], adenosine metabolism in those neurons may be different from that in neurons of other regions and similar to that in the leptomeningeal cells.

Adenosine in the subarachnoid space under the rostral basal forebrain regulates non-REM sleep

Radulovacki et al. [3] reported that intraperitoneal treatment of rats with the ADA inhibitor deoxycoformycin induced sleep. We extended their study and found that inhibition of ADA in the leptomeninges of the rostral basal forebrain by coformycin increased the adenosine level in the subarachnoid space and selectively induced non-REM sleep. These results are in agreement with previous findings that the adenosine concentration in the subarachnoid space of the rostral basal forebrain is critical for the induction of non-REM sleep by the endogenous hypnotic molecule prostaglandin (PG) D_2 [24]. PGD_2 is synthesized by the key enzyme LPGDS, predominantly expressed in the leptomeninges, choroid plexus, and oligodendrocytes, in adult rat brain [25]. PGD_2 receptors are also dominantly localized in the leptomeninges of the basal forebrain [24]. The leptomeninges is a membrane system that encloses the brain to form the subarachnoid space and is generally recognized only as a cushion to protect the brain from physical impacts. However, the colocalization of ADA and LPGDS in the leptomeninges suggests that this membrane system plays a crucial role in the regulation of sleep by producing two endogenous somnogenic substances, adenosine and PGD_2, both of which circulate in the subarachnoid space and act as humoral sleep regulators [13,26]. This suggestion is also supported by the facts that PGD_2 and adenosine A_{2A} receptor agonists exhibit potent hypnotic effects when administered to the subarachnoid space below the rostral basal forebrain rather than to any other brain regions [27,28].

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