Effects on sleep of microdialysis of adenosine A₁ and A₂A receptor analogs into the lateral preoptic area of rats

Methippara, Melvi M., Sunil Kumar, Md. Noor Alam, Ronald Szymusiak, and Dennis McGinty. Effects on sleep of microdialysis of adenosine A₁ and A₂A receptor analogs into the lateral preoptic area of rats. Am J Physiol Regul Integr Comp Physiol 289: R1715–R1723, 2005. First published August 18, 2005; doi:10.1152/ajpregu.00247.2005.— Evidence suggests that adenosine (AD) is an endogenous sleep factor. The hypnogenic action of AD is mediated through its inhibitory A₁ and excitatory A₂A receptors. Although AD is thought to be predominantly active in the wake-active region of the basal forebrain (BF), a hypnogenic action of AD has been demonstrated in several other brain areas, including the preoptic area. We hypothesized that in lateral preoptic area (LPOA), a region with an abundance of sleep-active neurons, AD acting via A₁ receptors would induce waking by inhibition of sleep-active neurons and that AD acting via A₂A receptors would promote sleep by stimulating the sleep-active neurons. To this end, we studied the effects on sleep of an AD transport inhibitor, nitrobenzyl-thio-inosine (NBTI) and A₁ and A₂A receptor agonists/antagonists by microdialyzing them into the LPOA. The results showed that, in the sleep-promoting area of LPOA: 1) A₁ receptor stimulation or inhibition of AD transport by NBTI induced waking and 2) A₂A receptor stimulation induced sleep. We also confirmed that NBTI administration in the wake-promoting area of the BF increased sleep. The effects of AD could be mediated either directly or indirectly via interaction with other neurotransmitter systems. These observations support a hypothesis that AD mediated effects on sleep-wake cycle are site and receptor dependent.

Sleep is also induced by the local administration of AD or its agonists into basal forebrain (BF) (20, 25). In BF, increasing extracellular AD levels by administration of nitrobenzyl-thio-inosine (NBTI), an AD transport inhibitor, also enhances sleep; sleep is unaffected after NBTI perfusion in thalamus even if it causes an increase in AD level (24). During sleep deprivation, AD levels increase in the BF, a region containing many wake-active neurons (40), but not in other sleep-wake-related regions such as the preoptic area (POA), pedunculopontine tegmental nucleus, or dorsal raphe nucleus (23). Therefore, AD is thought to have a predominant action in the wake-active region of the BF. However, additional targets for the hypnogenic action of AD have been suggested. Sleep is also induced by the administration of AD or its agonists into the preoptic area (POA) (17, 29, 30, 45), the subarachnoid space underlying the POA (33–35, 45), or the shell of nucleus accumbens (34).

AD acts via inhibitory A₁ and excitatory A₂A receptors (7, 11, 12). A₁ receptors are widespread in brain, whereas the distribution of A₂A receptors is limited (32). Both receptor types have been implicated in AD-mediated sleep promotion. Sleep induction by A₁ receptors is hypothesized to be mediated either by inhibition of BF wake-promoting neurons (1, 42) or by disinhibition of sleep-active neurons in the ventrolateral preoptic area (VLPOA; Refs. 5 and 19). Application of an A₂A receptor agonist in the nucleus accumbens or subarachnoid space below the rostral forebrain or VLPOA induces sleep (34, 35). In the subarachnoid space, A₂A receptor agonist application induces c-Fos in VLPOA (35), a region known to contain neurons that are predominantly active during sleep (37, 41). Although application of AD or NBTI to the BF consistently inhibits wake-active neurons, some sleep-active neurons were stimulated by AD and NBTI (1). This suggests that the action of AD could be determined by the AD receptor types in a given site. We hypothesized that AD acting via A₁ receptors would induce sleep by inhibiting the wake-active neurons in BF and would induce waking by inhibition of sleep-active neurons in lateral preoptic area (LPOA). We also reasoned that AD acting via A₂A receptors would promote sleep by stimulating the sleep-active neurons of LPOA. Therefore, we studied the effects on sleep of AD accumulation in a sleep-promoting (LPOA) vs. a wake-promoting area (BF). These experiments allowed us to confirm the earlier observations in the BF, which served as a benchmark.

MATERIALS AND METHODS

Subjects were male Sprague-Dawley rats (250–300 g at the time of surgery) maintained on a 12:12-h light-dark cycle (lights on at 0600 AM and lights off at 0600 PM) with food and water freely available. All experiments were conducted in accordance with the National Research Council’s “Guide for the Care and Use of Laboratory Animals,” and procedures were reviewed and approved by the Internal Animal Care and Use Committee of the Veterans Affairs Greater Los Angeles Healthcare System.

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Surgery

Under ketamine/xylazine anesthesia (80/10 mg/kg ip) and aseptic conditions, rats were surgically prepared for polygraphic recording of sleep-waking states as described previously (18). Briefly, four stainless-steel screw electrodes were implanted in frontal and parietal bones of the skull for EEG recording, and three stainless steel wires were inserted into nuchal muscles for electromyogram (EMG) recording. One screw electrode fixed on the nasal bone served as ground. All electrodes were connected to a plug and fixed on the skull with dental acrylic. A microdialysis guide assembly consisting of a 23 G guide cannula with a stylet was implanted unilaterally 1–2 mm above the hippocampus at AP = −6.0 mm; DV = 5.0 mm; L = 5.0 mm (22) to record brain temperature.

Recording

During recovery from surgery (1 wk) and subsequent recordings, animals were housed individually in Plexiglas cages placed in an electrically shielded, sound-attenuated, temperature-controlled recording chamber (temperature: 25 ± 2°C) and acclimatized to the recording environment. EEG signals were amplified and band-pass filtered at 0.3–100 Hz, and EMG activity was recorded from electrode pairs on the nuchal muscles after band pass filtering at 10–300 Hz (Model 78 D, Grass Instruments, Quincy, MA). For recording the brain temperature, signals from thermocouples were transmitted to Thermalert monitoring thermometers (Physitemp Instruments, Clifton, NJ) and through their analog output to a DC amplifier of the polygraph. All bioelectrical signals were analog-to-digital converted at 0.3–100 Hz, and EMG activity was recorded from electrode pairs on the nuchal muscles after band pass filtering at 10–300 Hz (Model 78 D, Grass Instruments, Quincy, MA). For recording the brain temperature, signals from thermocouples were transmitted to Thermalert monitoring thermometers (Physitemp Instruments, Clifton, NJ) and through their analog output to a DC amplifier of the polygraph. All bioelectrical signals were analog-to-digital converted using a 1401 Plus data acquisition interface and Spike2 software (Cambridge Electronic Design, Cambridge, UK) and stored on a PC for off-line analysis. Polygraphic data were digitized at a sampling rate of 128 Hz for EEG and 256 Hz for EMG and brain temperature.

Drugs

All drugs were purchased from Sigma (St. Louis, MO). The drugs used were the adenosine transport inhibitor, NBTI (20 μM or 50 μM), the AD A1 receptor agonist, N(6)-cyclopentyladenosine (CPA; 25 μM), the AD A2A receptor agonist, 2-[p-(2-carboxyethyl) phenylethylamino]-5′-N-ethylcarboxamido adenosine (CGS21680, 0.5 μM, 5.0 μM, and 50 μM), the AD A1 antagonist, 8-cyclopentyl-1, 3-dimethylxanthine (CPDX, 1 μM, 5 μM, and 10 μM) and the A2A antagonist, 8-(3-Chlorostyryl) caffeine (CSC, 1 μM, 5 μM, or 10 μM). Stock solutions of drugs were made as follows: NBTI was dissolved in DMSO to make 10 mM stock solution, CPA and CGS was dissolved in double-distilled water (1 mM stocks), CPDX was dissolved in 0.1 N NaOH (1 mM stock), and CSC was dissolved in DMSO (75.6 mM stock). All stock solutions were diluted in artificial cerebrospinal fluid (ACSF; in mM): 145 Na+, 2.7 K+, 1.0 Mg2+, 1.2 Ca2+, 1.5 Cl− and 2 Na2HPO4, pH 7.2 to their final working concentrations and kept at −20°C until further use.

Experiments

A total of 42 rats were used in three experiments (Table 1). In most experiments, rats were perfused with ACSF for 2 h, followed by 90 min of perfusion with one dose of drug or ACSF. For part of experiment 2, rats were microdialyzed for 90 min with a drug (A1 or A2A agonist), followed by 20 h of ACSF perfusion. The order of administration of drug doses and ACSF was randomized. ACSF perfusion was considered as the control treatment in all experiments. The amounts of DMSO in the final dilutions were 200 nl in 1 ml of 10 μM CSC (0.02% DMSO) and 2 μl in 1 ml of 20 μM of NBTI (0.2% DMSO). Because local application of DMSO into the POA at the higher doses of 5% (unpublished observations) and 25% (31) did not affect sleep, microdialysis with DMSO alone was not undertaken in this study.

One week after surgery, the stylet, which was 2 mm longer than the guide cannula, was replaced by a microdialysis probe with 1-mm-long membrane (EiCom, Kyoto, Japan), so that the probe projected into the site of drug delivery (BF or LPOA). At least 15–20 h after probe insertion, low dead volume Teflon tubing (100 cm, EiCom) was connected from inlet and outlet of the microdialysis probe to a remote pump, and the rats were connected to a polygraph cable for behavioral state recording. Rats were perfused with ACSF for at least an hour before the recordings started. The flow rate of the perfusate was set at

Table 1. Summary of experiments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Drug</th>
<th>Duration</th>
<th>Site</th>
<th>Drug Delivery</th>
<th>Dose, μM</th>
<th>n for Each</th>
<th>Total n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Adenosine transport inhibitor</td>
<td>NBTI</td>
<td>90 min</td>
<td>BF</td>
<td>Day and night</td>
<td>20</td>
<td>6</td>
<td>14</td>
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<td>50</td>
<td>8</td>
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<tr>
<td>2: AD A1 and A2A agonists</td>
<td>CPA (A1 agonist)</td>
<td>90 min followed by 20 h of ACSF</td>
<td>LPOA</td>
<td>Night</td>
<td>25</td>
<td>5</td>
<td>5</td>
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<td></td>
<td>CGS (A2A agonist)</td>
<td>90 min followed by 20 h of ACSF</td>
<td>LPOA</td>
<td>Night</td>
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<td>90 min</td>
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<td>3: AD A1 and A2A antagonists</td>
<td>CPDX (A1 antagonist)</td>
<td>90 min</td>
<td>LPOA</td>
<td>Night</td>
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NBTI, nitrobenzyl-thio-inoosine; CPA, N(6)-cyclopentyladenosine; CGS, 2-[p-(2-carboxyethyl) phenylethylamino]-5′-N-ethylcarboxamido adenosine; CPDX, 8-cyclopentyl-1, 3-dimethylxanthine; CSC, 8-(3-Chlorostyryl) caffeine.
2 μl/min, and the time taken by the perfusate to reach the rat from the reservoir vial was ~600 s.

Experiment 1. Effect of the adenosine transport inhibitor NBTI into LPOA vs. BF. It was previously shown that administration of NBTI increased extracellular AD levels (24). The objective of this experiment was to compare the effects on the sleep-wake state of increasing extracellular AD levels in the sleep-promoting LPOA vs. the wake-promoting BF. Because NBTI doses in the micromolar range are required to inhibit both NBTI-sensitive and -insensitive equilibrative AD transporters (44), we used 20- and 50-μM doses.

Two groups of rats were used: one group (n = 8) received two doses of NBTI in LPOA, and the other group (n = 6) was dialyzed with the two doses in the BF. Rats were microdialyzed with ACSF for 2 h followed by 90 min of perfusion with one dose of NBTI. The recordings were performed both during day (9:00 AM to 2:00 PM) and night (6:00 PM to 10:00 PM). NBTI was administered twice in 24 h; once during day and the other at night. Brain temperature was not recorded in this experiment.

Experiment 2. Effects of adenosine agonists into LPOA. The aim of these experiments was to study the short-term (90 min) and long-term (20 h) effects on sleep-wake behavior of agonists of AD A₁ and A₂ₐ receptors after administering them in LPOA. The rats were adapted for 10 days to a 4-h phase-advanced light-dark cycle with lights on at 2:00 AM and lights off at 2:00 PM. A₁ and A₂ₐ agonists were administered immediately after lights off and continued for 90 min. For studying the short-term effects, three doses of the A₂ₐ agonist were used: 0.5 μM, 5.0 μM, and 50 μM; for comparison, data from the 90-min microdialysis period with 25 μM of the A₁ agonist (CPA) was used.

For studying the long-term effects, after the 90-min microdialysis with the drug, ACSF was perfused for 20 h. For long-term studies, only one dose of either A₁ (CPA; 25 μM) or A₂ₐ (CGS; 50 μM) agonist was used.

Experiments 3. Effects of adenosine antagonists into LPOA. As in the experiment 2, rats were adapted for 10 days to a shifted light-dark cycle (2:00 AM lights on and 2:00 PM lights off). All antagonist
studies involved short-term drug perfusions lasting for 90 min followed by 2 h of ACSF perfusion. Microdialysis administration of A1 antagonist (CPDX) was performed in the dark phase (beginning at 3:00 PM) and of the A2A antagonist (CSC) in the light phase (8:00 AM). One of the three doses (1.0 \mu M, 5.0 \mu M, and 10.0 \mu M) of both drugs was given on any one day preceded or followed by ACSF.

Histology

After the completion of all recordings, rats were injected with heparin (500 U ip), followed by pentobarbital sodium anesthesia (100

Fig. 2. Effects of nitrobenzyl-thio-inosine (NBTI) on hypnograms. Effects on sleep-wake of 20 \mu M of NBTI into BF at night (A) and LPOA during day (B). Arrows indicate the beginning of NBTI microdialysis. Thin lines represent artificial cerebrospinal fluid (ACSF) and thick lines represent NBTI. Abscissa is the duration of experiment in minutes.

Fig. 3. Effects of NBTI on sleep-wake cycle of rats. A comparison of effects on sleep-wake of microdialysis of 20 \mu M of NBTI into BF (A) and LPOA (B) during both day and night. Asterisks indicate statistically significant differences (P ≤ 0.05) from the corresponding ACSF values (one-way ANOVA followed by Holm-Sidak’s post hoc test). Tsleep, total sleep.

Fig. 4. Short-term effects on sleep-wake during the microdialysis into LPOA of 20 \mu M of N(6)-cyclopentyladenosine (CPA) and 50 \mu M 2-[p-(2-carbonyl-ethyl) phenylethylaminio]-5’-N-ethylcarboxamido adenosine (CGS). The sleep-wake effects were averaged for the entire duration of recording (90 min). Microdialysis was performed in the dark phase. #Statistically significant difference for the CPA is P ≤ 0.05. *Statistically significant difference for the CGS (One-way ANOVA followed by Holm-Sidak or Dunnet’s post hoc test).
The animals were perfused transcardially with 50 ml of PBS (pH 7.4) followed by 300 ml of 4% paraformaldehyde and 100 ml each of 10% and 30% sucrose in PBS. The brains were removed and equilibrated in 30% sucrose, 40-μm-thick serial sections were cut on a freezing microtome through the coronal plane and stained for Nissl (cresyl violet). Sleep and temperature data used for analysis were selected from only those rats whose brain sections showed the dialysis probe tracks terminating in the LPOA or BF.

Data Analysis

Sleep and temperature. On the basis of EEG and EMG patterns (46), polysomnographic data were scored manually at every 10-s interval into five sleep-wake stages: active waking (AW) and quiet waking (WQ) comprising awake, slow-wave sleep 1 (SWS 1), and slow-wave sleep 2 (SWS 2) comprising non-rapid eye movement (NREM), and rapid eye movement (REM) sleep. Brain temperature data were averaged every 10 s to correspond to the sleep-scoring interval. The episode durations of each of five sleep-wake stages were calculated for three EEG bands: delta (0.75–4.0 Hz), theta (4.0–8.0 Hz), and alpha (8.0–12.8 Hz). A one-way ANOVA followed by a post hoc test of either Holm-Sidak or Dunnet’s method was used to determine significant differences between control (ACSF) and experimental (drug) treatments.

RESULTS

The tips of the microdialysis probes were located within the borders in BF in 6 rats (experiment 1) and in LPOA in 36 rats (all groups) (Table1, Fig. 1). In the LPOA, the rostrocaudal distribution of the location of the probe tips extended between the stereotaxic planes of 0.26 mm and 0.8 mm posterior to bregma: 5 probe tips were located at −0.26 mm plane, and 11 probe tips were located each at −0.30 mm, −0.40 mm, and −0.80 mm planes. All locations were within the boundaries of LPOA; in 12 rats, the probe tips touched or penetrated VLPOA (Fig.1B). The location of tips of the probes in LPOA generally followed the cluster of sleep-active neurons reported (41). In the BF, probe tips were located in horizontal limb of diagonal band at 0.20 mm, −0.30 mm, and −0.80 mm from bregma (Fig. 1D).

Table 2. Effects of AD A1 agonist (25 μM) and A2A antagonist (10 μM) into IPOA on sleep-wake episodes

<table>
<thead>
<tr>
<th>Episode Duration</th>
<th>AWAKE</th>
<th>QWAKE</th>
<th>SWS 1</th>
<th>SWS 2</th>
<th>REM</th>
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<tr>
<td></td>
<td>CSF</td>
<td>CPA</td>
<td>CSF</td>
<td>CPA</td>
<td>CSF</td>
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<tr>
<td>&lt;30 s</td>
<td>14.0±3.9</td>
<td>12.6±4.4</td>
<td>36.0±8.5</td>
<td>38.4±6.9</td>
<td>43.8±8.8</td>
</tr>
<tr>
<td>40–1 min</td>
<td>2.80±0.8</td>
<td>6.6±1.4</td>
<td>3.00±0.8</td>
<td>4.8±0.9</td>
<td>6.0±1.6</td>
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<tr>
<td>1–2 min</td>
<td>1.40±0.5</td>
<td>8.4±1.3</td>
<td>1.40±0.4</td>
<td>1.2±0.7</td>
<td>0.4±0.2</td>
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<tr>
<td>2–4 min</td>
<td>1.60±0.6</td>
<td>4.8±0.3</td>
<td>0.20±0.2</td>
<td>0.6±0.4</td>
<td>0.0±0.0</td>
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<tr>
<td>4–8 min</td>
<td>0.40±0.2</td>
<td>1.4±0.5</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
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<tr>
<th>Episode Duration</th>
<th>AWAKE</th>
<th>QWAKE</th>
<th>SWS 1</th>
<th>SWS 2</th>
<th>REM</th>
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<tr>
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<td>CSF</td>
<td>CPA</td>
<td>CSF</td>
<td>CPA</td>
<td>CSF</td>
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<tr>
<td>&lt;30 s</td>
<td>12.2±1.1</td>
<td>7.0±2.0</td>
<td>28.2±2.4</td>
<td>24.2±2.5</td>
<td>44.6±5.3</td>
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<tr>
<td>40–1 min</td>
<td>3.6±0.7</td>
<td>3.4±1.0</td>
<td>4.2±2.2</td>
<td>6.8±2.7</td>
<td>8.4±1.4</td>
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<tr>
<td>1–2 min</td>
<td>2.4±1.1</td>
<td>3.8±1.0</td>
<td>1.8±1.2</td>
<td>1.6±0.9</td>
<td>2.0±0.9</td>
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<tr>
<td>2–4 min</td>
<td>1.0±0.6</td>
<td>4.6±1.4</td>
<td>0.6±0.6</td>
<td>0.4±0.2</td>
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<td>4–8 min</td>
<td>0.0±0.0</td>
<td>1.4±0.4</td>
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Bold values indicate significant difference (P ≤ 0.05) from CSF control (one-way ANOVA, followed by Holm-Sidak post hoc test). CSF, cerebrospinal fluid.
onset of microdialysis, CPA induced a significant, but short-lasting (30 min) increase in brain temperature (Fig. 6).

\( A_{2A} \) AGONIST (CGS). In the first 90 min of microdialysis, CGS perfusion significantly \( (P < 0.05) \) reduced AW and increased SWS1; it did not affect QW, SWS2, or REM (Fig. 4). CGS perfusion did not affect the durations of sleep-wake episodes (data not shown). CGS microdialysis induced long-term effects on sleep-wake stages; 2 h after the termination of CGS perfusion, AW was significantly \( (P \leq 0.05) \) decreased, while there were significant increases in SWS1 and SWS2 (Fig. 5). CGS did not affect the brain temperature (Fig. 6). Of the three doses of CGS used for microdialysis, only 50 \( \mu \)M induced significant changes in sleep-wake stages, while concentrations of 0.5 \( \mu \)M and 5.0 \( \mu \)M were ineffective (data not shown). CGS significantly reduced power in the theta band of the EEG spectrum of NREM episodes during the drug perfusion, while power in the sigma and delta bands was unaffected compared with the ACSF control (Table 3).

Experiment 3. Effects of adenosine antagonists into LPOA. Microdialysis of 1- and 5-\( \mu \)M concentrations of both \( A_1 \) antagonist, CPDX, and the \( A_{2A} \) antagonist CSC did not affect sleep-wake structure compared with ACSF (Fig. 7, A–D); 10 \( \mu \)M of CPDX also did not affect the sleep-wake stages (Fig. 7E). On the other hand, the \( A_{2A} \) antagonist, CSC, markedly \( (P \leq 0.05) \) elevated AW and suppressed SWS 2, but it did not affect QW, SWS1, or REM (Fig. 7F). Microdialysis of 10-\( \mu \)M CSC significantly \( (P \leq 0.05) \) increased long-duration AW episodes (2–8 min) and reduced short-duration episodes of

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**Fig. 5.** Long-term (20 h) effects on sleep-wake cycle of microdialysis of 20 \( \mu \)M CPA and 50 \( \mu \)M CGS into LPOA. Statistically significant differences \( (P \leq 0.05; \) one-way ANOVA followed by Holm-Sidak or Dunnett’s post hoc test) from the ACSF are represented by \# for the CPA and by * for the CGS. Hatched bar on the abscissa indicates the duration of drug perfusion. Solid and open bars represent dark and light portions of L/D cycle. AW, active waking; QW, quiet waking; SWS 1, slow-wave sleep 1; SWS 2, slow-wave sleep 2; REM, rapid eye movement.

**Fig. 6.** Effects of \( A_1 \) and \( A_{2A} \) agonists on brain temperature. Comparison of effects of microdialysis into LPOA of 20 \( \mu \)M of CPA and 50 \( \mu \)M CGS. #Statistically significant difference for the CPA is indicated by \( P \leq 0.05 \) (One-way ANOVA followed by Holm-Sidak post hoc test).

**Fig. 7.** Effects on sleep of three doses of \( A_1 \) and \( A_{2A} \) antagonists. Comparison of dose responses on sleep-wake during microdialysis into LPOA of three doses (1.0 \( \mu \)M, 5.0 \( \mu \)M, and 10.0 \( \mu \)M) of 8-cyclopentyl-1, 3-dimethylxanthine (CPDX) \( (A, C, \) and \( E \)) and 8-(3-Chlorostyryl) caffeine (CSC) \( (B, D, \) and \( F \)). *Statistically significant difference \( (P \leq 0.05; \) one-way ANOVA followed by Holm-Sidak post hoc test) from the ACSF control.
Table 3. Effects of microdialysis of AD A1 and A2A agonists (CPA and CGS) and A2A antagonist (CSC) on non-rapid eye movement EEG spectral power

<table>
<thead>
<tr>
<th>Spectral Bands of EEG</th>
<th>CPA Difference From the Baseline (%)</th>
<th>CGS Difference From the Baseline (%)</th>
<th>CSC Difference From the Baseline (%)</th>
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<tbody>
<tr>
<td>Sigma (11–16 Hz)</td>
<td>70.06±13.69</td>
<td>79.47±3.91</td>
<td>101.19±10.66</td>
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<td>Theta (6.25–9.0 Hz)</td>
<td>81.39±12.17</td>
<td>79.31±4.63*</td>
<td>115.18±19.64</td>
</tr>
<tr>
<td>Delta (0.75–4.0 Hz)</td>
<td>108.12±13.72</td>
<td>90.73±11.34</td>
<td>112.67±19.22</td>
</tr>
</tbody>
</table>

*Significant difference (P < 0.05) from the baseline; one-way ANOVA followed by Holm-Sidak post hoc test.

DISCUSSION

**NBTI effects.** In this study, we compared the effects of the adenosine transport inhibitor NBTA in a wake-related (BF) vs. a sleep-related (LPOA) area. The microdialysis of NBTA induced opposite effects in the two regions; sleep was enhanced in the BF and wakefulness was enhanced in the LPOA. The effect induced in the BF replicated and confirmed earlier reports (24), indicating that AD accumulation in the BF could facilitate sleep. Sleep facilitation could be mediated through inhibition of wake-related neurons of the region (1, 42), including cholinergic neurons.

However, the observation of waking induced by NBTA in the LPOA is inconsistent with the hypothesis of AD as a general sleep factor (2, 24). This unexpected finding could be explained by a hypothesis that buildup of AD in the sleep-promoting area inhibits the sleep-active neurons via their resident A1 receptors (see below). A higher dose (50 μM) of NBTA application in BF and LPOA was ineffective. This may have resulted from coincident activation of opposing effects of AD at A1 and A2A receptors, nullifying the general inhibitory effect of AD buildup. At the lower dose, the A1 effect would predominate; at the higher dose, the A2A effect would counteract the A1 effect.

**A1 agonist/antagonist effects.** Earlier studies have indicated that A1 receptors could mediate the sleep-inducing action of AD by either postsynaptic inhibition of wake-active neurons in the BF (1, 42, 43) or presynaptic disinhibition of GABAergic inputs to sleep-active neurons of the ventrolateral POA (5, 19). Accordingly, stimulation of A1 receptors in the sleep-promoting LPOA would be expected to induce sleep. However, in this study, administration of A1 agonist or the AD transport inhibitor NBTA in the LPOA induced waking and suppressed sleep. This observation supports a hypothesis that AD in LPOA may inhibit sleep-active neurons, which are prominent in LPOA (41), via A1 receptors. Although A1 receptor stimulation of LPOA suppressed sleep, it did not affect sleep intensity during residual NREM, as indicated by the EEG markers of sleep depth in rat (Table 3). It has been shown that in hypothalamic neurons, glutamatergic neurotransmission is antagonized by A1 agonist actions (21). Possibly in LPOA, AD antagonizes the excitatory input to sleep-active neurons.

**A2A agonist/antagonist effects.** We have also examined the involvement of AD A2A receptors of LPOA in sleep-wake and body temperature regulation. Whereas stimulation of A1 receptors induced waking and suppressed sleep, A2A receptor activation produced sleep enhancement, particularly SWS1. The lack of effect of A2A receptor stimulation on sleep intensity indicated by delta and sigma power in the EEG spectrum could be due to the absence of its effect on SWS2, the stage most rich in these frequency bands in the rat. The effects of the A2A antagonist were opposite to those of the agonist, namely, an increase in waking with a concomitant decrease in sleep. These results complement the earlier reports of sleep induction by A2A agonist in subarachnoid space near the VLPOA and BF (33–35). However, microinjection of an A2A agonist, CV 1808 (2-phenylaminoadenosine) into POA of rats was reported not to induce significant changes in sleep, although the resulting amount of SWS 2 was strongly elevated (45). Differences between the two studies could be due to the fact CV 1808 exhibits more than 10-fold less selectivity as A2A ligand compared with CGS 21680, the A2A agonist used in this study (14).

In the rat hypothalamus, presence of mRNA for A2A receptors was indicated by RT PCR studies (6), and A2A receptors were weakly radiolabeled by the selective A2A antagonist [3H]SCH 58261 in mouse hypothalamus (8). Weak immunocytochemical labeling of A2A receptors was seen in the magnocellular preoptic nucleus (32), which is adjacent to our microdialysis probes. The strong effects observed after A2A agonist/antagonist application in LPOA in the present study...

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**Fig. 8. Schematic showing the hypothetical involvement of adenosine (AD) A1 and A2A receptors of BF wake active neurons and preoptic area (POA) sleep promoting neurons in state transitions. During waking to sleep transition, AD accumulation around the BF wake active neurons inhibits them by their resident A1 receptors and stimulates sleep active neurons of POA by A2A receptors precipitating sleep. Oval with a line across indicates inhibition of adenylate cyclase, and cone with serrations indicates stimulation. Ad ctc, adenylate cyclase; Gi, inhibitory G protein; Gs, stimulating G protein.**
also suggests the presence of A2A receptors in the LPOA. A2A agonist/antagonist-induced effects could also be mediated indirectly via metabotropic glutamate receptor 5 (mGlur5) in LPOA (49), which is known to interact with A2A receptors (9). Another possibility is that the effects observed could be via A2A receptor-mediated alteration of the hypothalamic blood flow (16). Involvement of A2A receptors of LPOA in sleep promotion is significant in view of the recent finding that caffeine-induced waking is greatly attenuated in A2A receptor knockout mice, but not in A1 receptor knockouts (26). Moreover, knockout mice lacking A2A receptors were also shown to have altered sleep (48), in contrast to the A1 receptor knockout mice whose homeostatic regulation of NREM sleep remained unaffected (38).

Methodological considerations. In this report, microdialysis was used for drug delivery to the brain parenchyma of LPOA and BF. The distinct advantage offered by microdialysis is the continuous and steady rate of drug administration without restraining or otherwise disturbing the animals, as opposed to the bolus delivery of the microinjection method (45), which may induce release of prostaglandins affecting the body temperature (27, 28) that, in turn, can alter the sleep-wake stages. In this study, the effects of drugs were consistent and reversible; microdialysis of ACSF alone did not affect the sleep-wake stages; and the effects of the adenosine A2A agonist were opposite to those of the antagonist.

Schematic of the mechanism of sleep-promoting action of AD. During waking, activity of the cholinergic wake-active neurons increases the extracellular AD levels. AD acts upon A1 receptors on these wake-active neurons inhibiting them through their negative coupling to adenylate cyclase. Simultaneously, extracellular AD excites nearby sleep-active neurons via their resident A2A receptors by increasing adenylate cyclase activity, causing the transition to sleep (Fig. 8).

In conclusion, the results of the present study show that, in the LPOA sleep-promoting area, A1 receptor activation induces waking, and A2A receptor activation induces sleep. These observations support the hypothesis that AD-mediated effects on sleep-wake states are site and receptor dependent.

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