Adenosine: A Mediator of the Sleep-Inducing Effects of Prolonged Wakefulness

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Both subjective and electroencephalographic arousal diminish as a function of the duration of prior wakefulness. Data reported here suggest that the major criteria for a neural sleep factor mediating the somnogenic effects of prolonged wakefulness are satisfied by adenosine, a neuromodulator whose extracellular concentration increases with brain metabolism and which, in vitro, inhibits basal forebrain cholinergic neurons. In vivo microdialysis measurements in freely behaving cats showed that adenosine extracellular concentrations in the basal forebrain cholinergic region increased during spontaneous wakefulness as contrasted with slow wave sleep; exhibited progressive increases during sustained, prolonged wakefulness; and declined slowly during recovery sleep. Furthermore, the sleep-wakefulness profile occurring after prolonged wakefulness was mimicked by increased extracellular adenosine induced by microdialysis perfusion of an adenosine transport inhibitor in the cholinergic basal forebrain but not by perfusion in a control noncholinergic region.

Abundant experimental evidence supports the commonsense notion that prolonged wakefulness decreases the degree of arousal, which is usually measured as electroencephalographic activation (EEG arousal). Both the propensity to sleep and the intensity of delta EEG waves upon falling asleep have been demonstrated to be proportional to the duration of prior wakefulness (*1*). What might be the neural mediator of this effect of prior wakefulness? Our laboratory has provided evidence that the basal forebrain and mesopontine cholinergic neurons whose discharge activity plays an integral role in EEG arousal (*2*) are under the tonic inhibitory control of endogenous adenosine, an inhibition that is mediated postsynaptically by an inwardly rectifying potassium conductance and by an inhibition of the hyperpolarization-activated current (*3*). Adenosine is of particular interest as a putative sleep-wakefulness neuromodulator (*4*) because (i) the production and concentration of adenosine in the extracellular space have been linked to neuronal metabolic activity (*5*); (ii) neural metabolism is much greater during wakefulness (W) than during delta slow wave sleep (SWS) (*6*); and (iii) caffeine and theophylline are powerful blockers of electrophysiologically relevant adenosine receptors, promoting both subjectively and EEG-defined arousal while suppressing recovery sleep after deprivation (*7*). Our laboratory has recently demonstrated that microdialysis perfusion of adenosine in the cholinergic basal forebrain and the mesopontine cholinergic nuclei reduces wakefulness and EEG arousal (*8*).

Although the preceding evidence is consistent with adenosine as a neural sleep factor mediating the somnogenic effects of prolonged EEG arousal and wakefulness, key questions relevant to a demonstration of this role have remained unaddressed. (i) Are brain extracellular adenosine concentrations higher in spontaneous W than in SWS? (ii) Do adenosine concentrations increase with increasing duration of W and then decline slowly as recovery sleep occurs after W? (iii) Do pharmacological manipulations increasing brain adenosine concentrations produce changes in sleep and wakefulness that mimic those seen during recovery from prolonged wakefulness? (iv) Are adenosine sleep-wakefulness effects mediated selectively by neurons implicated in EEG arousal, such as cholinergic neurons, rather than stemming from widespread neuronal populations, each with relatively similar influence?

Under pentobarbital anesthesia, cats were implanted with electrodes for recording EEG, electromyogram, electro-oculogram, and ponto-geniculo-occipital waves for determination of behavioral state (*9*) and with guide cannulae for insertion of microdialysis probes (*10*). Probes were targeted to the cholinergic basal forebrain and, as a control region, to the thalamic ventroanterior/ventrolateral (VA/VL) complex, which was selected for contrast because it is not cholinergic and, as a relay nucleus, does not have cortical projections as widespread as those of the basal forebrain cholinergic neurons (*11*).

Brain extracellular adenosine concentrations were measured in the basal forebrain and the thalamus with the use of high-performance liquid chromatography and ultraviolet (UV) detection from samples collected by in vivo microdialysis (Fig. 1A) (*12*). Adenosine concentrations in consecutive samples over one complete sleep cycle [that is, a cycle containing W, SWS, rapid eye movement (REM) sleep, and W again at the end] are shown in Fig. 1B. The initial cluster of successive W episodes has consistently high values, whereas the following cluster of sleep states has generally much lower SWS values, especially as SWS becomes more consolidated. In some experiments (Fig. 1B), samples were collected during REM sleep episodes, and the adenosine concentrations measured appeared similar to the concentrations seen in adjacent SWS samples. However, we did not pursue the analysis of REM sleep samples, because the focus of the present study was not on REM sleep. Furthermore, it was relatively difficult to get pure REM samples, and there was some evidence that the short-duration REM episodes did not allow full equilibrium of adenosine with the extracellular fluid. As predicted, adenosine concentrations were less in SWS than in W, being significantly reduced by 21% in both regions [paired t test, $t(4) = 6.53$ and $P < 0.01$ for the basal forebrain and $t(4) = 2.80$ and $P < 0.05$ for the thalamus]. The grand mean (±SEM) of adenosine concentrations was 30.6 ± 5.1 nM during W versus 24.1 ± 4.4 nM during SWS (*13*).

To study the effect of prolonged wakefulness on brain extracellular adenosine concentrations, we atraumatically kept the cats awake by playing with or handling them. During the 6-hour waking period and the 3-hour subsequent recovery sleep period, EEG activity was continuously monitored, and three 10-min microdialysis samples were analyzed per hour from the basal forebrain site. The mean adenosine concentrations for six animals for each hour of the experiment were expressed as a percentage of the second-hour values (adenosine concentration at 2 hours was 30.0 ± 9.5 nM) (Fig. 2). As predicted, during the extended waking period, the extracellular adenosine concentration increased progressively with increasing duration of waking, reaching, at 6 hours, about twice that (58.9 ± 15.7 nM) seen at the onset of the experiment (Fig. 2, $P < 0.05$). During the 3-hour recovery period, adenosine declined slowly, and, at the end of the 3-hour recording window, it still had not declined to the values at the experiment's onset, although values approximated the baseline value in one cat that was recorded for 6 hours of recovery sleep.

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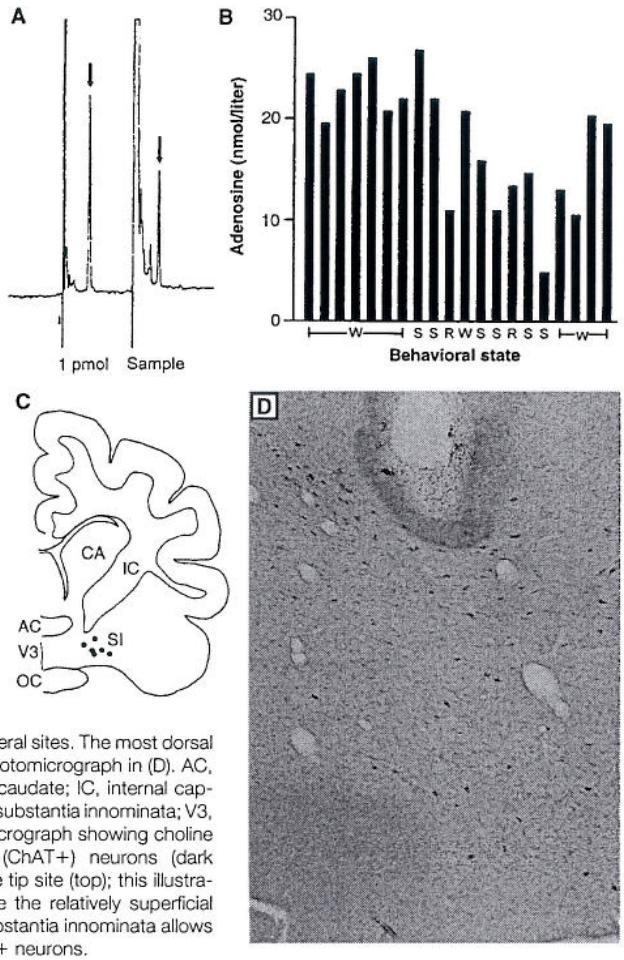

Fig. 1. Extracellular adenosine concentrations during spontaneous wakefulness and sleep. (**A**) Chromatograms of the adenosine reference standard (left) and the basal forebrain microdialysis sample (right), both of which show peaks (arrows) at 8-min retention time. (**B**) Adenosine concentrations in 10-min consecutive samples from an individual microdialysis probe in the basal forebrain. Labels indicate the predominant behavioral state: W, wakefulness; S, slow wave sleep; and R, REM sleep. (**C**) Coronal schematic of the basal forebrain showing the sites of the tips of the six probes used for the prolonged wakefulness and NBTI perfusion experiments. All sites are mapped onto this one section, including homotopic mapping for contralateral sites. The most dorsal site is that shown in the photomicrograph in (D). AC, anterior commissure; CA, caudate; IC, internal capsule; OC, optic chiasm; SI, substantia innominata; V3, third ventricle. (**D**) Photomicrograph showing choline acetyltransferase–positive (ChAT+) neurons (dark spots) surrounding a probe tip site (top); this illustration was selected because the relatively superficial location of the tip in the substantia innominata allows clear visualization of ChAT+ neurons.

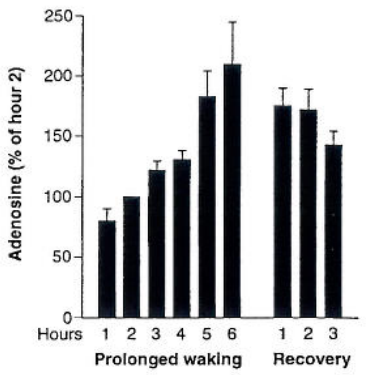

Fig. 2. Prolonged wakefulness and recovery sleep. Mean extracellular adenosine values increased in the basal forebrain during 6 hours of prolonged wakefulness [0900 to 1500; repeated measures of the analysis of variance (ANOVA) between treatments gave values of $F(8, 5) = 7.0$ and $P < 0.0001$, and the paired t test between the second and the last hour of wakefulness gave values of $t(5) = 3.14$ and $P < 0.05$]. The adenosine values decreased in the subsequent 3 hours of spontaneous recovery sleep ($n = 6$). Values are normalized relative to the second hour of deprivation (due to technical problems, three first-hour values were missing).

We next addressed the question of whether there was site specificity for adenosine effects on sleep and wakefulness. To achieve local increases in adenosine that would allow comparison of the sleep-wakefulness effects of elevated adenosine in the basal forebrain and in the thalamus, we used unilateral microdialysis perfusion of the adenosine transport inhibitor S-(4-nitrobenzyl)-6-thioinosine (NBTI, 1 μM) (*14*), in the basal forebrain and thalamus. NBTI increased adenosine concentrations about equally (to about twice the control values) in both the basal forebrain and thalamus (Fig. 3A). Despite the similar NBTI-induced increases in adenosine in the basal forebrain and thalamus, only the adenosine increases in the basal forebrain induced a decrease in wakefulness and an increase in SWS (Fig. 3B). Similarly, a power spectral analysis of the EEG revealed that the relative power in the delta band (0.3 to 4 Hz) was increased and the relative power in the gamma band was decreased after NBTI infusion in the basal forebrain but not in the thalamus (*15*) (Fig. 3C). NBTI perfusion in the basal forebrain also increased REM sleep, a finding similar to the effects of microdialysis perfusion of adenosine (*8*) (Fig. 3B).

Our final analysis examined how closely the increase of basal forebrain adenosine concentrations by NBTI mimicked the sleep-wakefulness changes associated with the increased basal forebrain adenosine concentrations caused by prolonged wakefulness. Prolonged wakefulness and NBTI infusion in the basal forebrain induced adenosine increases in the basal forebrain of almost the same magnitude, which were slightly more than twice the control values (*16*) (Figs. 2 and 3A). We noted that this congruence of adenosine concentrations afforded a useful opportunity (i) to determine if the same increase in adenosine, whether from NBTI or prolonged wakefulness, produced similar sleep-wakefulness changes, a finding that would be compatible with adenosine's acting as a sleep factor modulating the somnogenic effects of prolonged wakefulness, and (ii) to determine how closely a local basal forebrain increase in adenosine produced the same sleep-wakefulness effects as the presumptively global adenosine increases induced by deprivation, thus allowing an estimate of the potency of local, unilateral, basal forebrain changes. Both prolonged wakefulness and NBTI infusion in the basal forebrain produced the same pattern of sleep-wakefulness changes, with a reduction in wakefulness and an increase in SWS (Fig. 3D). Power spectral analysis showed that both prolonged wakefulness and NBTI infusion, compared with control values of spontaneous sleep-wakefulness states with artificial cerebrospinal fluid (ACSF) perfusion, produced the same pattern of relative power changes, as discussed in the previous paragraph (*17*).

What might be the mechanism of the observed changes in extracellular concentrations of adenosine that occur in association with sleep-wakefulness changes? Mechanisms that influence extracellular adenosine concentrations include modulation of adenosine anabolic and catabolic enzyme activity and adenosine transport rate constants or activities (*18*). For example, increases in metabolic activity during wakefulness could increase intracellular adenosine concentrations and, by altering the transmembrane adenosine gradient, reduce or even reverse the direction of the inward diffusion of adenosine via its facilitated nucleoside transporters (*19*). Similar adenosine increases may occur in other central nervous system regions, and diurnal variations of adenosine concentrations in the frontal cortex and hippocampus have

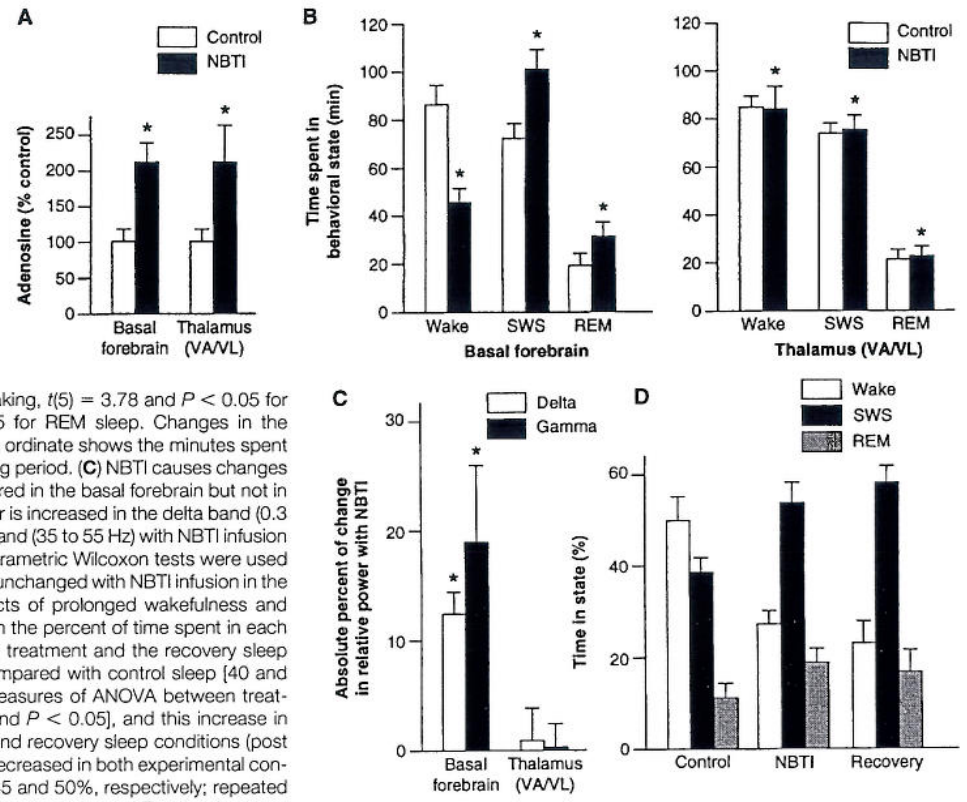

Fig. 3. Effects of local perfusion of the adenosine transport inhibitor NBTI (1 μM). (**A**) Microdialysis perfusion of NBTI increases adenosine concentrations in both the basal forebrain (paired t test, $t(5) = 4.79$ and $P < 0.01$) and the thalamus (paired t test, $t(4) = 3.92$ and $P < 0.05$) by about twofold (the means of the last three samples before and the means of the first three samples after onset of NBTI perfusion are compared). (**B**) NBTI administration causes sleep-wakefulness changes in the basal forebrain (left panel) but not in the VA/VL thalamus (right panel). In the basal forebrain, the paired t test gave values of $t(5) = 3.47$ and $P < 0.05$ for waking, $t(5) = 3.78$ and $P < 0.05$ for SWS, and $t(5) = 2.76$ and $P < 0.05$ for REM sleep. Changes in the thalamus are P = NS for all states. The ordinate shows the minutes spent in each state during the 3-hour recording period. (**C**) NBTI causes changes in the power spectrum when administered in the basal forebrain but not in the VA/VL thalamus. The relative power is increased in the delta band (0.3 to 4 Hz) and decreased in the gamma band (35 to 55 Hz) with NBTI infusion in the basal forebrain ($P < 0.04$; nonparametric Wilcoxon tests were used because of nonnormality of data) but is unchanged with NBTI infusion in the thalamus. (**D**) Comparison of the effects of prolonged wakefulness and NBTI perfusion in the basal forebrain on the percent of time spent in each behavioral state. During both the NBTI treatment and the recovery sleep conditions, SWS was increased as compared with control sleep [40 and 50%, respectively; $n = 5$; repeated measures of ANOVA between treatments gave values of $F(2, 4) = 5.92$ and $P < 0.05$], and this increase in SWS did not differ between the NBTI and recovery sleep conditions (post hoc Neuman Keul). Wakefulness was decreased in both experimental conditions as compared to control sleep [45 and 50%, respectively; repeated measures of ANOVA between treatments gave values of $F(2, 4) = 9.41$ and $P < 0.01$], whereas the two experimental conditions did not differ from each other. REM sleep in the NBTI-treated and recovery sleep groups had similar percentage increases (65 and 50%).

indeed been reported, although these studies did not measure behavioral state–related changes (*20*). We suggest that adenosine's powerful state-altering effects in the cholinergic basal forebrain region occur primarily because of the cholinergic neurons' widespread and strategic efferent targets in the thalamic and cortical systems that are important for the control of EEG arousal (*21*). Increased adenosine concentrations in the cholinergic basal forebrain zone would thus decrease EEG arousal, increase drowsiness, and promote EEG delta wave activity during subsequent sleep. We suggest that extracellular adenosine concentrations decrease in SWS because of the reduced metabolic activity of sleep, especially in delta wave sleep, when cholinergic neurons are relatively quiescent. This postulate is congruent with the observed declining exponential time course of delta wave activity over a night's sleep (*1*).

Taken together, these results suggest that adenosine is a physiological sleep factor that mediates the somnogenic effects of prior wakefulness. The duration and depth of sleep after wakefulness appear to be profoundly modulated by the elevated concentrations of adenosine.

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8. C. M. Portas, M. Thakkar, D. G. Rainnie, R. W. Greene, R. W. McCarley, *Neuroscience*, in press. This study also demonstrated a concentration-response relation over a four-log-unit concentration range between adenosine perfused into the cholinergic basal forebrain and the extent of reduction of wakefulness.
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10. Intracerebral guide cannulae (CMA 10 guide; CMA/Microdialysis, Stockholm, Sweden) were implanted 12 mm above the target. The coordinates for the basal forebrain (substantia innominata) were AP 15.5, ML 5, and DV −1.5, for the thalamus they were (VA/VL) AP 11, ML 5, and DV 2.5 [A. L. Berman and E. G. Jones, *The Thalamus and Basal Telencephalon of the Cat* (Univ. of Wisconsin Press, Madison, WI, 1982)]. After surgery, the animals were allowed to recover for 2 weeks. Histological processing was on 40-μm sections of formaldehyde-fixed brain tissue processed for immunohistochemistry with an antibody for choline acetyltransferase [P. J. Shiromani, S. Winston, R. W. McCarley, *Mol. Brain Res.* **38**, 77 (1996)].
11. We wished to test the hypothesis that adenosine exerts a selectively stronger influence on neurons that are intimately related to sleep-wakefulness control; we chose cholinergic neurons for study because our in vitro data indicate that adenosine exerts powerful inhibitory effects on them.
12. The mobile phase consisted of 8 mM NaH_2PO_4 in 8% methanol (pH = 4), with a flow rate of 80 μl/min produced by a Bioanalytical Systems (BAS, West Lafay-

ette, IN) PM-80 pump. Separation was achieved by a BAS microbore column (MF-8949; 1 × 100 mm, with C18 packing of 3-μm particle size), which was attached directly to the injector (Rheodyne 9125) and to the UV detector (Waters 486 UV detector, outfitted with a Waters microbore cell kit). Adenosine was detected at a wavelength of 258 nm. Chromatographic data were recorded on a chart recorder, and the peak heights of microdialysis samples were compared to the peak heights of adenosine standards (1 pmol/10 μl) for quantification. The detection limit of the assay was 50 fmol (based on a signal-to-noise ratio of 3:1). Repeated assays of standards and pooled samples showed less than 10% variability. Custom-made CMA 10 probes from CMA/Microdialysis had a polycarbonate membrane (20,000-dalton cutoff), a 500-μm outer diameter, a 2-mm microdialysis membrane length, and a 35-mm shaft length. During the experiment, ACSF (composed of 147 mM NaCl, 3 mM KCl, 1.2 mM $CaCl_2$, and 1.0 mM $MgCl_2$, at a pH of 6.6) was pumped through the probe at a flow rate of 1.5 μl/min, the same flow rate used for drug perfusion. Consecutive 10-min dialysis samples were collected throughout the day via tubing with a low dead space volume (1.2 μl per 10 cm, FEP tubing; CMA/Microdialysis) and correlated with electrographically defined sleep-wakefulness states. Adenosine from a microdialysis sample produced a sharp chromatogram peak with a high signal-to-noise ratio and the same 8-min retention time as the adenosine standard (Fig. 1A).

13. For the analysis of the group data, a sleep cycle was defined as a continuous period that contained all of the behavioral states (W, SWS, and REM sleep), and began and ended with waking periods; the validity of comparisons over time was ensured by rejection of any cycles where there were suggestions of nonstationarity (adenosine values with >25% change between the first and last waking epochs). Of the samples in this comparison of W and SWS, 65% were 100% in a single state, and the remaining 35% had less than 20% of another state. The mean cycle duration was not different in the basal forebrain and thalamus samples.
14. NBTI actions are discussed in G. Sanderson and C. N. Scholfield [*Pfluegers Arch. Eur. J. Physiol.* **406**, 25 (1996)] and H. L. Haas and R. W. Greene [*Naunyn-Schmiedeberg's Arch. Pharmacol.* **337**, 561 (1988)]. These references and our preliminary data confirmed 1 μM as the lowest dose producing maximal effect. To ensure the presence of normal sleep, the 3-hour baseline period was not started until 30 min after the first REM episode (typically 1 to 2 hours after the animal was connected to the polygraph and microdialysis lines). Basal extracellular concentrations of adenosine were determined during the 3-hour baseline period that preceded the drug administration.
15. EEG power spectral analysis was performed during ACSF perfusion, during perfusion with 1 μM NBTI in the basal forebrain and thalamus, and during recovery sleep after 6 hours of wakefulness. Parietal EEG screw electrodes were used for EEG acquisition. The data were filtered at 70 Hz (low-pass filter) and 0.3 Hz (high-pass filter) with a Grass electroencephalograph and were continuously sampled at 128 Hz by a Pentium microprocessor computer with a Data-Wave (Data-Wave Technology, Longmont, CO) system. Absolute total power was calculated for the frequency range between 0.3 and 55 Hz. Five different frequency bands were used to calculate the relative power: delta, 0.3 to 4 Hz; theta, 4.1 to 9 Hz; alpha, 9.1 to 15 Hz; beta, 15.1 to 25 Hz; and gamma, 25.1 to 55 Hz. After basal forebrain NBTI perfusion, the relative power was significantly increased in the delta and decreased in the theta, alpha, beta, and gamma bands ($P < 0.04$; nonparametric Wilcoxon matched pairs signed-ranks test, used because of nonnormality of data). There was no change in power in any frequency band after NBTI infusion in the thalamus.
16. In evaluating the physiological relevance of adenosine at various concentrations, it is important to note that in vitro data from our laboratory (*3*) demonstrated that endogenous adenosine had a consistent inhibitory effect on cholinergic neurons. These data imply that adenosine's physiological effects in vivo are to be expected at baseline that is, without sleep deprivation or NBTI. Rainnie *et al.* (*3*) did not measure endogenous adenosine concentrations, and thus the precise in vitro effects of doubling adenosine concentrations have not yet been specified, although it is known that there are progressive increases in inhibition of cholinergic neurons (beyond that seen from the endogenous inhibitory effect) with increasing concentrations of exogenously applied adenosine. Furthermore, we believe that the actions of adenosine that we have found in animal studies apply also to humans. First, the increase in EEG sleepiness with increasing duration of wakefulness has been documented in humans (*1*). Second, the adenosine physiology and pharmacology of experimental animals and of humans appear to be comparable [see reviews in (*4*–*7*) and also L. J. Findley, M. Boykin, T. Fallon, L. Belardinelli, *J. Appl. Physiol.* **65**, 556 (1988); and H. L. Haas, R. G. Greene, M. G. Yasargil, V. Chan-Palay, *Neurosci. Abstr.* **13**, 155 (1987)]. Finally, the adenosine antagonist caffeine increases wakefulness in formal experimental studies [see (*7*) and H. P. Landolt, D. J. Dijk, S. E. Gaus, A. A. Borbely, *Neuropsychopharmacology* **12**, 229 (1995)] and, as with the adenosine antagonist theophylline, constitutes the sleep-delaying ingredient in coffee and tea.
17. Changes in the entire relative power spectrum with NBTI infusion and in recovery sleep after prolonged wakefulness were, for each band, in the same direction (n = four animals).
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19. This possibility has recently been reviewed by J. M. Brundege and T. V. Dunwiddie [*J. Neurosci.* **16**, 5603 (1996)], who also provided direct evidence for the possibility that an increase in intracellular adenosine (either by exogenous adenosine or inhibiting metabolism of endogenous adenosine) could lead to an increase in extracellular adenosine and its actions on receptors.
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21. It is also possible that adenosine's effects in the neocortex may be directly attenuated by cholinergic receptor activation, as has been shown in the hippocampus [P. F. Worley, J. M. Baraban, M. McCarren, S. H. Snyder, B. E. Alger, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3467 (1987)]. Thus, adenosine's direct inhibitory effects on cholinergic somata might be enhanced by a consequent disinhibition of adenosine's effects on neocortical neurons. The specificity of sleep-wakefulness effects of NBTI does not support the idea that adenosine's effects result from a global action on brain neurons, as suggested by J. H. Benington and H. C. Heller [*Prog. Neurobiol.* **45**, 347 (1995)].

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