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Research report

The adenosine kinase inhibitor ABT-702 augments EEG slow waves in rats

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Abstract

ABT-702 is a novel and selective non-nucleoside adenosine kinase (AK) inhibitor that produces increases in endogenous extracellular adenosine. Adenosine (ADO) is thought to be an important neuromodulator of sleep, therefore, the effects of ABT-702 and AK inhibition were examined on rat EEG and sleep, and compared to ADO receptor agonists to further evaluate the role of ADO receptor activation on sleep related EEG patterns. ABT-702 (10.0–30.0 μmol/kg, i.p.) increased the amplitude of the 1–4 Hz band (Fast Fourier Transform (FFT) analysis, $p<0.05$), which is indicative of augmented sleep-related slow waves. Theophylline (5.0 μmol/kg, i.p.), a centrally active, non-selective adenosine receptor antagonist, attenuated the effects of ABT-702 (20.0 μmol/kg, i.p.) on EEG, whereas 8-(*p*-sulfophenyl)-theophylline (8-PST, 150.0 μmol/kg, i.p.), a peripherally active antagonist, did not, indicating that the EEG effects of ABT-702 are mediated by a central ADO receptor mechanism. The selective A_1 agonist N6-cyclopentyladenosine (CPA, 30.0 μmol/kg, i.p.) also increased the amplitude of 1–4 Hz band, but was not as efficacious as ABT-702. In contrast, the A_{2A} agonist CGS-21680 (1.0–10.0 μmol/kg, i.p.) and the non-selective agonist, N^6-ethylcarboximidoadenosine (NECA, 0.03–0.1 μmol/kg, ip.), lowered 1–4 Hz amplitude for 2 h after injection. Finally, ABT-702 (10.0 μmol/kg, i.p.) was found to significantly increase slow wave sleep and decrease REM sleep in rats implanted with both EEG and EMG electrodes for evaluation of sleep. These studies demonstrate that increased extracellular adenosine through AK inhibition can elicit modulatory effects on EEG slow waves via an interaction with central ADO receptor subtypes.

Keywords: EEG; Adenosine; Adenosine kinase inhibitor; Slow waves; Sleep

1. Introduction

Extracellular levels of adenosine (ADO) increase in response to tissue trauma during conditions such as ischemia, hypoxia, and neuronal hyperexcitability [29,38]. In neural tissue, extracellular ADO is a potent inhibitor of dopamine, GABA, glutamate, acetylcholine, serotonin and norepinephrine release acting primarily via presynaptic A_1 receptors with preferential effects on excitatory as opposed to inhibitory neurotransmitter release [52]. Postsynaptically, ADO also modulates neuronal excitability by hyperpolarizing the postsynaptic membrane [38]. These actions of ADO mediate the ability of this endogenous autocoid to limit seizure activity, attenuate pain and inflammation, and impair motor performance [21,52].

The neuroinhibitory actions of ADO may also play a role in the regulation of sleep [37]. One hypothesis suggests that during waking, through a breakdown of ATP, ADO levels increase until a state of net inhibitory influence is exerted on ascending cerebral activating systems, thus promoting sleep [5,33,35]. Exogenous ADO and several ADO A_1 and A_{2A} receptor agonists have been shown to promote electroencephalographic (EEG) signs of sleep [6,38,39]. In contrast, ADO antagonists such as caffeine promote EEG signs of neural activation and arousal [23,32,36,51,53]. The view that ADO is involved in sleep regulation is further supported by studies showing that local injections of ADO into key structures controlling EEG arousal, such as the

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cholinergic mesopontine nucleus and basal forebrain, promote physiological and behavioral signs of sleep [1,37].

Thus far, pharmacological sleep studies have relied heavily on exogenously applied ADO agonists to demonstrate the association between ADO-receptor mediated neuromodulation and sleep regulation. A way to endogenously stimulate ADO receptors is to increase extracellular ADO by blocking or attenuating its degradation. Endogenous ADO concentrations in the CNS extracellular space are controlled by reuptake and metabolism in neurons [2]. Intracellular metabolism of ADO is under the control of adenosine kinase (AK) that catalyzes the phosphorylation of ADO to AMP and is the rate-limiting enzyme regulating extracellular ADO concentrations [2]. Increased intracellular ADO by AK inhibition creates an unfavorable concentration gradient for the reuptake of ADO into the cell, thus increasing extracellular ADO [11,15,31]. Compounds that inhibit AK increase basal extracellular ADO levels both in vivo and in vitro [9,10,42]. AK inhibitors also potentiate evoked release of ADO in the brain [8]. Thus, AK inhibitors may be useful in modulating endogenous extracellular ADO to levels that mimic natural fluctuations as a result of the breakdown of ATP. Given the likely role of endogenous ADO in the control of somnolence and arousal, it is reasonable to think that AK inhibition by AK inhibitors, and a resulting elevation of ADO, could elicit or augment physiological signs of sleep. In recent years, considerable effort has been given toward exploiting AK inhibition to modulate the beneficial analgesic and anti-inflammatory effects of ADO, while relatively little has been reported investigating the effects of AK inhibition on arousal.

ABT-702 is a novel and selective non-nucleoside AK inhibitor [16]. Consistent with its in vitro activity, ABT-702 has been shown to potently attenuate nociception and inflammation in vivo [7,16,22,48]. ABT-702 is selective for AK inhibition over binding directly to ADO A_1, A_{2a}, and A_3 receptors, the adenosine transporter, and adenosine deaminase [16]. Additionally, the in vivo effects of this compound are blocked or attenuated by non-selective ADO antagonists [17,22]. Thus, the effects of ABT-702 are likely due to increased extracellular ADO as a result of AK inhibition. The purpose of the present study was to determine whether increases of endogenous ADO by AK inhibition affect arousal as measured by recording the EEG after administration of ABT-702. Several ADO receptor agonists including N^6-cyclopentyladenosine (CPA, A_1 receptor selective), CGS-21680 (A_{2A} receptor selective), and *N*-ethylcarboxamidoadenosine (NECA, nonselective) were also tested for comparison to ABT-702 since these agonists bind to adenosine receptor subtypes that have been previously implicated in modulating sleep [3,6,18,25,40,41]. A quantitative frequency–amplitude analysis using Fast Fourier Transform (FFT) was applied to EEG records from rats with an emphasis on 1–4 Hz EEG activity, levels of which are known to be a good indicator of slow wave (non-REM) sleep. In additional experiments, two ADO receptor antagonists, the CNS penetrating 8-cyclopentyltheophylline (theophylline) and the non-CNS penetrating 8-(*p*-sulfophenyl)-theophylline (8-PST), were co-administered with ABT-702 and the EEG recorded. The purpose of co-administration of theophylline with ABT-702 was to determine whether antagonism of central ADO receptors could attenuate the effects of ABT-702. Since ABT-702 has little binding potency at any of the known ADO receptor subtypes, attenuation of ABT-702 by theophylline could implicate a mechanism by which ABT-702 secondarily affects ADO receptors by elevating endogenous extracellular ADO. The lack of attenuation of ABT-702 EEG effects by co-administration with the non-CNS penetrating 8-PST would further implicate a central, rather than a peripheral site of action for ABT-702. Finally, in rats implanted with both EEG and EMG electrodes, ABT-702 was specifically evaluated for effects on slow wave and REM sleep.

2. Methods

Male Sprague–Dawley rats (6 months) were obtained from Charles River (Portage, MI), and were maintained on a 12-h light/dark cycle, fed ad libitum, and treated in accordance with approved AAALAC approved procedures. EEG recording electrodes were bilaterally implanted under pentobarbital anesthesia (50 mg/kg, i.p.) over the parietal cortex (−2.0 mm AP, 4.0 mm L). A reference electrode was placed 11 mm posterior to bregma. A miniature connector was affixed to the skull and the rats were allowed 2 weeks recovery from the surgery. In a separate group of Sprague–Dawley rats, in addition to EEG recording electrodes, thin silver wire electrodes were placed in neck muscles to record EMG (electromyogram) for evaluation of sleep patterns.

The EEG (sampling rate 256 Hz) was recorded inside sound-attenuating chambers. Before experiments began, a flexible cable was attached to the implanted miniature connector that allowed unrestricted movement during the recording sessions. EEG amplifiers (Grass Instrument Division, Astro-Med, West Warwick, RI) and a computer-based system (Stellate Systems, Montreal, Canada) were used to acquire and analyze data. Before any experiments began, implanted rats were habituated to the sleep recording chambers for 2–5 h on 5–6 consecutive days. All experiments and habituation sessions were conducted during the light phase of the circadian cycle.

Dose–response effects on EEG were determined for ABT-702 (3.0–30.0 μmol/kg, i.p.), CGS-21680 (1.0–10.0 μmol/kg, i.p.), CPA (1.0–30.0 μmol/kg, i.p.) and NECA (0.1–1.0 μmol/kg, i.p.). The selected doses for these compounds are in the range that reduce exploratory motor activity but lower than those required to disrupt motor coordination [17,22,27]. The treatments were administered in a random order on different days with one treatment per day and 3 days between treatments. On one of these treatment days the rat would receive a vehicle control

treatment. This within subjects design allowed each rat to serve as its own control. EEG recordings were begun within 5 min after injection and recording sessions lasted for 120 min. The time of day for injections and subsequent recordings were between 10:00 AM and 12:00 PM.

The effects of theophylline (5.0 μmol/kg, i.p.) or 8-(*p*-sulfophenyl)-theophylline (8-SPT, 150.0 μmol/kg, i.p.) on ABT-702 (20 μmol/kg, i.p.)-induced EEG changes were determined in another group of 8 rats. Theophylline (10.0 μmol/kg, i.p.) has been shown to significantly attenuate the antinociceptive effects of ABT-702 [22]. We chose a lower theophylline dose of 5.0 μmol/kg in this experiment since, in preliminary dose finding experiments (not shown), a dose of 15.0 μmol/kg began to elicit a stimulant effect on the EEG. Theophylline has been previously reported to lower 1–4 Hz EEG and sleep in rats, however, the dose used in this experiment is about 10-fold lower than those doses reported to produce a stimulant effect on EEG [36,51] Choosing a non-stimulant dose of theophylline for this experiment is important for showing pharmacological antagonism. Attenuating ABT-702-induced slow waves by a stimulant dose of theophylline could be a summation of opposing physiological effects, rather than due to functional receptor antagonism. The dose of 8-PST chosen for this experiment is well above that necessary to block peripheral ADO receptor activation but does not attenuate the centrally mediated antinociceptive effects of ABT-702 [22]. Each rat received 6 different treatments on separate days, each treatment being a combination of two injections. The treatment groups are listed in Table 1. The first injection was administered 20 min before the second injection. The EEG recordings were begun within 5 min after this second injection. The treatments were administered in random order across days with 3 days between treatments. Again, each rat served as its own control.

The average EEG amplitude in microvolts (μV) was determined for 30 s epochs using Fast Fourier Transform (FFT) analysis for the frequency bands of 1–4 (delta), 4–8 (theta), 8–13 (alpha), 13–25 (beta1), 25–50 (beta2), and 1–50 Hertz (Hz). Thus, for an analysis of the entire 120 min recording session, 240–30 s FFT analyzed epochs were averaged together, and for the analysis of 20 min bins, 20–30 s FFT analyzed epochs were averaged together. Epochs that contained movement artifact in the EEG were excluded from this averaging. A repeated measure, one-way ANOVA was utilized for statistical evaluation of FFT data with treatment or time as the repeated measure. A Fishers protected least significant difference (PLSD) post-hoc test was used for comparisons between treatments.

For the evaluation of sleep patterns, a within subjects design was also employed by administering ABT-702 (10.0 μmol/kg, i.p.) or vehicle to rats on separate days. These rats were, in addition to being recorded for EEG/EMG, visually monitored for overall activity level by the use of video cameras installed in the recording chambers. EEG and EMG recordings, as well as video monitoring continued for 5 h after injection. A computer-based scoring system classified Fourier transformed EEG/EMG states as those being awake, slow wave sleep (SWS), and rapid eye movement (REM) sleep for each 4 s epoch during the entire 5 h recording session. The awake condition was identified as high amplitude EMG and low amplitude 1–4 Hz EEG. SWS was identified as low amplitude EMG and high amplitude 1–4 Hz EEG, and REM was identified as low amplitude EMG, and low amplitude 1–4 Hz EEG. Behaviorally, the awake EEG/EMG state corresponded to the rats actively exploring, grooming, or sitting still with eyes open. The sleeping EEG/EMG corresponded to the rats being immobile, eyes closed, and frequently in a curled position. The rats would also show brief motor twitches during the REM state. The sleep data was expressed as averaged time spent in either the awake, SWS, or REM state during 1 h bins or for the total 5 h recording session. A repeated measure (time) ANOVA and student *t*-test were utilized for statistical evaluation of sleep data.

All treatments were administered by the intraperitoneal (i.p.) route of administration. ABT-702 was synthesized as previously described [24]. CPA, NECA, 8-PST, and theophylline (8-cyclopentyltheophylline) were purchased from Sigma (St. Louis, MO). CGS-21680 was obtained from Research Biochemicals (Natick, MA). All compounds were dissolved in sterile hydroxypropyl-β-cyclodextrin (Aldrich Chemical, Milwaukee, WI) with 10% DMSO (Sigma) prior to injection.

Table 1
Treatment groups for antagonist experiments

Treatments	Injection 1	Injection 2
1	Vehicle	Vehicle
2	ABT-702, 20.0 μmol/kg	Vehicle
3	Vehicle	Theophylline, 5.0 μmol/kg
4	Vehicle	8-PST, 150.0 μmol/kg
5	ABT-702, 20.0 μmol/kg	Theophylline, 5.0 μmol/kg
6	ABT-702, 20.0 μmol/kg	8-PST, 150.0 μmol/kg

3. Results

Fig. 1 shows representative examples of EEG recordings and associated FFT spectral analysis from individual rats in experiments examining the effects of ABT-702. Early in the EEG recording session, the low-voltage activated EEG pattern shown in trace 1 is common in rats treated with vehicle and the lower doses of ABT-702 (3.0 and 10.0 μmol/kg, i.p.). Vehicle treated rats, after a within session period of habituation, begin to express higher voltage EEG slow waves (trace 2). After administration of ABT-702 at 10.0 μmol/kg, rats also express a higher voltage slow wave EEG pattern similar to that of slow wave sleep (Trace 3). Slow wave episodes seen in vehicle treated rats are fewer and shorter in duration compared to the ABT-702 treated rats. At the higher dose of ABT-702 (30.0 μmol/kg), slow waves

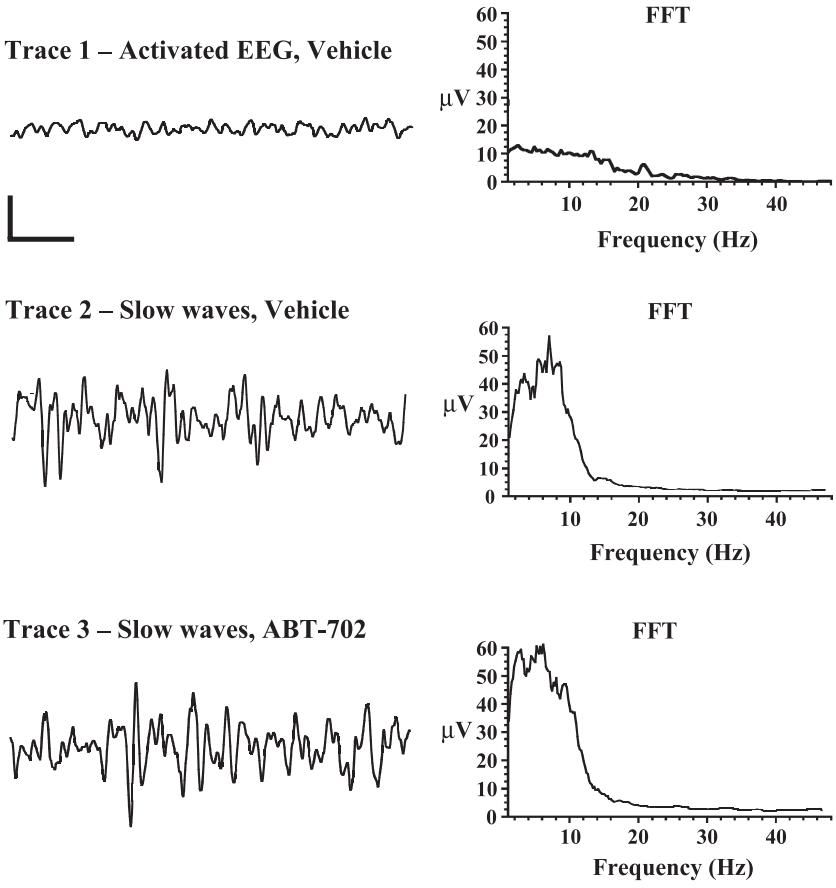

Fig. 1. Examples of EEG recordings from individual rats with corresponding spectral FFT histograms to the right of each trace. Trace 1: Rats express a low voltage (activated) EEG pattern during the early portions of the recording session under vehicle treatment. After this initial activation, rats begin to express higher voltage slow waves associated with drowsiness and sleep. This activated pattern is also expressed in rats treated with ABT-702, but the amount of activated EEG expressed is reduced. Trace 2: After an initial expression of activated EEG patterns, vehicle treated rats express high amplitude slow waves. Trace 3: After an initial expression of activated EEG patterns similar to vehicle treatment, rats treated with ABT-702 (10.0 μmol/kg) express sleep-like slow waves. At this 10.0 μmol/kg dose, slow wave activity appears qualitatively similar to slow waves expressed in vehicle treated rats. At the higher dose of ABT-702 (30.0 μmol/kg), slow waves become higher in amplitude, more synchronous, and have a more rapid onset. These sections of EEG were taken approximately 25 min after injection of ABT-702 or vehicle. Calibration: vertical 500 μV, horizontal 0.5 s.

became higher in amplitude, were more synchronous, and had a more rapid onset. However, even though synchronous activity predominated the EEG of rats treated with 30.0 μmol/kg ABT-702, a normal low voltage activated pattern appeared during occasional bouts of locomotor activity. Additionally, these rats appeared to be as reactive to random ambient acoustic noise as rats administered vehicle or lower doses of ABT-702. Thus, ABT-702 treated rats did not appear to be heavily sedated or anesthetized at any of the doses tested.

Table 2 shows that during the 2 h following administration, the average amplitude of 1–4 Hz delta slow wave activity was significantly increased by administration of ABT-702 ($F(3,21)=59.892$, $p<0.0001$). Likewise, activity in the 4–8 Hz theta band was also significantly increased ($F(3,21)=46.848$, $p=0.0018$), although the magnitude of this 4–8 Hz increase was not as great as that seen in the lower 1–4 Hz band. In contrast to the effects of the 1–4 and 4–8 Hz bands, significant decreases of EEG amplitude were seen in the 8–13 Hz alpha band ($F(3,21)=8.757$, $p<0.0006$),

Table 2
Effects of ABT-702 on EEG frequency bands—120 min averaged activity

EEG amplitude (μV)—mean ± SEM						
μmol/kg	1–4 Hz	4–8 Hz	8–13 Hz	13–25 Hz	25–50 Hz	1–50 Hz
0.0	35.4±3.0	32.4±3.1	21.6±2.3	12.8±1.1	4.9±0.4	100.9±9.6
3.0	35.6±2.9	31.2±2.9	19.9±1.9	11.9±1.0	4.7±0.4	103.4±8.9
10.0	44.2±4.8	35.5±3.2	19.3±2.0	11.3±1.1	4.1±0.3	112.1±11.3
30.0	72.6±5.2*	36.9±3.0	17.1±1.5	9.4±0.8*	3.1±0.2*	138.8±10.6*

*$p<0.05$, ANOVA Fishers post hoc vs. 0.0 group, $n=8$.

the 13–25 Hz beta1 band ($F(3,21)=19.546$, $p<0.0001$), and the 25–50 Hz beta2 band ($F(3,21)=64.587$, $p<0.0001$). Despite the decrease in high frequency EEG amplitude, the total EEG frequency spectrum of 1–50 Hz was significantly increased ($F(3,21)=19.152$, $p<0.0001$) because of the increases in the higher energy 1–4 and 4–8 Hz bands. Fig. 2 shows the time course of effects on 1–4 Hz activity of ABT-702. The treatment effect ANOVA was significant ($F(3,28)=11.5$, $p<0.001$) as well as the time-treatment repeated measures interaction ($F(15,140)=2.3$, $p=0.0056$). The effects of ABT-702 (30.0 μmol/kg, i.p.) had a rapid onset of action (<20 min) and persisted for the entire 2-h recording session.

Fig. 3 shows the effects of the centrally active adenosine receptor antagonist theophylline on slow waves induced by ABT-702. An ABT-702 dose of 20.0 μmol/kg was selected for this experiment because, based on the effects presented in Table 2, it falls between the minimally effective 10.0 μmol/kg and maximally effective 30.0 μmol/kg dose. The 1–4 Hz EEG amplitude was averaged over the 2 h recording session and the overall treatment effect ANOVA was significant ($F(3,28)=7.6$, $p=0.0007$). Fisher post-hoc ANOVA revealed a significant increase of EEG slow waves with vehicle-ABT-702 and theophylline (5.0 μmol/kg)-ABT-702 treatment compared to vehicle–vehicle treatment. However, the post-hoc analysis also shows that the theophylline (5.0 μmol/kg)-ABT-702 treatment group was significantly different from the vehicle-ABT-702 group, indicating that the antagonist attenuated the effect of ABT-702 on slow waves. The EEG was not affected by treatment with vehicle followed by the 5.0 μmol/kg dose of theophylline, which concurred with our preliminary dose finding studies. Thus, it is concluded that the reduced level of slow wave activity observed in the ABT-702-theophylline treatment group compared to ABT-702-vehicle treatment is not the sum of opposing actions of theophylline and ABT-702, rather, it is more likely the result of functional antagonism of central ADO receptors. 8-PST (150.0 μmol/kg), an antagonist that does not readily cross the blood–brain barrier, did

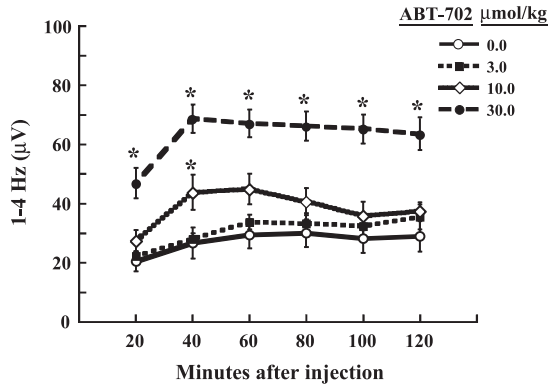

Fig. 2. Time Course for the Effects of ABT-702 on EEG; 1–4 Hz Amplitude. ABT-702 significantly increases 1–4 Hz amplitude. The onset of this effect was less than 20 min after administration. *ANOVA Fishers post hoc analysis, $p<0.05$ vs. 0.0 group, $n=8$.

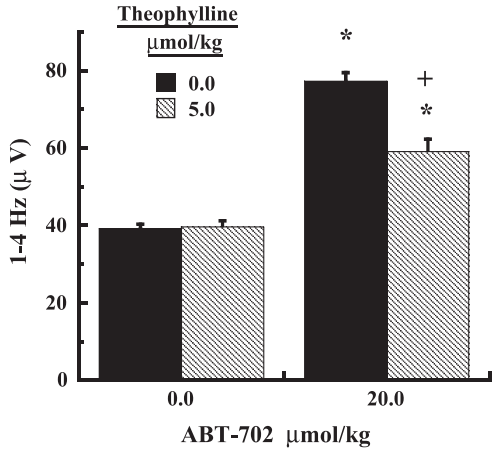

Fig. 3. Effects of the non-selective, centrally active adenosine antagonist theophylline on ABT-702-induced increase of 1–4 Hz EEG. The antagonist was administered 20 min following ABT-702. Theophylline attenuates the effects of ABT-702 on 1–4 Hz EEG. ANOVA, Fishers post hoc analysis, *$p<0.05$ vs. 0.0/0.0, + $p<0.05$ vs. ABT-702 (20.0)/0.0, $n=8$.

not attenuate ABT-702 slow waves (Fig. 4). The dose of 8-PST in this experiment has been shown to completely block peripheral ADO receptors (22). The inability of 8-PST to block or attenuate the effects of ABT-702, in contrast to theophylline which did attenuate ABT-702, strongly implicates a central site of action for the effects of ABT-702. Like theophylline, 8-PST has no effect alone on the EEG at the doses used in this pharmacological study.

Fig. 5 shows the dose–response effects of ABT-702 (data derived from the experiment shown in Table 1) on 1–4 Hz EEG compared to direct acting agonists of central ADO receptors. Since each compound is tested in different groups of rats, the data are expressed in this graph as a percent change from vehicle control to be able to compare the magnitude of the agonist effects across several different experiments. We have found that, although absolute

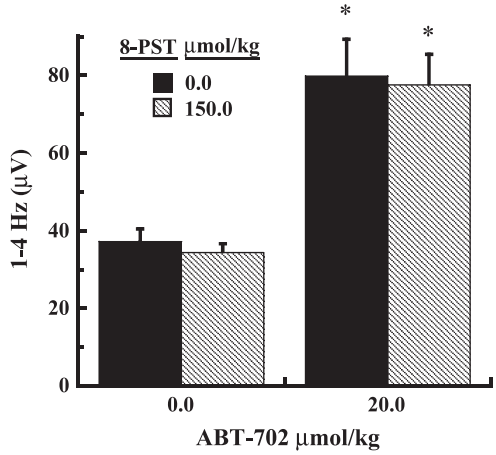

Fig. 4. The effects of 8-PST, a peripherally active adenosine antagonist, on ABT-702-induced increase of 1–4 Hz amplitude. The antagonist was administered 20 min following ABT-702. 8-PST did not attenuate the ABT-702 effect. *ANOVA, Fishers post hoc analysis, $p<0.05$ vs. 0.0/0.0 group, $n=8$.

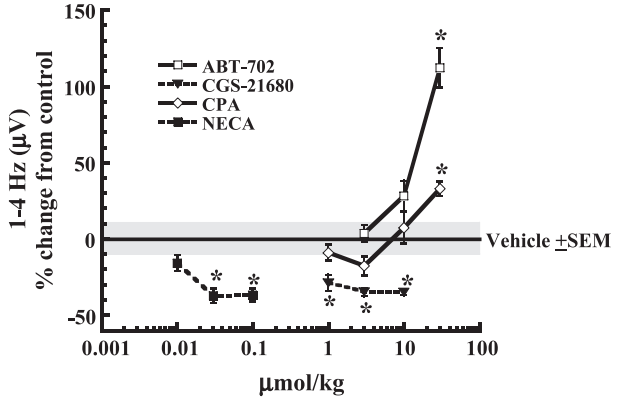

Fig. 5. A comparison of the effects of ABT-702 to A_1 and A_{2A} selective agonists. The effects of the compounds are expressed as a percent change from vehicle control. ABT-702 produced an effect on 1–4 Hz EEG, which is similar to the A_1 agonist CPA, but can be differentiated from acute administration of the A_{2A} agonist CGS-21680. It is also dissimilar from the non-selective agonist NECA, which produced a pattern similar to CGS-21680. *ANOVA, Fishers post hoc analysis vs. vehicle, $p<0.05$.

amplitudes of the EEG can vary from rat to rat, the magnitude (% change) of drug effect is comparable across subjects, regardless of baseline amplitude EEG variances. The selective A_1 ADO receptor agonist CPA produced a significant treatment effect ($F(4,20)=9.7$, $p=0.0002$) and a post-hoc significant increase of 1–4 Hz activity was achieved at the dose of 30.0 μmol/kg. While both the A_{2A} selective agonist CGS-21680 ($F(3,15)=12.9$, $p=0.0002$) and the non-selective agonist NECA ($F(3,15)=14.4$, $p=0.0001$) produced significant treatment effects, they differed from both ABT-702 and CPA in that they produced decreases in 1–4 Hz slow wave amplitude.

Fig. 6A, B, and C show the effects of a dose of 10 μmol/kg ABT-702 on sleep in rats. ABT-702 significantly decreased wakefulness ($F(1,14)=6.381$, $p=0.0242$) and Fisher's post hoc PLSD revealed significant differences from control during hours 1 and 5. In contrast, ABT-702 significantly increased SWS ($F(1,14)=13.5$, $p=0.0025$) and differences from controls were seen during hours 1, 4, and 5. In our lab, it is common to see the highest SWS or 1–4 Hz activity during the middle time periods of an extended recording session. This can be seen in Fig. 6B during hours 2 and 3. The effect of ABT-702 on SWS did not significantly exceed SWS levels in control rats during hours 2 and 3 of treatment, rather, it increased SWS during periods when vehicle treated rats are expressing relatively lesser amounts of SWS. Fig. 6C shows that REM sleep was significantly decreased ($F(1,14)=6.7$, $p=0.02$), however, by

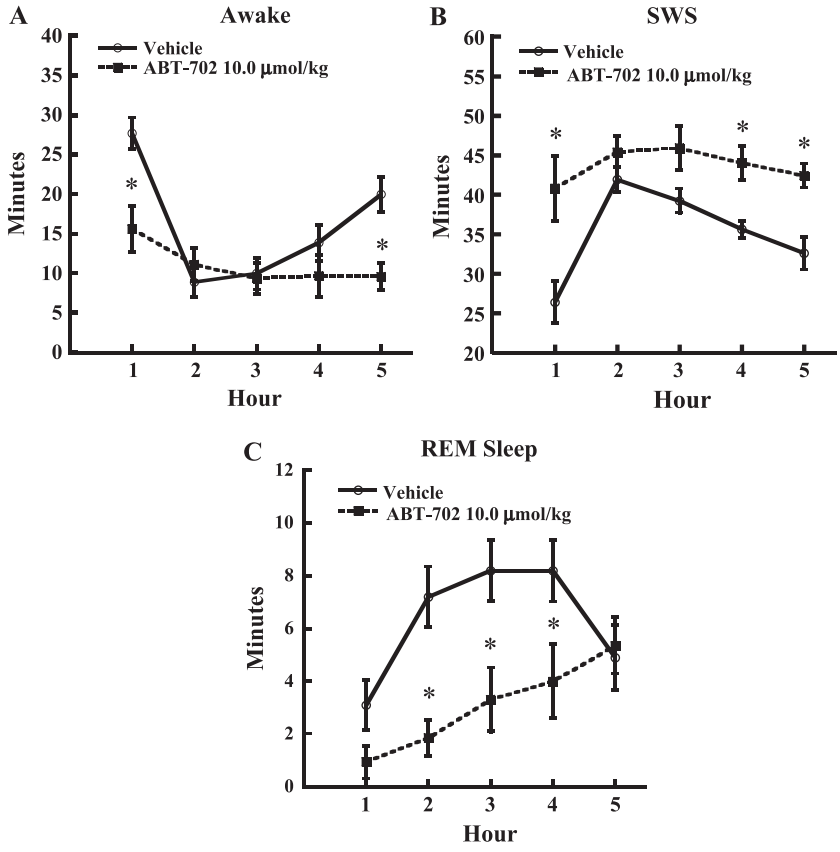

Fig. 6. Effects of ABT-702 on waking, slow wave sleep (SWS) and REM sleep. (A) ABT-702 significantly decreased waking during hours 1 and 5 of the recording session. *ANOVA, Fishers post hoc analysis vs. vehicle, $p<0.05$. (B) ABT-702 significantly increased SWS during hours 1, 4, and 5 of the recording session. The SWS time of ABT-702 treated rats did not exceed the maximal SWS time seen in vehicle treated rats during hours 2 and 3. *ANOVA, Fishers post hoc analysis vs. vehicle, $p<0.05$. (C). ABT-702 significantly decreased REM sleep during hours 2–4 of the recording session, but was back to control levels during hour 5. *ANOVA, Fishers post hoc analysis vs. vehicle, $p<0.05$.

hour 5 of the recording session, REM sleep recovered to control levels while SWS still remained elevated. Fig. 7A shows 1–4 Hz FFT amplitude profile for SWS and waking epochs during the sleep recording sessions. The data in this graph is represented as a percent change from vehicle. There is no statistically significant repeated measures (time) ANOVA effect for ABT-702 ($F(1,14)=1.3$, $p=0.27$), but a significant time–dose interaction ($F(1,14)=2.7$, $p=0.04$) was achieved. When this data is averaged into one value over the 5 h recording session, ABT-702 significantly increases 1–4 Hz amplitude during SWS (Fig. 7B, paired $t(7)=-3.5$, $p=0.01$). In contrast, there was no significant repeated measures effect ($F(1,14)=0.014$, $p=0.91$), no dose–time interaction ($F(1,14)=1.1$, $p=0.38$), or averaged 5 h ABT-702 effect (paired $t(7)=0.75$, $p=0.48$) on 1–4 Hz activity during awake epochs (Fig. 7A and B). Thus, ABT-702 had two main effects on sleep; one is to increase total SWS time and the other is to increase the amplitude of EEG slow waves during non-REM sleep. No effect, however, was seen on the EEG during waking.

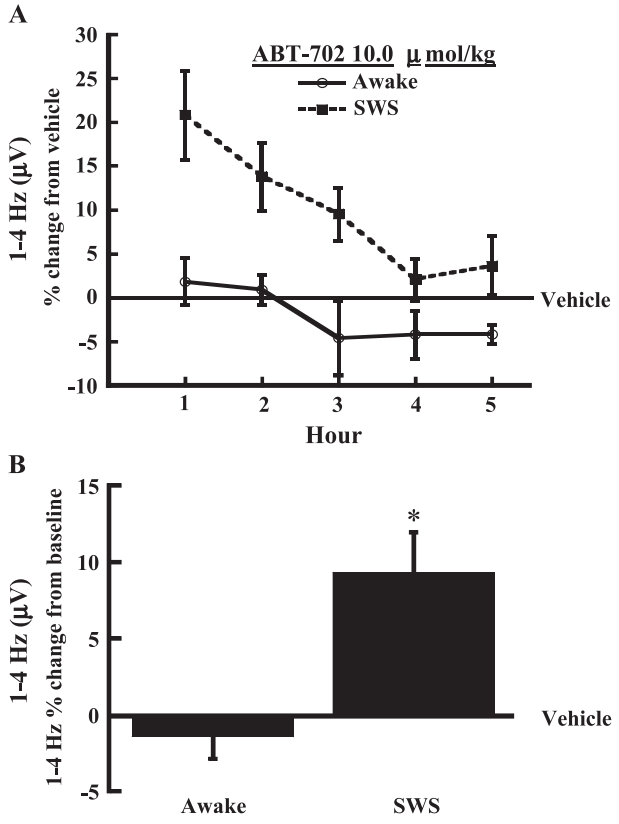

Fig. 7. The effects of ABT-702 (10.0 μmol/kg) on 1–4 Hz amplitude during waking and slow wave sleep. The data are presented as a percent change from vehicle control. (A) ABT-702 tended to increase 1–4 Hz amplitude during epochs of EEG/EMG identified as slow wave (Non-REM) sleep, although this did not achieve significance using a repeated measures (time) ANOVA. ABT-702 did not increase waking 1–4 Hz power. (B) Data from Fig. 7A is averaged over the entire 5 h recording session. By collapsing the data in this way, ABT-702 shows a significant increase in 1–4 Hz activity during SWS, but not during waking. *Students paired t-test, $p<0.05$.

4. Discussion

These results represent the first demonstration of a central effect of AK inhibition on cortical arousal levels in vivo. The increase of 1–4 Hz low frequency EEG and decrease of fast activity (>13 Hz) by the AK inhibitor ABT-702 further substantiate the concept that endogenous ADO plays a role in the synchronization of neural activity, a function believed to be associated with SWS EEG patterns [33,35]. Inhibition of AK appears to be the most likely mechanism for these effects since ABT-702 has greater than 2000-fold selectivity for AK inhibition over ADO A_1, A_{2a}, and A_3 receptors, the adenosine transporter, and adenosine deaminase [16]. The effects of ABT-702 on EEG, especially the high dose of 30.0 μmol/kg, are somewhat like the effects of muscarinic antagonists, such as atropine, which synchronize the EEG [13,14,25]. However, ABT-702 (10.0 μmol/kg) increased 1–4 Hz activity only during SWS and not during waking EEG, an effect unlike atropine [50]. ABT-702 is 7000-fold selective for AK over a variety of other neurotransmitter and peptide receptors, including muscarinic subtypes, ion channel proteins, reuptake sites, and enzymes [16]. The enhancement of EEG slow waves produced by ABT-702 was also attenuated by the non-selective, centrally active ADO receptor antagonist theophylline. Thus, it is unlikely that the effects of ABT-702 on EEG are the result of a direct interaction with non-adenosinergic neurotranasmitter systems. These results are consistent with a proposed central mechanism whereby AK inhibition leads to enhanced extracellular ADO concentrations that can interact with ADO receptor subtypes [8].

The increase in slow wave sleep shown in Fig. 7A corresponds to the overall effect seen on the 1–4 Hz EEG band (Table 2). A concurrent ABT-702-induced decrease of high frequency activity, particularly in the band represented by frequencies above 25 Hz, is an EEG state associated with SWS [44]. Studies have shown that systemic or intracerebroventricular injections of ADO agonists increase slow wave amplitude specifically during SWS, an effect we see with ABT-702 [6,41]. The REM state, with considerable similarities to the waking state with regard to EEG activity, was decreased by ABT-702. Adenosine inhibits cholinergic mesopontine and basal forebrain neurons, whose firing rate is high during waking and REM sleep [33,34,37,45,46]. Moreover, it has been suggested that ADO may act to inhibit GABAergic REM-on neurons in the laterodorsal tegmentum (LTD) [3]. Thus, it would not be surprising that AK inhibition would increase SWS while decreasing REM. However, the reported effects of ADO on REM are varied. For example, local injection of adenosine receptor agonists into the pontine reticular formation have been shown to increase REM sleep, an effect that may be locally mediated by enhanced acetylcholine release or inhibition of GABA transmission [25,26]. Likewise, local application of ADO in the basal forebrain and LTD also increase REM [47]. In

contrast, decreased REM sleep is observed with subarachnoid infusion of CPA in the region of the basal forebrain and with systemic CPA injections [40,41]. This may suggest that there are local opposing effects of ADO on the REM phase of sleep. The report by Schwierin et al. [41] that systemically administered CPA, together with our report that systemically administered ABT-702, both decrease REM may be indicative of a net CNS action in which decreased REM is the predominant effect.

Although our report demonstrates the in vivo effects of AK inhibition and AK inhibitors on arousal, previous in vitro findings are also supportive of AK inhibition modulating sleep. Arrigoni et al. [3] demonstrated that the effects of another AK inhibitor, 5-iodotubercidin, were indistinguishable from ADO itself in inhibiting LTD neurons in slices. Cholinergic neurons of the LTD discharge during waking and REM, but are less active during non-REM sleep [43,49]. Reduced LTD neuron activity is thought to play a permissive role in the transition from waking to sleep [45]. In addition to AK inhibitors, other modulators of extracellular ADO concentrations also have effects on EEG, sleep, and arousal. Erythro-9-(hydroxy-2, nonyl-3) adenine, a specific inhibitor of adenosine deaminase, decreases wakefulness and increases sleep in rats [39]. In contrast, soluflazine, a nucleoside transport and ADO reuptake inhibitor, increased waking and decreased sleep [39]. The mechanism underlying the effects of soluflazine remains unclear. It should be noted, however, that AK inhibition has been shown to be more effective than either inhibition of adenosine deaminase or inhibition of ADO uptake in enhancing extracelluar ADO concentrations, as well as enhancing functional correlates like antinociception and anticonvulsant activity [15,19,54].

In the present study, differential effects on EEG were observed between ABT-702 and ADO receptor agonists. The A_{2A} receptor selective agonist, CGS-21680, and the non-selective agonist NECA decreased slow wave amplitude while a high dose of the A_1 receptor-selective agonist, CPA, produced an augmentation in 1–4 Hz amplitude that was similar to ABT-702. It has been reported that ADO A_1 receptors play a primary role in the generation of sleep and EEG slow waves, and the similarity of ABT-702 to CPA in this study is further support for this theory [3,47]. The differential affinity of ADO for A_1 (nM) and A_{2A} (μM) receptors might explain, in part, why an apparent A_1 receptor-like effect was observed for ABT-702 [22]. A predominant role for A_1 receptor activation in sleep induction is also supported by biochemical evidence for selective changes in A_1 receptor signaling during sleep deprivation and the phenotype of receptor gene disrupted mice which show decreased slow wave activity [4,18]. However, a role for affects on A_{2A} receptors with AK inhibition must be considered. First, CGS 21680 (A_{2A} agonist) and the AK inhibitor 5-iodotubercidin can elicit the excitatory actions of BDNF in hippocampal slices [12]. Thus, evidence exists for the ability of AK inhibitor-induced increases of ADO to activate A_{2A} receptors. Secondly, CGS 21680 is reported to augment sleep when given as local injections in the pontine reticular formation and basal forebrain [26,40]. A similarity between EEG patterns generated by ABT-702 and CPA can only be suggestive of a common underlying mechanism. Pharmacological experiments utilizing antagonists that are selective for the A_1 and A_{2A} could further support or refute a predominant effect of AK inhibition for one subtype or another.

Both AK inhibitors and ADO receptor agonists show similar behavioral profiles in that they are more potent to reduce inflammatory hyperalgesia as compared to their ability to reduce motor performance [17,22,27]. Additionally, the pattern of motor impairment produced by AK inhibitors and ADO receptor agonists is different than that observed for other classical CNS sedatives like the benzodiazepines and ethanol, where the dose–response curves for impairment of rotorod performance are either left of, or superimposed on, the dose–response curves for impairment of exploratory motor activity [unpublished observations]. ABT-702 is also distinguishable from anesthetics, which can shift the EEG toward greater expression of slow wave activity [20]. While ABT-702 increased SWS, the treated rats were sensitive to ambient acoustic stimuli during which a waking, activated EEG pattern and EMG muscle tone were expressed. Furthermore, it has been reported that the ED50 for ABT-702 to disrupt rotarod performance is >100 μmol/kg, more than threefold higher than the highest dose tested for effects on EEG [17]. Likewise, the pattern of effects of barbiturates and benzodiazepines on EEG can be differentiated from that produced by AK inhibition observed in this study [20,30]. The rotarod and EEG findings, taken together, do not suggest an anesthetic, or for that matter a sedative-hypnotic profile for ABT-702 at the doses tested. Thus, AK inhibitors and ADO receptor agonists appear to affect aspects of motor performance and arousal that are distinctly different from those produced by classical CNS sedatives. These findings are consistent with other data indicating that AK inhibitor-mediated antinociception is primarily mediated by actions at spinal sites, whereas effects on EEG and motor performance are mediated via supraspinal mechanisms [28,48,55].

In summary, the present data further support the role of ADO in modulating EEG measures of arousal. AK inhibition by ABT-702 was able to increase EEG slow waves as measured by quantitative EEG. It also increased sleep time and increased amplitude of EEG slow waves during sleep, a possible indication of a deeper sleep being elicited. The effect of ABT-702 treatment on REM sleep was similar to controls by hour 5 of recording, a time when SWS was still increased. However, a caveat remains that REM sleep was significantly decreased by ABT-702 during earlier time points. Nonetheless, our results suggest that further study of AK inhibitors as potential pro-somnolent agents is warranted. The effects of AK inhibition on EEG slow waves and pain together also raises the possibility of

being able to achieve clinically relevant analgesia at the same time as having a beneficial effect on sleep.

References

[1] M.N. Alam, R. Szymusiak, H. Gong, J. King, D. McGinty, Adenosinergic modulation of rat basal forebrain neurons during sleep and waking: neuronal recording with microdialysis, J. Physiol. 521 (3) (1999) 679–690.

[2] J.R.S. Arch, E.A. Newsholme, The control of the metabolism and the hormonal role of adenosine, Essays Biochem. 14 (1978) 82–123.

[3] E. Arrigoni, D.G. Rainnie, R.W. McCarley, R.W. Greene, Adenosine-mediated presynaptic modulation of glutamatergic transmission in the laterodorsal tegmentum, J. Neurosci. 21 (3) (2001) 1076–1085.

[4] R. Basheer, L. Halldner, L. Alanko, R.W. McCarley, B.B. Fredholm, T. Porkka-Heiskanen, Opposite changes in adenosine A1 and A2A receptor mRNA in the rat following sleep deprivation, NeuroReport 12 (8) (2001) 1577–1580.

[5] J.H. Benington, H.C. Heller, Restoration of brain energy metabolism as the function of sleep, Prog. Neurobiol. 45 (1995) 347–360.

[6] J.H. Benington, S.K. Kodali, H.C. Heller, Stimulation of A1 adenosine receptors mimic the electroencephalographic effects of sleep deprivation, Brain Res. 692 (1–2) (1995) 79–85.

[7] D.L. Boyle, E.A. Kowaluk, M.F. Jarvis, C.H. Lee, S.S. Bhagwat, M. Williams, G.S. Firestein, Anti-inflammatory effects of ABT-702, a novel adenosine kinase inhibitor, in rat adjuvant arthritis, J. Pharmacol. Exp. Ther. 296 (2) (2001) 495–500.

[8] D.R. Britton, J. Mikusa, C.H. Lee, M.F. Jarvis, M. Williams, E.A. Kowaluk, Site and event specific increase in striatal adenosine release by adenosine kinase inhibition in rats, Neurosci. Lett. 266 (1999) 93–96.

[9] J.M. Brundege, T.V. Dunwiddie, Modulation of excitatory synaptic transmission by adenosine released from single hippocampal pyramidal neurons, J. Neurosci. 16 (18) (1996) 5603–5612.

[10] J.M. Brundege, T.V. Dunwiddie, Role of adenosine as a modulator of synaptic activity in the central nervous system, Adv. Pharmacol. 39 (1997) 353–391.

[11] L.P. Davies, D.D. Jamieson, J.A. Baird-Lambert, R. Kaslauzkas, Halogenated pyrrolopyrimidine analogues of adenosine from marine organisms: pharmacological activities and potent inhibition of adenosine kinase, Biochem. Pharmacol. 33 (1984) 347–355.

[12] M.J. Diogenes, C.C. Fernandes, A.M. Sebastiao, J.A. Ribeiro, Activation of adenosine A2A receptor facilitates brain-derived neurotrophic factor modulation of synaptic transmission in hippocampal slices, J. Neurosci. 24 (12) (2004) 2905–2913.

[13] H.C. Dringenberg, M.L. Rubenstien, H. Solty, S. Tomaszek, A. Bruce, Electroencephalographic activation by tacarine, deprenyl, and quipazine; cholinergic vs. non-cholinergic contributions, Eur. J. Pharmacol. 447 (1996) 43–50.

[14] M.G. Frank, J. Page, H.C. Heller, The effects of REM sleep-inhibiting drugs in neonatal rats: evidence for a distinction between neonatal active sleep and REM sleep, Brain Res. 778 (1995) 64–72.

[15] K. Golembiowska, T.D. White, J. Sawyok, Adenosine kinase inhibitors augment release of adenosine from spinal cord slices, Eur. J. Pharmacol. 307 (1996) 157–162.

[16] M.F. Jarvis, H. Yu, K. Kohlhaas, K. Alexander, C.H. Lee, M. Jiang, S.S. Bhagwat, M. Williams, E.A. Kowaluk, ABT-702 (4-amino-5-(-bromophenyl)-7-(6-morpholino-pyridin-3-yl)pyrido[2,3-*d*]pyimidine), a novel orally effective adenosine kinase inhibitor with analgesic and anti-inflammatory properties: I. In vitro characterization and acute antinociceptive effects in the mouse, J. Pharmacol. Exp. Ther. 295 (3) (2000) 1156–1164.

[17] M.F. Jarvis, J. Mikusa, K. Chu, S. McGaraughty, M.P. Honore, Comparison of the effects of adenosine kinase inhibitors and adenosine receptor agonists to attenuate thermal hyperalgesia and reduce motor performance, Pharmacol. Biochem. Behav. 73 (3) (2002) 573–581.

[18] N. Kaushal, B. Johansson, E. Arrigoni, L. Halldner, B. Fredholm, R. Greene, Reduced slow wave activity in the adenosine A1 receptor knockout mice, Abstr.-Soc. Neurosci. 27 (2001) 523.2.

[19] G.J. Keil, G.E. Delander, Spinally mediated antinociception is induced in mice by an adenosine kinase, but not an adenosine deaminase inhibitor, Life Sci. 51 (1992) 171–176.

[20] F.N. Krijzer, R. van der Molen, Classification of psychotropic drugs by rat EEG analysis: the anxiolytic profile in comparison to the antidepressant and neuroleptic profile, Neuropsychobiology 18 (1) (1987) 51–56.

[21] E.A. Kowaluk, M.F. Jarvis, Therapeutic potential of adenosine kinase inhibitors, Expert Opin. Investig. Drugs 9 (2000) 551–564.

[22] E.A. Kowaluk, J. Mikusa, C.T. Wismer, C.Z. Zhu, E. Schweitzer, J.J. Lynch, C.H. Lee, M. Jiang, S.S. Bhagwat, A Gomtsyan, J. McKie, B.F. Cox, J. Polakowski, G. Reinhart, M. Williams, M.F. Jarvis, ABT-702 (4-amino-5-(-bromophenyl)-7-(6-morpholino-pyridin-3-yl)pyrido[2,3-*d*]pyimidine), a novel orally effective adenosine kinase inhibitor with analgesic and anti-inflammatory properties: II. In vivo characterization in the rat, J. Pharmacol. Exp. Ther. 295 (3) (2000) 1165–1174.

[23] H.P. Landolt, E. Werth, A.A. Borbely, D.J. Dijk, Caffeine intake (200 mg) in the morning affects human sleep and EEG power spectra at night, Brain Res. 675 (1–2) (1995) 67–74.

[24] C.H. Lee, M. Jiang, M. Cowart, G. Gfesser, R. Perner, K.H. Kim, Y.G. Gu, M. Williams, M.F. Jarvis, E.A. Kowaluk, A.O. Stewart, S.S. Bhagwat, Discovery of 4-amino-5-(3-bromophenyl)-7-(6-morpholino-pyridin-3-yl)pyrido[2,3-*d*]pyrimidine, an orally active, non-nucleoside adenosine kinase inhibitor, J. Med. Chem. 44 (13) (2001) 2133–2138.

[25] G.A. Marks, C.B. Birabil, Enhancement of rapid eye movement sleep in the rat by cholinergic and adenosenergic agonists infused into the pontine reticular formation, Neuroscience 86 (1) (1998) 29–37.

[26] G.A. Marks, J.P. Shaffery, S.G. Speciale, C.G. Birabil, Enhancement of rapid eye movement sleep in the rat by actions at A1 and A2a adenosine receptor subtypes with a differential sensitivity to atropine, Neuroscience 116 (3) (2003) 913–920.

[27] H.M. Marston, K. Finlayson, T. Maemoto, H.J. Olverman, A. Akahane, J. Sharkey, S.P. Butcher, Pharmacological characterization of a simple behavioral response mediated selectively by central adenosine A1 receptors, using in vivo and in vitro techniques, J. Pharmacol. Exp. Ther. 285 (1998) 1023–1030.

[28] S. McGaraughty, K.L. Chu, C.T. Wismer, J. Mikusa, C.Z. Zhu, M. Cowart, E.A. Kowaluk, M.F. Jarvis, Effects of A-134974, a novel adenosine kinase inhibitor, on carrageenan-induced inflammatory hyperalgesia and locomotor activity in rats: evaluation of the sites of action, J. Pharmacol. Exp. Ther. 296 (2) (2001) 501–509.

[29] A.C. Newby, Adenosine and the concept of "retaliatory metabolites", Trends Biochem. Sci. 2 (1984) 42–44.

[30] B. Nikel, I. Szelenyi, Comparison of changes in the EEG of freely moving rats induced by enciprazine, buspirone, and diazepam, Neuropharmacology 28 (8) (1989) 799–803.

[31] M.A. Pak, H.L. Haas, U.K.M. Decking, J. Schrader, Inhibition of adenosine kinase increases endogenous adenosine and depresses neuronal activity in hippocampal slices, Neuropharmacology 33 (1994) 1049–1053.

[32] P. Popoli, S. Sagretella, A. Scotti de Carolis, An EEG and behavioural study on the excitatory properties of caffeine in rabbits, Arch. Int. Pharmacodyn. Ther. 290 (1) (1987) 5–15.

[33] T. Porkka-Heiskanen, R.E. Strecker, M. Thakkar, A.A. Bjorkum, R.W. Greene, R.W. McCarley, Adenosine-mediator of the sleep-inducing effects of prolonged wakefulness, Science 276 (5316) (1997) 1265–1268.

[34] C.M. Portas, M. Thakkar, D.G. Rainnie, R.W. Greene, R.W. McCarley, Role of adenosine in behavioral state modulation: a

microdialysis study in the freely moving cat, Neuroscience 79 (1) (1997) 225–235.
[35] M. Radulovacki, Role of adenosine in sleep in rats, Rev. Clin. Basic Pharmacol. 5 (1985) 327–339.
[36] M. Radulovacki, R.M. Virus, Dose–response effects of 8-cyclopropyltheophylline on sleep and wakefulness in rats, Psychopharmacology 94 (3) (1988) 417–420.
[37] D.G. Rainnie, H.C. Grunze, R.W. McCarley, R.W. Greene, Adenosine inhibition of mesopontine cholinergic neurons: implications for EEG arousal, Science 263 (5147) (1994) 689–692.
[38] V. Ralevic, G. Burnstock, Receptors for purines and pyrimidines, Pharmacol. Rev. 50 (1998) 413–492.
[39] N. Sarda, R. Cespuglio, A. Gharib, D. Reynaud, Role of hypothalamic adenosinergic systems in regulating states of wakefulness in the rat, C. R. Acad. Sci., Sér. III 308 (17) (1989) 473–478.
[40] S. Satoh, H. Matsumura, O. Hayaishi, Involvement of adenosine A_{2a} receptors in sleep promotion, Eur. J. Pharmacol. 351 (1998) 155–162.
[41] B. Schwierin, A.A. Borbely, I. Tobler, Effects of *N*6-cycolopentyladenosine and caffeine on sleep regulation in the rat, Eur. J. Pharmacol. 300 (1996) 163–171.
[42] V.M. Sciotti, D.G. Van Wylen, Increases in interstitial adenosine and cerebral blood flow with inhibition of adenosine kinase and adenosine deaminase, J. Cereb. Blood Flow Metab. 13 (2) (1993) 201–207.
[43] K. Semba, The cholinergic basal forebrain: a critical role in cortical arousal, Adv. Exp. Med. Biol. 295 (1991) 197–218.
[44] F.Z. Shaw, J.H. Chew, Dynamic changes of gamma activities of somatic cortical evoked potentials during wake–sleep states in rats, Brain Res. 5:983 (1–2) (2003) 152–161.
[45] M. Steriade, Changing concepts on localization of brain function; behavioral states of vigilance, The Intact and Sliced Brain, MIT Press, London, 2001, pp. 18–23.
[46] M. Steriade, S. Datta, D. Pare, G. Oakson, R.C. Curro Dossi, Neuronal activities in brain-stem cholinergic nuclei related to tonic activation processes in thalamocortical systems, J. Neurosci. 10 (8) (1990) 2541–2559.
[47] R.E. Strecker, S. Morairty, M.M. Thakkar, T. Porkka-Heiskanen, R. Basheer, L.J. Dauphin, D.G. Rainnie, C.M. Portas, R.W. Greene, R.W. McCarley, Adenosinergic modulation of basal forebrain and preoptic/anterior hypothalamic neuronal activity in the control of behavioral state, Behav. Brain Res. 115 (2) (2000) 183–204.
[48] R. Suzuki, L.C. Stanfa, E.A. Kowaluk, M. Williams, M.F. Jarvis, A.H. Dickenson, The effect of ABT-702, a novel adenosine kinase inhibitor, on the responses of spinal neurones following carrageenan inflammation and peripheral nerve injury, Br. J. Pharmacol. 132 (7) (2001) 1615–1623.
[49] R. Szymusiak, T. Iriye, D. McGinty, Sleep-waking discharge of neurons in the posterior lateral hypothalamic area of cats, Brain Res. Bull. 23 (1–2) (1989) 111–120.
[50] R. Szymusiak, D. McGinty, M.N. Shouse, D. Shepard, M.B. Sterman, Effects of systemic atropine sulfate administration on the frequency content of the cat sensorimotor EEG during sleep and waking, Behav. Neurosci. 104 (1) (1990) 217–225.
[51] R.M. Virus, S. Ticho, M. Pilditch, M. Radulovacki, A comparison of the effects of caffeine, 8-cyclopentyltheophylline, and alloxazine on sleep in rats. Possible roles of central nervous system adenosine receptors, Neuropsychopharmacology 3 (4) (1990) 243–249.
[52] M. Williams, M.F. Jarvis, Purinergic and pyrimidinergic receptors as potential drug targets, Biochem. Pharmacol. 59 (2000) 1173–1185.
[53] G.M. Yanik, S. Glaum, M. Radulovacki, The dose–response effects of caffeine on sleep in rats, Brain Res. 403 (1987) 177–180.
[54] G. Zhang, P.H. Franklin, T.F. Murray, Manipulation of endogenous adenosine in the rat prepiriform cortex modulates seizure susceptibility, J. Pharmacol. Exp. Ther. 264 (3) (1993) 1415–1424.
[55] C.Z. Zhu, J. Mikusa, K.L. Chu, M. Cowart, E.A. Kowaluk, M.F. Jarvis, S. McGaraughty, A-134974: a novel adenosine kinase inhibitor, relieves tactile allodynia via spinal sites of action in peripheral nerve injured rats, Brain Res. 905 (1–2) (2001) 104–110.