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Dialysis delivery of an adenosine A_{2A} agonist into the pontine reticular formation of C57BL/6J mouse increases pontine acetylcholine release and sleep

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Abstract

In vivo microdialysis in C57BL/6J (B6) mouse was used to test the hypothesis that activating adenosine A_{2A} receptors in the pontine reticular formation (PRF) increases acetylcholine (ACh) release and rapid eye movement (REM) sleep. Eight concentrations of the adenosine A_{2A} receptor agonist 2-*p*-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride (CGS 21680; CGS) were delivered to the PRF and ACh in the PRF was quantified. ACh release was significantly increased by dialysis with 3 μM CGS and significantly decreased by dialysis with 10 and 100 μM CGS. Co-administration of the adenosine A_{2A} receptor antagonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385; 30 nM) blocked the CGS-induced increase in ACh release. In a second series of experiments, CGS (3 μM) was delivered by dialysis to the PRF for 2 h while recording sleep and wakefulness. CGS significantly decreased time in wakefulness (−51% in h 1; −54% in h 2), increased time in non-rapid eye movement (NREM) sleep (90% in h 1; 151% in h 2), and increased both time in REM sleep (331% in h 2) and the number of REM sleep episodes (488% in h 2). The enhancement of REM sleep is consistent with the interpretation that adenosine A_{2A} receptors in the PRF of the B6 mouse contribute to REM sleep regulation, in part, by increasing ACh release in the PRF. A_{2A} receptor activation may promote NREM sleep via GABAergic inhibition of arousal promoting neurons in the PRF.

Keyword: microdialysis.

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Cholinergic neurotransmission at muscarinic receptors in the pontine reticular formation (PRF) of cat (reviewed in Baghdoyan and Lydic 2002; Lydic and Baghdoyan 2005) and rat (Imeri *et al*. 1994; Bourgin *et al*. 1995; Kubin 2001) plays an important role in generating the rapid eye movement (REM) phase of sleep. Microinjection of the acetylcholinesterase inhibitor neostigmine into the PRF of C57BL/6J (B6) mouse causes a REM sleep-like state (Lydic *et al*. 2002) that is concentration-dependent (Douglas *et al*. 2005) and blocked by the muscarinic receptor antagonists methoctramine (Coleman *et al*. 2004a) and atropine (Douglas *et al*. 2005). These data demonstrate that in the B6 mouse a REM sleep-like state is also produced by activation of muscarinic receptors in the PRF. Cholinergic input to the PRF originates from the laterodorsal tegmental and pedunculopontine tegmental (LDT/PPT) nuclei (reviewed in Steriade and McCarley 2005). Acetylcholine (ACh) release in cat PRF is significantly greater during REM sleep than during wakefulness and non-rapid eye movement (NREM) sleep (Kodama *et al*. 1990; Leonard and Lydic 1995, 1997). Additional evidence supporting a role for pontine cholinergic neurotransmission in REM sleep generation is provided by studies demonstrating that electrical stimulation of the LDT/PPT increases ACh release in the PRF (Lydic and Baghdoyan 1993) and increases REM sleep (Thakkar *et al*. 1996). In addition, neurotoxic lesions of cholinergic LDT/PPT neurons

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Abbreviations used: ACh, acetylcholine; CGS 21680, CGS, 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride; d.f., degrees of freedom; EEG, electroencephalogram; EMG, electromyogram; FFT, Fast Fourier transformation; LDT, laterodorsal tegmental nucleus; NREM, non-rapid eye movement; PPT, pedunculopontine tegmental nucleus; PRF, pontine reticular formation; PnC, pontine reticular nucleus, caudal part; PnO, pontine reticular nucleus, oral part; REM, rapid eye movement; ZM 241385, ZM, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol.

cause a long-lasting inhibition of REM sleep (Webster and Jones 1988).

Since the 1950s, the neuromodulator adenosine repeatedly has been shown to promote NREM sleep (reviewed in Benington and Heller 1995; Porkka-Heiskanen *et al*. 2002; Basheer *et al*. 2004; Radulovacki 2005). Consistent with these findings, adenosine antagonists such as caffeine and theophylline enhance wakefulness and disrupt sleep (Fredholm *et al*. 1999; Shaw *et al*. 2000). There are four adenosine receptor subtypes (A_1, A_{2A}, A_{2B} and A_3), all of which couple to G proteins (Ribeiro *et al*. 2003). Inhibitory G protein-coupled adenosine A_1 receptors and stimulatory G protein-coupled adenosine A_{2A} receptors in the brain mediate the sleep-inducing effects of adenosine (reviewed in Porkka-Heiskanen *et al*. 2002; Basheer *et al*. 2004). Sleep is increased by forebrain administration of adenosine, adenosine receptor agonists or drugs that increase adenosine. These forebrain sites include the substantia innominata (Porkka-Heiskanen *et al*. 1997; Portas *et al*. 1997), basal forebrain area (Basheer *et al*. 1999), rostral basal forebrain and underlying subarachnoid space (Satoh *et al*. 1996, 1998, 1999; Hong *et al*. 2005), ventrolateral preoptic area (Morairty *et al*. 2004), subarachnoid space anterior to the ventrolateral preoptic area (Scammell *et al*. 2001), medial preoptic area (Ticho and Radulovacki 1991; Mendelson 2000), and lateral pre-optic area (Methippara *et al*. 2005).

Fewer studies have investigated a sleep-related role for adenosine in the brain stem (Rainnie *et al*. 1994; Portas *et al*. 1997; Porkka-Heiskanen *et al*. 2000; Arrigoni *et al*. 2001), and very few studies have focused specifically on REM sleep (Marks and Birabil 1998; Marks *et al*. 2003). Adenosine A_1 and A_{2A} receptor agonists have been shown to increase REM sleep when microinjected into rat PRF (Marks and Birabil 1998; Marks *et al*. 2003). REM sleep enhancement by an adenosine A_{2A} receptor agonist, but not by an adenosine A_1 receptor agonist, was blocked by the muscarinic receptor antagonist atropine (Marks *et al*. 2003).

Enhancing cholinergic neurotransmission at muscarinic receptors in the PRF is thought to be one common mechanism by which a variety of neurotransmitters and neuromodulators regulate REM sleep (reviewed in Baghdoyan and Lydic 2002; Lydic and Baghdoyan 2003, 2005). Microdialysis studies in the striatum of freely moving rat have shown that an adenosine A_{2A} receptor agonist stimulates ACh release (Kurokawa *et al*. 1996). *In vitro* studies in striatum, hippocampus, motor nerve terminals and cortex also have shown that adenosine A_{2A} receptor agonists increase ACh release (reviewed in Sebastiao and Ribeiro 1996). It is not known whether activation of adenosine A_{2A} receptors in mouse PRF increases ACh release and increases REM sleep. The present study used *in vivo* microdialysis and electrographic recordings of sleep and wakefulness to test the hypothesis that dialysis administration of the adenosine A_{2A} receptor agonist 2-*p*-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride (CGS 21680; CGS) into the PRF of B6 mouse increases both ACh release and REM sleep. Portions of these data have been presented in abstract form (Coleman *et al*. 2004b).

Experimental procedures

Animals and surgery

All experiments used adult male B6 mice ($n = 42$; mean weight = 26 g; Jackson Laboratory, Bar Harbor, ME, USA) and were approved by the University of Michigan Committee on Use and Care of Animals. The studies were performed in accordance with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals (National Institutes of Health Publication 80–23). Mice were housed individually in a temperature-controlled room under constant illumination with free access to food and water.

Mice were anesthetized with isoflurane (induction: 2.5%; maintenance: 1.2%; delivered in 100% oxygen) (Abbott Laboratories, North Chicago, IL, USA) and placed in a David Kopf (Tujunga, CA, USA) Model 962 stereotaxic frame with a Model 923 mouse head holder adaptor and anesthesia mask. Two groups of mice were surgically implanted for subsequent neurochemical studies, as previously described in detail (Coleman *et al*. 2004a,c). Mice in the first group were used to measure PRF ACh release during drug delivery to the PRF. The PRF includes the pontine reticular nucleus, oral part (PnO) and the pontine reticular nucleus, caudal part (PnC) (Paxinos and Franklin 2001). To access the PRF, a craniotomy was made 4.72 mm posterior to bregma and 0.75 mm lateral to the sagittal suture according to the mouse brain atlas (Paxinos and Franklin 2001). A CMA/7 guide cannula (CMA Microdialysis, North Chelmsford, MA, USA) was lowered 4.2 mm below the skull surface and secured in place with dental acrylic. A second group of mice was used to record states of sleep and wakefulness during dialysis drug delivery to the PRF. In addition to a guide cannula aimed for the PRF, mice in the second group were implanted with electrodes for recording the electroencephalogram (EEG) and electromyogram (EMG). The guide cannula, two stainless steel anchor screws (Small Parts Inc., Miami Lakes, FL, USA), and a plastic pedestal (Plastics One, Roanoke, VA, USA) containing the electrodes were bonded to the skull with dental acrylic. Following a 1-week recovery period, mice were conditioned for 7 days by daily handling and/or adapting them to a Plexiglas recording chamber (BAS Raturn system; Bioanalytical Systems Inc., West Lafayette, IN, USA).

Materials

Drugs delivered to the PRF by dialysis included the selective adenosine A_{2A} receptor agonist CGS (Sigma-Aldrich, St Louis, MO, USA) and the selective adenosine A_{2A} receptor antagonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a] [1,3,5] triazin-5-yl-amino]ethyl)phenol (ZM 241385; ZM; Tocris Cookson, Ballwin, MO, USA). CGS was dissolved in Ringer's solution (147 mM NaCl; 2.4 mM $CaCl_2$; 4.0 mM KCl). Neostigmine bromide (0.1 μM; Sigma-Aldrich) was present in the Ringer's solution only for the experiments that measured ACh. Eight concentrations of CGS (0.1, 0.3, 1, 3, 10, 30, 100 and 1000 μM) were used to

perfuse the dialysis probe. ZM (30 nM) was co-delivered to the probe with CGS (3 μM).

Microdialysis and ACh quantification

CMA/7 dialysis probes, used for all experiments, have a 1-mm long by 0.24-mm diameter cuprophane membrane with a 6-kDa cut-off (CMA Microdialysis). A dialysis probe was inserted into the guide cannula and perfused continuously with Ringer's solution at a flow rate of 2 μL/min. The probe membrane extended 1 mm below the end of the implanted guide cannula. Six dialysis samples (25 μL/sample) were collected on ice while perfusing the probe with Ringer's solution (control). Using a CMA/110 liquid switch, the perfusate was then changed to Ringer's containing CGS or CGS plus ZM and six additional dialysis samples were collected. Only one concentration of CGS was administered per experiment, and only one experiment was performed per mouse. All experiments began between 10:00 and 11:00 h.

ACh in the dialysis samples was quantified using high performance liquid chromatography with electrochemical detection (HPLC/EC; Bioanalytical Systems Inc.). A sample was injected into the HPLC/EC system immediately after collection and carried in a 50-mM Na_2HPO_4 mobile phase (pH 8.5) through an analytical column that separated ACh and choline. An immobilized enzyme reactor column then proportionally converted the amount of ACh and choline in the sample to hydrogen peroxide. The hydrogen peroxide was detected by a platinum electrode (500 mV applied potential) referenced to an Ag^+/AgCl electrode. Chromgraph® software (Bioanalytical Systems Inc.) was used to digitize the resulting signal and to generate chromatographic peaks. The amount of ACh in a dialysis sample was determined by comparing the chromatographic peak area produced by the dialysis sample to a standard curve created immediately before each experiment.

The percent of ACh recovered by the dialysis probe was determined *in vitro* before and after every experiment and compared by *t*-test. If the pre- and post-experimental probe recovery values were significantly different ($p < 0.05$), then the results from that experiment were not included in the final data set. For all CMA/7 probes used to quantify ACh release, the average ± SD percent recovery of ACh was 5.85 ± 0.35%.

Microdialysis drug delivery and recordings of sleep and wakefulness

After being conditioned to the Plexiglas recording chamber, mice exhibited normal amounts of wakefulness, NREM sleep and REM sleep (Franken *et al*. 1999). The day before the experiment, a mouse was placed in the recording chamber between 16:00 and 17:00 h and left overnight. The next day the recording session began between 10:00 and 11:00 h. One hour before the recording session, the stylet of the microdialysis probe guide cannula was replaced with a microdialysis probe. The probe was perfused with Ringer's solution until the start of the recording session. A CMA/110 liquid switch then was turned to deliver Ringer's solution (control) or Ringer's containing CGS for 2 h. During the 2-h dialysis period, continuous recordings of EEG and EMG were obtained. The EEG and EMG signals were amplified with a Grass Instruments (West Warwick, RI, USA) model 15RXi amplifier. Signals were then filtered (EEG, 0.3–30 Hz; EMG, 10–6K Hz), digitized at 128 Hz, and recorded using Icelus software, as described previously (Coleman *et al*. 2004a). Each mouse was used for only one dialysis experiment.

Electrographic data and EEG power spectral analysis

The electrographic recordings were scored as wakefulness, NREM sleep or REM sleep according to previously described criteria (Lydic *et al*. 2002; Coleman *et al*. 2004a; Douglas *et al*. 2005). Dependent measures of arousal state included mean 1-h values of per cent of time spent in wakefulness, NREM sleep and REM sleep; the number of REM sleep episodes; and the duration (min) of REM sleep episodes. Latency to onset (min) of the first REM sleep episode also was quantified.

EEG signals were evaluated using Fast Fourier transformation (FFT) to reveal power spectra for consecutive 10-s epochs. Analyses were conducted in 0.5 Hz increments for frequencies ranging from 0.5 to 25 Hz. The bins were averaged over five 1-min intervals of EEG recording.

Histological evaluation of dialysis sites

Mice were deeply anesthetized and decapitated 3–5 days following microdialysis. Brains were quickly removed and frozen. Serial coronal brain stem sections were cut at a thickness of 40 μm, fixed in hot (80°C) paraformaldehyde vapor and stained with cresyl violet. Dialysis probe sites were assigned posterior, lateral, and ventral stereotaxic coordinates (mm) by comparing the stained tissue sections to the mouse brain atlas (Paxinos and Franklin 2001).

Statistical analyses

Descriptive statistics (mean ± SEM) were used to summarize dependent measures of ACh release and arousal state. ANOVA and post-hoc comparisons with Bonferroni correction were used to evaluate drug main effects on pontine ACh release using Statistical Analysis System programs (release 9.1, SAS Institute, Cary, NC, USA). Statistically significant differences in wakefulness, NREM sleep and REM sleep between the experimental and control conditions were evaluated by *t*-test. A probability value of $p < 0.05$ indicated a statistically significant difference.

Results

CGS caused biphasic, concentration-dependent changes in pontine reticular formation ACh release

The per cent change in ACh release during dialysis delivery of CGS to the PRF is summarized in Fig. 1(a). One-way ANOVA showed a significant CGS main-effect ($F = 8.24$; d.f. = 7,16; $p < 0.001$). The numerator degrees of freedom (d.f.) equals the number of CGS concentrations minus 1, and the denominator d.f. equals the number of animals used minus the number of CGS concentrations. Post-hoc multiple comparisons test with Bonferroni correction showed that 3 μM CGS significantly ($p < 0.001$) increased ACh release and 100 and 1000 μM CGS significantly ($p < 0.005$) decreased ACh release compared with control levels.

Figure 1(b) demonstrates that all microdialysis probe sites ($n = 24$) were localized within the PRF. Mean ± SEM

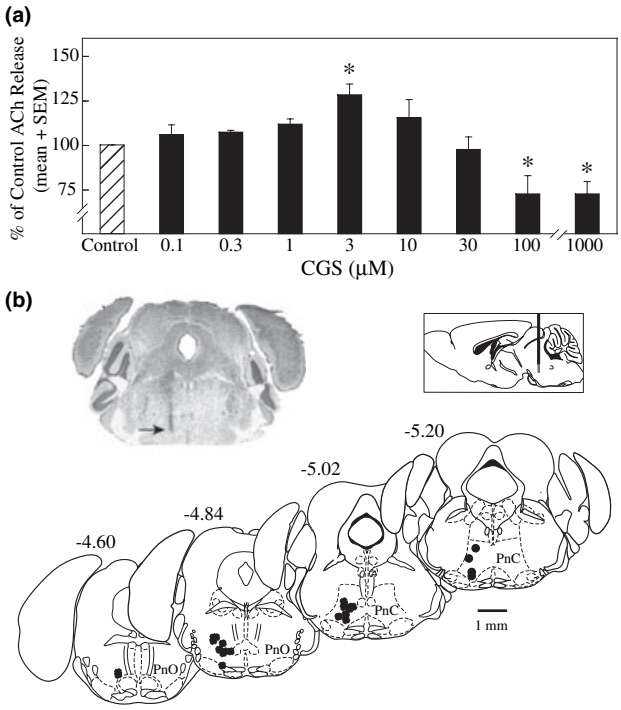

Fig. 1 CGS caused a biphasic, concentration-dependent change in pontine reticular formation ACh release. (a) Eight concentrations of CGS were administered to the PRF by dialysis. Three mice were used per concentration of CGS, and each mouse contributed six samples during dialysis with Ringer's (Control) and six samples during dialysis administration of CGS (filled bars). Asterisks indicate a significant ($p < 0.005$) difference from mean control level (hatched bar) of ACh release. (b) The histological section illustrates a representative dialysis probe site localized to the PnO. The arrow marks the most ventral part of the 1-mm long dialysis membrane. The sagittal diagram at right (modified from Paxinos and Franklin 2001) schematizes a dialysis probe in the PRF. The coronal diagrams at bottom are from the mouse brain atlas (Paxinos and Franklin 2001) and illustrate microdialysis sites in the PRF. Filled circles summarize dialysis sites from 24 mice used to obtain the ACh release data in (a). The number at the top of each coronal diagram indicates mm posterior to bregma.

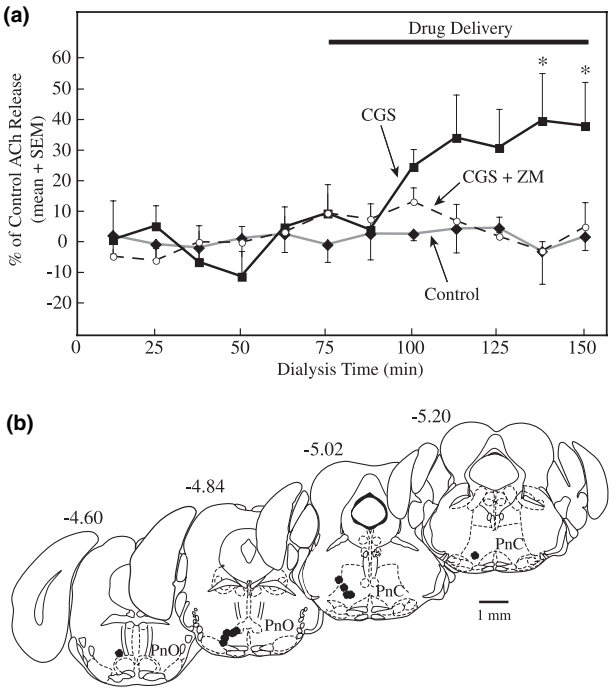

Fig. 2 Co-administration of CGS and ZM to the pontine reticular formation prevented the CGS-induced increase in ACh release. (a) Sequential dialysis samples collected every 12.5 min from the PRF show the time course of ACh release for Ringer's (Control; $n = 3$ mice), CGS (3 μM; $n = 3$ mice), and co-administration of CGS (3 μM) and ZM (30 nM; $n = 5$ mice). These data summarize ACh measured in 132 dialysis samples obtained from 11 experiments. Control levels of ACh release were established during the first 75 min of dialysis with Ringer's solution. The horizontal bar indicates that dialysis drug delivery to the PRF occurred during min 75–150. CGS caused a significant ($p < 0.005$) increase in ACh release over control levels (asterisks). (b) Filled circles indicate the microdialysis sites for the 11 mice used in (a). The number at the top left of each diagram indicates mm posterior to bregma (Paxinos and Franklin 2001).

stereotaxic coordinates for the microdialysis sites were 4.95 ± 0.04 mm posterior to bregma, 0.73 ± 0.03 mm lateral to the midline and 5.01 ± 0.06 mm ventral to the surface of the skull.

The adenosine A_{2A} antagonist ZM blocked the CGS-induced increase in pontine reticular formation ACh release

Figure 2(a) illustrates the time course of ACh release during PRF dialysis with Ringer's (control), CGS (3 μM), and CGS (3 μM) plus ZM (30 nM). Control levels of ACh were stable and not significantly different between experiments. Two-way repeated measures ANOVA revealed a significant drug main-effect ($F = 7.78$; d.f. = 2,8; $p < 0.05$), time main-effect ($F = 2.75$; d.f. = 11,88; $p < 0.005$), and drug by time interaction ($F = 1.88$; d.f. = 22,88; $p < 0.05$). The numerator d.f. for the main effect of each factor (drug and time) equals the number of factor levels minus 1. The numerator d.f. for the drug by time interaction equals the d.f. for drug multiplied by the d.f. for time. The denominator d.f. for the drug main-effect equals the number of animals minus the number of drug conditions. The denominator d.f. for the time main-effect and drug by time interaction equals the total number of dialysis samples minus the d.f. for the effects of time and the drug by time interaction. Post-hoc multiple comparisons statistic with Bonferroni correction showed that 3 μM CGS significantly ($p < 0.005$) increased ACh release after 50 min of delivery (Fig. 2a, dialysis time 136.5 min). Co-administration of CGS and ZM prevented the CGS-induced increase in pontine ACh release.

All microdialysis sites ($n = 11$) for the antagonist blocking studies were localized to the PRF and are illustrated in

Fig. 2(b). Mean ± SEM stereotaxic coordinates for the Fig. 2(b) microdialysis sites were 4.91 ± 0.06 mm posterior to bregma, 0.7 ± 0.05 mm lateral to the midline, and 5.08 ± 0.07 mm ventral to the surface of the skull.

EEG power spectra during CGS delivery were similar to baseline

Figure 3 summarizes the averaged EEG power spectra for four mice during wakefulness, NREM sleep and REM sleep. Insets show representative EEG and EMG recordings from one mouse obtained during dialysis delivery of 3 μM CGS. Figure 3(a) indicates that wakefulness was characterized by a low amplitude, mixed frequency EEG (0.5–25 Hz) and a high amplitude EMG. Figure 3(b) shows that NREM sleep was characterized by increased delta power (0.5–4 Hz) compared with wakefulness and REM sleep, high amplitude EEG and an EMG of lower amplitude than the waking EMG. Figure 3(c) illustrates that REM sleep was comprised of increased theta power (5–9 Hz) with rhythmic low amplitude EEG waves and a low amplitude EMG. Figure 3 shows that the EEG power spectra computed for wakefulness, NREM sleep and REM sleep did not differ when recordings without dialysis (baseline) were compared with recordings during microdialysis (CGS). During pontine reticular formation dialysis with CGS, sleep architecture was normal and REM sleep always emerged from NREM sleep.

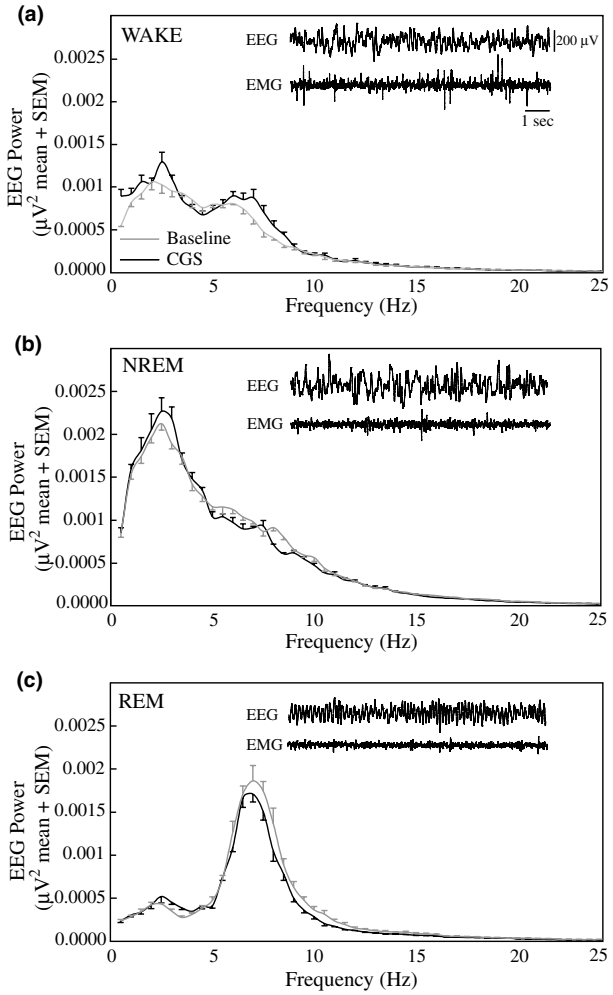

Fig. 3 EEG power spectra for sleep and wakefulness during baseline (no microdialysis) recordings and during drug delivery to the PRF by dialysis (CGS). The data summarize averaged EEG power in 0.5 Hz frequency intervals ($n = 4$ mice). Insets: 10-s intervals of EEG and EMG recordings during CGS administration show electrographic features that are similar to those of baseline recordings during wakefulness (a), NREM sleep (b), and REM sleep (c). There were no significant differences in EEG power comparing baseline and CGS.

Pontine reticular formation administration of CGS decreased wakefulness and increased both NREM sleep and REM sleep

Figure 4 summarizes group data showing the effects of CGS on per cent time spent in wakefulness, NREM sleep and REM sleep. In the first hour (Fig. 4a), CGS significantly decreased wakefulness by 51% ($t = 3.07$; d.f. = 8; $p < 0.05$) and increased NREM sleep by 90% ($t = 3.74$; d.f. = 8; $p < 0.01$). There was no difference between time spent in REM sleep during dialysis with CGS and Ringer's (control) in the first hour. In the second hour (Fig. 4b), CGS significantly decreased wakefulness by 54% ($t = 3.32$; d.f. = 8; $p < 0.05$), increased NREM sleep by 151% ($t = 3.1$; d.f. = 8; $p < 0.05$), and increased REM sleep by 331% ($t = 2.94$; d.f. = 8; $p < 0.05$) compared with control.

The number of REM sleep episodes in the first hour of CGS administration was not significantly different from control (Fig. 5a). In the second hour, CGS caused a significant increase ($t = 2.83$; d.f. = 8; $p < 0.05$) in the number of REM sleep episodes (Fig. 5b). Pontine CGS had no effect on REM sleep latency and no effect on duration of individual REM sleep episodes in the first or the second hour (data not shown).

Figure 5(c) shows that all microdialysis sites for the CGS sleep studies were localized to the PRF. Mean ± SEM stereotaxic coordinates for the microdialysis sites were 4.92 ± 0.05 mm posterior to bregma, 0.79 ± 0.06 mm lateral to the midline, and 5.2 ± 0.09 mm ventral to the surface of the skull. The microdialysis sites for the sleep studies (Figs 3–5) did not differ from the microdialysis sites for the ACh quantification studies (Figs 1 and 2).

Discussion

Dialysis administration of the adenosine A_{2A} receptor agonist CGS into the PRF of awake B6 mouse caused a concentration-dependent change in ACh release. The increase in ACh release evoked by 3 μM CGS was blocked by co-adminis-

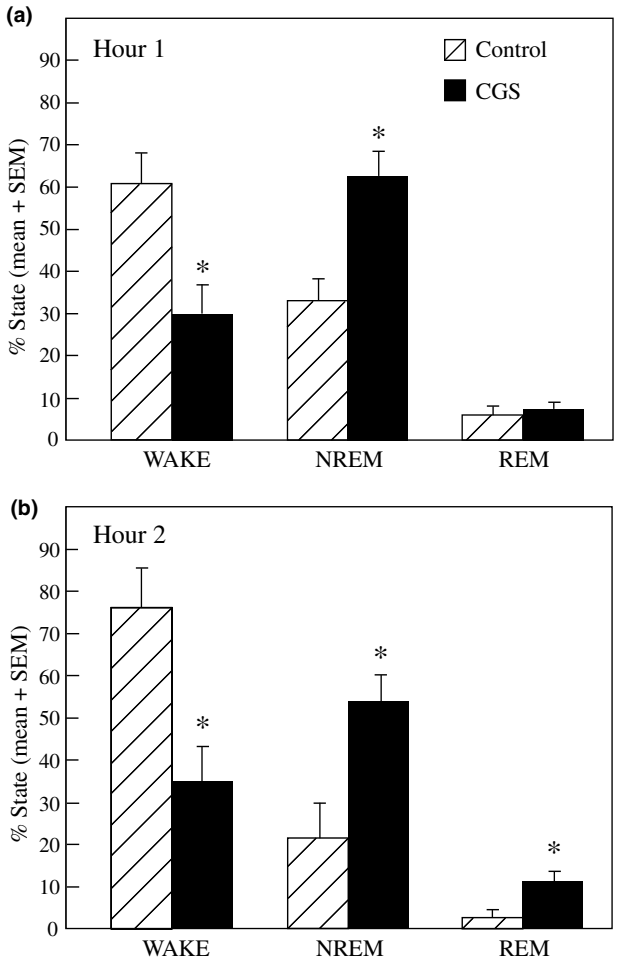

Fig. 4 CGS altered the per cent of time spent in wakefulness, NREM sleep and REM sleep. Per cent state values represent data from five mice during dialysis with Ringer's (Control) and five mice during dialysis with CGS (3 μM). Asterisks indicate a significant ($p < 0.05$) difference from control. (a) In the first hour of CGS delivery, NREM sleep was significantly increased and wakefulness was significantly decreased. (b) During the second hour, CGS delivery caused a significant increase in REM sleep. NREM sleep also was increased and wakefulness was decreased.

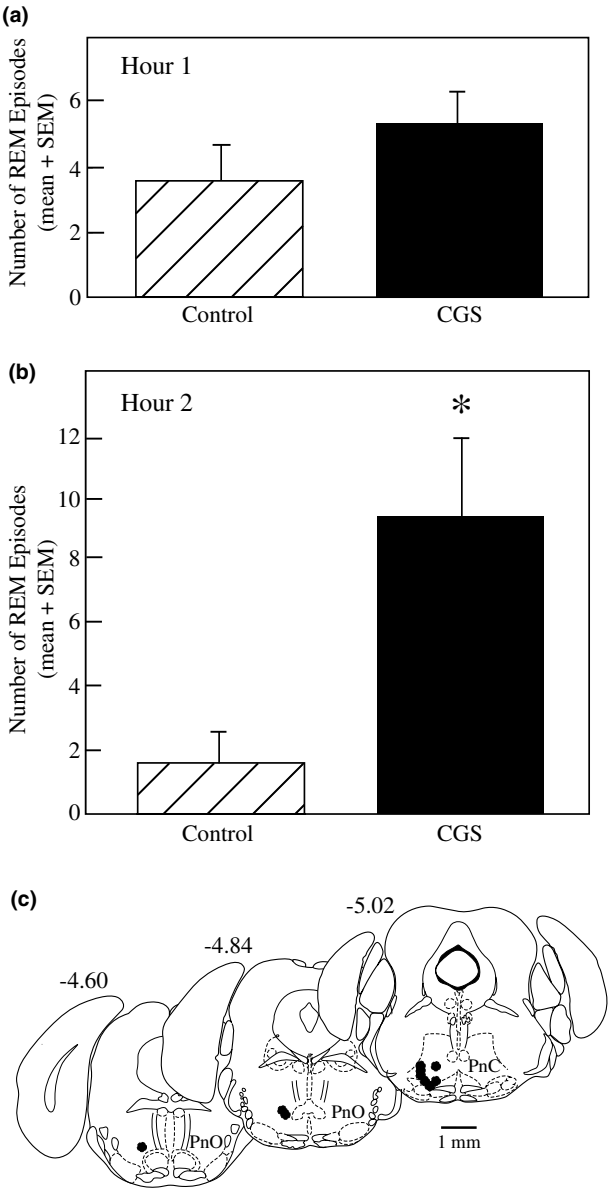

Fig. 5 CGS increased the number of REM sleep episodes. (a) In the first hour of pontine reticular formation dialysis with CGS (3 μM; $n = 5$ mice), the number of REM sleep episodes was not different from Ringer's (Control; $n = 5$ mice). (b) The number of REM sleep episodes was significantly ($p < 0.05$) increased over control values in the second hour of dialysis with CGS. Asterisk indicates a significant ($p < 0.05$) difference from mean control level. (c) Filled circles localize microdialysis sites for the 10 mice used for the CGS sleep studies (Figs 4 and 5). The number at the top left of each diagram indicates mm posterior to bregma (Paxinos and Franklin 2001). All drug delivery sites were in the PRF.

tering the adenosine A_{2A} receptor antagonist ZM. These findings support the interpretation that adenosine A_{2A} receptors in the PRF stimulate ACh release. Additional data demonstrated that the same concentration of CGS that increased ACh release when administered into the PRF also increased REM sleep. This finding is consistent with previous data showing that microinjection of CGS into rat PRF caused REM sleep enhancement (Marks *et al*. 2003). The present results show for the first time that administering CGS to the PRF caused a significant increase in NREM sleep. The mechanisms by which adenosine receptors in the PRF contribute to the regulation of sleep are considered below.

Modulation of ACh release in the PRF by adenosine A_{2A} and A_1 receptors

Dialysis administration of CGS (3 μM) into the PRF significantly increased ACh release in the PRF (Fig. 1). This

finding is similar to the effect of 3 μM CGS on ACh release in rat striatum (Kurokawa *et al*. 1996). Co-administration of CGS and the adenosine A_{2A} receptor antagonist ZM into the PRF blocked the increase in ACh release caused by CGS (Fig. 2). This finding supports the conclusion that the CGS-evoked increase in ACh release is mediated by adenosine A_{2A} receptors in the PRF.

The present study also found that dialysis with higher concentrations of CGS (100 and 1000 μM) significantly decreased ACh release in the PRF (Fig. 1). CGS has an approximately 200-fold higher affinity for adenosine A_{2A} receptors than for adenosine A_1 receptors (Jarvis *et al*. 1989). Thus, at high concentrations, CGS binds to adenosine A_1 receptors (Klotz 2000). Functional evidence indicates that both adenosine A_1 and A_{2A} receptors can coexist on the same nerve terminal to, respectively, inhibit or enhance neurotransmitter release (Sebastiao and Ribeiro 2000; Wardas 2002). Therefore, the decrease in ACh release caused by higher concentrations of CGS (Fig. 1) may have resulted from activation of adenosine A_1 receptors in the PRF. This interpretation is reinforced by studies showing that dialysis administration of the adenosine A_1 agonist N^6-p-sulfophenyladenosine to cat PRF decreased ACh release in the PRF (Tanase *et al*. 2003). This interpretation also fits with recent data showing that, in the lateral preoptic area, adenosine has opposite effects on sleep and wakefulness, depending upon the adenosine receptor subtype activated. Activation of lateral preoptic area adenosine A_1 receptors increases wakefulness, whereas activation of adenosine A_{2A} receptors increases sleep (Methippara *et al*. 2005).

Pontine reticular formation CGS inhibits wakefulness and promotes sleep

PRF administration of CGS (3 μM) decreased the time spent awake and increased the time spent asleep (Fig. 4). The EEG power spectra (Fig. 3) and the EEG and EMG recordings (Fig. 3 insets) for each arousal state during CGS administration were similar to those obtained during dialysis with Ringer's (control) and to those reported previously for B6 mouse (Franken *et al*. 1998; Lydic *et al*. 2002). Thus, the enhanced sleep produced by CGS is electrographically homologous to natural sleep.

Dialysis administration of 3 μM CGS into the PRF increased REM sleep during the second hour (Figs 4 and 5). This increase in REM sleep time and in the number of REM sleep episodes corresponds well with the time-course of ACh release evoked by PRF dialysis administration of CGS (Fig. 2). These results suggest that the increase in REM sleep was caused, in part, by the significant increase in ACh release elicited by CGS. This interpretation is consistent with data showing that muscarinic receptors are present in mouse PRF and contribute to REM sleep generation (DeMarco *et al*. 2003; Coleman *et al*. 2004a; Douglas *et al*. 2005). In the same area of the mouse PRF where administration of CGS increased ACh release and increased REM sleep, microinjection of the acetylcholinesterase inhibitor neostigmine produces a REM sleep-like state that is blocked by muscarinic receptor antagonists (Lydic *et al*. 2002; Coleman *et al*. 2004a; Douglas *et al*. 2005). CGS-evoked REM sleep enhancement in rat is blocked by the muscarinic receptor antagonist atropine (Marks *et al*. 2003). Considered together, these data indicate that the increase in REM sleep caused by CGS involves increased cholinergic neurotransmission at muscarinic receptors in the PRF.

Dialysis administration of CGS (3 μM) also caused an increase in NREM sleep (Fig. 4). This result was surprising, because the PRF is not known to regulate NREM sleep, and microinjection of CGS into rat PRF did not cause an increase in NREM sleep (Marks *et al*. 2003). The differences in the effect of CGS on NREM sleep between the previous (Marks *et al*. 2003) and present (Fig. 4) studies may be because of the route of drug administration (microinjection vs. microdialysis), species (rat vs. mouse), and/or site of drug administration within the PRF. Microinjection sites where CGS was administered to rat were localized to caudal portions of the PnO (Marks *et al*. 2003), whereas most of the dialysis sites used for the present study were localized to rostral portions of the PnC (Fig. 5c). A systematic mapping study is required to determine if sites in the PnC are more effective for producing NREM sleep following CGS administration than sites in the PnO.

Interestingly, NREM sleep was significantly increased during the first hour of CGS delivery. In contrast, REM sleep did not significantly increase until the second hour of CGS delivery (Fig. 4). The neurochemical mechanisms underlying these temporal differences in the response to CGS are not addressed by the present data. Sleep is a genetically modulated phenotype (Tafti and Franken 2002) and it is not known to what extent the Fig. 4 data are true for other strains of mice. The temporal differences in the NREM sleep and REM sleep responses to CGS may, in part, reflect the fact that B6 mice have seven times more NREM sleep than REM sleep (Franken *et al*. 1999). The h 1 versus h 2 effects of CGS on sleep may also vary as a function of species. B6 mice spend only 5 or 6% of total sleep time in the REM phase of sleep (Franken *et al*. 1999). In contrast, cats spend 15 to 20% of total sleep time in REM sleep. Thus, the finding that CGS caused an increase in NREM sleep before it increased REM sleep may also reflect the comparatively small amount of REM sleep characteristic of mice.

One mechanism by which adenosine A_{2A} receptors in the PRF may promote NREM sleep is by inhibiting the activity of wakefulness generating neurons (Methippara *et al*. 2005). Adenosine A_{2A} receptors are located on GABAergic neurons (Hettinger *et al*. 2001), and *in vivo* and *in vitro* data demonstrate that adenosine A_{2A} receptor stimulation increases GABA release (Mayfield *et al*. 1993; Ferre *et al*. 1994; Mayfield *et al*. 1996). The dorsal raphe nucleus promotes

wakefulness and receives GABAergic input from the PRF (Luppi *et al*. 1999). In addition to adenosine causing sleep by inhibiting neurons that promote arousal, there is support for the view that adenosine may disinhibit neurons that actively promote sleep (Morairty *et al*. 2004). These data support the speculation that the excitatory actions of adenosine A_{2A} receptors in the PRF would enhance NREM sleep if adenosine A_{2A} receptors are localized to GABAergic neurons that project to and inhibit serotonergic neurons in the dorsal raphe nucleus. Adenosine has been shown to inhibit the discharge of wakefulness-active neurons in the basal forebrain of rat (Alam *et al*. 1999) and cat (Thakkar *et al*. 2003). NREM sleep is positively correlated with FOS activation in the ventrolateral preoptic nucleus (Scammell *et al*. 2001). For a subset of ventrolateral preoptic nucleus neurons there is evidence that adenosine A_{2A} receptors cause neuronal activation (Gallopin *et al*. 2005).

Conclusion

The results indicate for the first time that acetylcholine release in the pontine reticular formation and sleep are altered by an adenosine A_{2A} receptor agonist. This study provides novel evidence that dialysis delivery of CGS to the PRF significantly increases NREM sleep. Whether pontine CGS increases NREM sleep by inhibiting wakefulness-promoting systems can be addressed by future studies. The finding that adenosine agonists and antagonists alter sleep and wakefulness are consistent with the view that endogenous adenosine contributes to arousal state control. This interpretation agrees with the fact that the arousal-promoting effects of caffeine result from blockade of A_{2A} receptors (Huang *et al*. 2005). The results presented here support the interpretation that sleep is increased by endogenous adenosine, acting at A_{2A} receptors in the PRF.

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