Adenosine A$_{2A}$ Receptors Regulate the Activity of Sleep Regulatory GABAergic Neurons in the Preoptic Hypothalamus

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Running head: Adenosine and GABAergic neurons of the preoptic area
ABSTRACT

The median preoptic nucleus (MnPN) and the ventrolateral preoptic area (VLPO) are two hypothalamic regions that have been implicated in sleep regulation, and both nuclei contain sleep-active GABAergic neurons. Adenosine is an endogenous sleep regulatory substance, which promotes sleep via A₁ and A₂₄ receptors (A₂₄R). Infusion of A₂₄R agonist into the lateral ventricle or into the subarachnoid space underlying the rostral basal forebrain (SS-rBF), has been previously shown to increase sleep. We examined the effects of an A₂₄R agonist, CGS-21680, administered into the lateral ventricle and the SS-rBF on sleep and Fos protein immunoreactivity (Fos-IR) in GABAergic neurons in the MnPN and VLPO. Intracerebroventricular (ICV) administration of CGS-21680 during the second half of lights-on phase increased sleep and increased the number of MnPN and VLPO GABAergic neurons expressing Fos-IR. Similar effects were found with CGS-21680 microinjection into the SS-rBF. The induction of Fos-IR in preoptic GABAergic neurons was not secondary to drug-induced sleep, since CGS-21680 delivered to the SS-rBF significantly increased Fos-IR in MnPN and VLPO neurons in animals that were not permitted sleep. ICV of infusion of ZM-241385, an A₂₄R antagonist, during the last 2 h of a 3 h period of sleep deprivation caused suppression of subsequent recovery sleep and reduced Fos-IR in MnPN and VLPO GABAergic neurons. Our findings support a hypothesis that A₂₄R mediated activation of MnPN and VLPO GABAergic neurons contributes to adenosinergic regulation of sleep.

Key words: Median preoptic nucleus, Ventrolateral preoptic area, Subarachnoid space, Fos immunoreactivity, GABAergic neurons.
INTRODUCTION

Adenosine (AD) is an endogenous sleep factor that has been shown to promote nonREM sleep (for review, see refs. 6, 7, 36 and 39). Extracellular concentrations of adenosine in the basal forebrain increase during wakefulness and decrease during sleep (37). Adenosine and adenosine agonists influence the activity of putative sleep- and wake-regulatory neurons (3, 40). Sleep is increased by administration of adenosine, adenosine receptor agonists or drugs that increase extracellular adenosine levels in forebrain sites including the substantia innominata (37, 38), magnocellular basal forebrain area (5), subarachnoid space ventral to the rostral basal forebrain (SS-rBF) (15, 43-45, 46) and median and lateral preoptic areas (28, 29, 30, 55).

Two brain regions implicated in the regulation of sleep are the median preoptic nucleus (MnPN) and the ventrolateral preoptic area (VLPO) of the hypothalamus. A subset of MnPN neurons exhibit sleep-related discharge with low discharge during waking, increased activity with the onset of sleep, and the highest discharge during nonREM and REM sleep (51). The number of MnPN neurons exhibiting c-Fos protein immunoreactivity (Fos-IR), a marker of neuronal activation, increases in proportion to the amount of preceding sleep and with increasing homeostatic sleep pressure (11-13). VLPO neurons also exhibit increased discharge during nonREM and REM sleep, as well as sleep-associated Fos-IR (48, 52). VLPO neurons appear critical for sleep induction and sleep maintenance, as excitotoxic VLPO lesions markedly decrease both nonREM and REM sleep (27). Excitotoxic lesion of preoptic area (POA) that primarily involves the medial region also causes chronic sleep loss (18).

There are four adenosine receptor subtypes, A₁, A₂A, A₂B and A₃, all of which are coupled to G proteins (42). Inhibitory G protein-coupled adenosine A₁ receptors and stimulatory G protein-coupled adenosine A₂A receptors (A₂AR) in the brain mediate the sleep-inducing effects
of adenosine (reviewed in refs. 6 and 36). Intracerebroventricular (ICV) infusion of an $A_{2A}$R agonist, 2-p-(2-carboxyethyl) phenylethylamino-5-N-ethylcarboxyamidoadenosine (CGS-21680), promotes sleep (10, 43-45, 46, 56). The largest increase in sleep occurs when CGS-21680 is administered to the SS-rBF (44). CGS-21680-induced sleep is associated with an increase in Fos-IR in VLPO neurons (46). A subset of VLPO neurons recorded in hypothalamic slice is excited by adenosine acting on the $A_{2A}$R (9). Previous in vivo studies have not identified the neurotransmitter phenotype(s) of the neurons that are activated by $A_{2A}$R agonist. The effects of $A_{2A}$R agonists on MnPN neurons have not been previously reported.

We hypothesized that $A_{2A}$R-dependent modulation of sleep is mediated through the activation of sleep-regulatory GABAergic neurons in the preoptic hypothalamus. We recorded sleep/wake behavior and quantified Fos-IR in GABAergic neurons in the MnPN and VLPO to 1) determine if central administration of an $A_{2A}$R receptor agonist, CGS-21680, promotes sleep and activates preoptic GABAergic neurons; 2) determine if CGS-21680 administration activates MnPN and VLPO neurons independently of increased sleep, i.e., in animals that are not permitted sleep after drug treatment; and 3) determine if ICV infusion of an $A_{2A}$R antagonist, ZM-241385, during sleep deprivation suppresses subsequent recovery sleep and attenuates the activation of preoptic GABAergic neurons during recovery sleep. Preliminary results have appeared previously in abstract form (21, 22).

**METHODS**

Experiments were performed on male, Sprague-Dawley rats, weighing between 300-350g at the time of surgery. These rats were maintained on 12:12 h light-dark cycle (lights on at 06:00 h) and with food and water available ad libitum. All experiments were conducted in accordance
with the National Research Council Guide for the Care and Use of Laboratory Animals and were approved by Institutional Animal Care and Use Committee, V.A. Greater Los Angeles Healthcare System.

**Surgical Procedures**

All surgical procedures were done under anesthesia (Ketamine + Xylazine: 80:10 mg/kg; i.p.) and aseptic conditions. Details of the surgical procedure were described previously (23). In brief, rats were implanted with electroencephalogram (EEG) and dorsal neck electromyogram (EMG) electrodes for recording sleep-wake behavior. In addition rats were implanted with a microinjection guide cannula (23G stainless steel tube) in the lateral ventricle at coordinates, AP = -0.8 mm; L = 1.4 mm and H = 3.6 mm (34), or the SS-rBF, at co-ordinates, AP = +1.5 mm; L = 1.8 mm and H = 8.3 mm (44).

Following implantation a blocking stylet was inserted into the guide cannula to maintain patency until microinjection. Rats were permitted a 10-12 day post-surgical recovery period, after which they were connected to recording cables and placed in temperature controlled (23±2°C) and sound attenuated recording chambers for adaptation.

**Experimental Protocols**

**Experiment-1: Effects of microinjection of CGS-21680 into the lateral ventricle on sleep and Fos-IR**

Three days before the experiments, the placement of ICV cannula was tested by ICV injection of angiotensin (200 ng in a volume of 5 µl). Only rats exhibiting a short-latency (<2 min) drinking response to angiotensin were used.

Control rats (n=7) received microinjection of vehicle containing 4% dimethyl sulfoxide (DMSO) in artificial cerebrospinal fluid (aCSF), of composition in mM, 145 NaCl, 2.7 KCl, 1.3
MgSO₄, 1.2 CaCl₂, and 2 Na₂HPO₄; pH, 7.2. Experimental rats received microinjection of either 8 nmol (n=7) or 24 nmol (n=7) CGS-21680 (Sigma, USA) in a volume of 5μl over 10 minutes. All ICV injections were initiated at 14:00 (lights-on at 06:00). Drug or vehicle was injected during second half of the light phase because baseline sleep amounts are reduced compared to the early light phase, increasing the likelihood of detecting drug-induced increases in sleep. The rats were left undisturbed for 2 hours following ICV injection, while EEG and EMG were continuously recorded.

**Experiment-2: Effects of microinjection of CGS-21680 into the SS-rBF on sleep and Fos-IR**

In this experiment, rats received microinjection of vehicle (n=7, 4% DMSO in aCSF) or 8 nmol (n=7) CGS-21680 in a volume of 5μl over 10 minutes into the SS-rBF at 14:00. Rats were left undisturbed for 2 hours, while EEG and EMG were continuously recorded.

**Experiment-3: Effects of microinjection of CGS-21680 into the SS-rBF, followed by 2h of sleep deprivation, on Fos-IR.**

Rats were microinjected with 8 nmol of CGS-21680 (n=7) or vehicle (n=7) in a volume of 5μl delivered over 10 minutes in the SS-rBF. To dissociate the direct effect of CGS-21680 on Fos-IR from the effect of increased sleep, groups of drug- and vehicle-treated rats were sleep deprived for 2 hours post injection. Rats were initially adapted to stimuli used to prevent sleep (tapping the cage and/or gentle movement of the cage) for about 20 min a day for 5 days before the experimental day. On the experimental day both drug and vehicle treated rats were subjected to sleep deprivation by delivering arousing stimuli within 10 s of the appearance of nonREM sleep detected by visualizing the EEG. Animals were euthanized immediately after the 2 hour sleep deprivation period.
Experiment-4: Effects of infusion of A$_{2A}$R antagonist, ZM-241385 into the lateral ventricle on sleep and Fos-IR

Three groups of rats were infused with either vehicle (n=6) or one of two doses of ZM-241385, an adenosine A$_{2A}$R antagonist (5 nmol, n=6 or 25 nmol, n=6), delivered into the lateral ventricle during the last 2h of 3h of sleep deprivation. Sleep deprivation was initiated 2h after lights on at 08:00. The rate of infusion was 0.4 µl/min. Following the end of sleep deprivation, ICV infusion was discontinued and rats were then left undisturbed and permitted recovery sleep while EEG and EMG were continuously recorded for 2h. Rats were euthanized immediately after the 2h recovery sleep period.

Histology and Immunohistochemistry

At the end of all experiments, rats were given a lethal dose of pentobarbital (100 mg/kg, IP). Immediately after anesthetization, rats were injected with heparin (500U, i.p.), and perfused transcardially with 30-50 ml of 0.1 M phosphate buffered saline (PBS; pH 7.2) followed by 500 ml of 4% paraformaldehyde in PBS, containing 15% saturated picric acid solution (a final concentration of the picric acid was 0.2%). The brains were removed, post-fixed for 20 min and then equilibrated in 30% sucrose. Coronal sections encompassing the MnPN and VLPO were freeze-cut at 30µm thickness and immunostained for c-Fos and GAD.

c-Fos immunostaining: Sections were first immunostained for c-Fos protein. Free-floating sections were incubated in 0.3% H$_2$O$_2$ in tris-buffered saline (TBS) at room temperature (RT) for 30 min and then rinsed 3 times for 10 min each in TBS. The sections were placed in blocking solution (8% goat serum in TBS) for 1 hr at RT. Sections were incubated in rabbit anti-c-Fos (PC-38, Calbiochem, EMD Millipore, 1:20,000, CA, USA) in 4% goat serum in TBS, for 40-48 h at 4°C. After rinsing in TBS 3 times for 10 min each, sections were incubated in biotinylated
goat anti-rabbit secondary antibody (1:1000 Vector Laboratories, CA, USA) in 4% goat serum in TBS, for 2 h at RT followed by rinsing with TBS. The sections were then incubated in avidin-biotin complex (1:500, Vector Laboratories) for 2 h at RT and then visualized with nickel-3,3’-diaminobenzidine tetrahydrochloride (DAB, Sigma, USA). Black staining confined to the nucleus indicated presence of Fos-IR.

**GAD Immunostaining:** Sections were then washed in TBS followed by incubation in blocking solution (10% horse serum in TBS) containing avidin, (1:50, Vector Laboratories) for 1 h at RT. Sections were then incubated in the primary antibody, monoclonal mouse anti GAD (MAB 5406; 1:400, Millipore, Temecula, CA, USA) with Biotin (1:50, Vector Laboratories) for 40-48 h at 4°C. The use of avidin and biotin (SP 2001, Vector Laboratories) in the GAD staining procedure reduced terminal staining substantially (20). The sections were then incubated in biotinylated horse anti-mouse secondary antibody (BA 2001; 1:400 Vector Laboratories) for 1 h at RT followed by rinsing with TBS. The sections were then incubated in avidin-biotin complex (1:150, Vector Laboratories) for 2h at RT followed by DAB visualization to give a brown product.

All sections were rinsed with TBS, mounted on gelatin-coated microscope slides, dehydrated in graded alcohols, cleared in xylene and cover slipped with DPX mounting medium. Tissues from control and experimental groups of each experiment were processed together using the same batch of reagents. Omission of the primary antibodies (anti-Fos and anti-GAD) in control sections did not yield staining in processes, proximal dendrites or cell bodies.

Serial coronal sections were stained for Nissl (cresyl violet) for localization of microinjection sites. Sections stained for Fos-GAD were used for localization of microinjection sites for ICV experiments.
Data Analysis

Sleep-wake scoring: Bioelectric signals were amplified and band pass filtered at 0.3–100 Hz (EEG) or 0.10–300 Hz (EMG) (Model 78 D, Grass Instruments, Quincy, MA). Bioelectrical signals were digitized at a sampling rate of 128 Hz for EEG and 256 Hz for EMG using a 1401 Plus data acquisition interface and Spike2 software (Cambridge Electronic Design, Cambridge, UK) and stored on a PC for off-line analysis.

The predominant sleep-wake states for each 10-s epoch were visually determined by an experienced scorer, blind to experimental condition and group identity of the animals. Wake was defined as low-voltage, high frequency activity combined with elevated neck muscle tone. NonREM sleep was defined as high-amplitude EEG with prominent activity in the 0.75- to 4.0-Hz range and relatively reduced muscle tone. REM sleep was defined as moderate-amplitude EEG with dominant theta frequency activity (6–8 Hz) combined with minimal neck EMG tonus except for occasional brief twitches. Digitized EEG signals were subjected to a fast Fourier transform algorithm, after which a power spectrum was computed for the delta frequency range of 0.75–4.0 Hz. This was done for each 10-s epoch of scored nonREM sleep and waking in the 2 h recording period. Epochs containing artifacts were omitted from spectral analysis. For individual animals, nonREM sleep EEG delta power was expressed as a percentage of waking EEG delta power values computed for the 2h of recording period. NonREM sleep latency was defined by the time elapsed from the end of sleep deprivation period to the onset of six or more consecutive epochs of NREM sleep (≥60 seconds). REM sleep latency was defined by the time between the end of sleep deprivation and the onset of at least two consecutive epochs of REM sleep (≥20 seconds). Sleep data were averaged through the entire 120-min recording period for statistical analysis.
**Cell counting and analyses:** A single person blind to the treatment conditions performed the counting and plotting of the immunoreactive neurons using the Neurolucida computer-aided plotting system (Micro Bright Field). Section outlines were drawn with 20x magnification, whereas the identification and counting of different neuronal types, i.e., single and dual labeled neurons were done manually under 400x magnification. Fos-IR was recognized by black stain localized to the nucleus, whereas brown-stained soma and dendrites identified GAD-containing neurons. Neurons having a black nucleus and a brown cytoplasm were identified as double labeled neurons.

Counts of Fos+, GAD+, and GAD+/Fos+ cells in MnPN and VLPO were performed using grids corresponding to the four areas of interest; rostral MnPN (rMnPN), caudal MnPN (cMnPN), core VLPO (cVLPO) and extended VLPO (eVLPO), as shown in Fig. 3 and described previously (21). For both the rMnPN and the cMnPN, cell counts were made in three sections and averaged to yield a single value for each rat. For VLPO, cell counts were made bilaterally in three sections containing the largest part of the VLPO. Those six counts were then averaged to yield a single value for both the cVLPO and eVLPO (dorsal and medial combined boxes) for each rat.

**Statistical analysis**

The responses of various doses of CGS-21680 and ZM-24135 on single-labeled Fos+, GAD+, and double-labeled GAD+/Fos+ neurons in the MnPN and VLPO were compared with those obtained after vehicle treatments, using one-way ANOVA followed by Holm-Sidak test for pair-wise multiple comparisons. One-way ANOVA followed by Holm-Sidak tests were also used to determine the effects of CGS-21680 and ZM-241385 on sleep-wake parameters. The responses of an optimum dose of CGS-21680 into the SS-rBF on Fos-IR in GAD+ MnPN and
VLPO neurons as well as sleep-wake parameters were compared with those observed after vehicle treatment using independent t-test.

**RESULTS**

*Effects of ICV injection of CGS-21680*

ICV administration of 24 nmol of CGS-21680 caused a significant decrease in % time awake and an increase in % time in nonREM sleep, compared to both the 8 nmol dose and vehicle (Figure 1A). The 24 nmol dose also increased % time in REM sleep compared to the vehicle. There were no significant differences in sleep-wake amounts between 8 nmol CGS-21680 and vehicle. Delta power in nonREM sleep significantly increased in response to the higher dose of CGS-21680 (Figure 2A). Latencies to nonREM and REM sleep onset decreased significantly after 24 nmol CGS-21680 administration, compared to both the 8 nmol dose and vehicle (Figure 2B and 2C).

Examples of GAD and Fos immuno-staining and locations of counting boxes in the preoptic area are shown in Figure 3. Table 1 shows the single GAD-immunoreactive (IR), single Fos-IR and dual GAD+Fos-IR cell counts in the MnPN and VLPO in animals receiving ICV injections of CGS-21680 or vehicle. Plotted in Figure 4 is the percentage of GAD-IR neurons that were dual labeled for Fos-IR for the three groups. The numbers of single GAD-IR and single Fos-IR neurons in the MnPN and in the VLPO in vehicle and drug treated animals were comparable (Tables 1). However, the % GAD+ neurons double-labeled and the number of Fos/GAD dual immunoreactive neurons in both the MnPN and the VLPO were elevated in rats treated with CGS-21680 (24 nmol), compared to those treated with vehicle (Figure 4 and Table 1). The number of Fos/GAD dual immunoreactive neurons and the %GAD+ neurons double-
labeled did not differ significantly between vehicle and 8 nmol CGS-21680 treated animals in all of the four areas examined (Table 1 and Figure 4).

**Effects of SS-rBF injection of CGS-21680**

Microinjection of an A2AR agonist into the SS-rBF has been shown to have more potent sleep-promoting effects than microinjection in the lateral ventricle (Satoh et al., 1999) so we applied only the lower dose of CGS-21680 (8 nmol) in this site.

SS-rBF administration of 8 nmol CGS-21680 significantly decreased waking and increased nonREM and REM sleep compared to vehicle (Figure 1B). Latencies to both nonREM and REM sleep onset decreased significantly in response to SS-rBF administration of CGS-21680 (Figure 2B and 2C). The nonREM delta power was also significantly increased in response to drug (Figure 2A).

The %GAD+ neurons double-labeled and number of dual Fos/GAD-IR neurons in the MnPN (rostral and caudal) and in the VLPO (core and extended) were elevated in CGS-21680 injected rats compared to those injected with vehicle (Figure 4 and Table 2). No significant changes were detected in the number of single GAD-IR or single Fos-IR neurons in the VLPO and MnPN in the CGS-21680 treated group compared to the vehicle group (Table 2).

**c-Fos expression in preoptic neurons in CGS-21680 treated sleep-deprived rats**

To determine if treatment with CGS-21680 could activate MnPN and VLPO GABAergic neurons independent of increasing sleep, groups of drug- and vehicle-treated rats (n=7) were subjected to sleep deprivation for 2h post-injection, and then sacrificed without opportunity for recovery sleep. Drug and vehicle injections were made into the SS-rBF. Time (in min) spent awake and in nonREM sleep during the sleep deprivation period are shown in Figure 5A. There
were no significant differences between CGS-21680- and vehicle-treated rats. REM sleep was completely suppressed in both groups during sleep deprivation (data not shown).

In spite of the comparable reductions in sleep achieved in the two groups, the percentage of GAD-IR neurons dual immunolabeled for Fos-IR was significantly elevated in all MnPN and VLPO subregions examined in CGS-2160-treated versus vehicle-treated rats (Figure 4). The number of GAD/Fos dual IR cells was also elevated in the MnPN and VLPO of animals treated with drug (Table 2).

**Effects of ICV infusion of A2A R antagonist ZM-241385**

ICV infusion of ZM-241385 or vehicle (n=6/group) was performed during the final 2 h of a 3 h period of sleep deprivation. Drug and vehicle were infused at a rate of 0.4µl/min. Total amount of drug delivered was 5 nmol in one experimental group and 25 nmol in the other. Time spent awake and in nonREM sleep during the 3h sleep deprivation period are shown in Figure 5B.

Following the end of sleep deprivation and discontinuation of ICV infusion, all animals were permitted 2 hours of undisturbed opportunity for recovery sleep. ICV infusion of ZM-241385 at 5 and 25 nmol, caused dose-dependent increases in % time awake during the 2h recovery sleep opportunity (Figure 6). The % time spent in nonREM and REM sleep during the recovery period were significantly reduced in response to both doses of ZM-241385 compared to vehicle (Figure 6). The latency to sleep onset following the end of sleep deprivation was significantly increased in ZM-241385-treated rats (5 nmol; 23.1±3.4 min and 25 nmol; 26.6±1.7 min) compared to vehicle-treated rats (12.4±2.7 min; F(2,15)=7.54, p<.02). In addition, EEG delta power during nonREM sleep was significantly lower during the first hour of recovery sleep in both groups receiving drug, compared to vehicle (Figure 6B).
ICV infusion of ZM-241385 resulted in significant decreases in the %Fos+/GAD+ neurons in both the rostral and caudal portions of the MnPN (Figure 7). Significant decreases in %GAD+ neurons double-labeled were also observed in the core and extended VLPO in response to drug (Figure 7). Dual Fos/GAD-IR cell counts were also reduced in these preoptic nuclei in response to drug (Table 3). There were no significant differences in single GAD-IR or single Fos-IR cell counts between drug- and vehicle-treated animals in any of the preoptic nuclei examined (Table 3).

**DISCUSSION**

The ICV administration of an adenosine A2A R agonist, CGS-21680, increased both nonREM and REM sleep amounts, increased delta power within nonREM sleep, and increased Fos-IR in GABAergic neurons in the MnPN and VLPO. Similar effects were observed after SS-rBF administration of this A2A R agonist, albeit at a lower dose. CGS-21680-induced increases in Fos-IR in MnPN and VLPO GABAergic neurons were observed even in animals that were not allowed to sleep after drug administration. We also found that ICV infusion of the adenosine A2A R antagonist, ZM-241385, during sleep deprivation caused decreased recovery sleep and suppression of Fos-IR in MnPN and VLPO GABAergic neurons. Given that GABAergic neurons in the MnPN and VLPO play a crucial role in the generation of sleep, these findings support the hypothesis that activation of MnPN and VLPO GABAergic neurons are involved in the adenosinergic regulation of sleep.

This is the first study to demonstrate that *in vivo* central administration of A2A R agonists and antagonists alters c-Fos expression in GABAergic neurons in the VLPO and MnPN. This is
also the first demonstration that activation of preoptic sleep-regulatory neurons in response to central A2AR agonists can occur independently of drug-induced sleep.

Adenosine is one of the several endogenous neuromodulators, including prostaglandin D2 (14, 43), interleukin-1 (4, 19) and growth hormone releasing hormone (31, 35), that have been implicated in sleep regulation. The sleep-promoting effects of adenosine have been shown to involve A1-mediated inhibition of cholinergic and noncholinergic arousal-related neurons in the basal forebrain (3, 5, 15, 29, 37, 43-45, 46, 53). Additional sites of A1-mediated inhibition of arousal systems include hypocretin neurons in the perifornical lateral hypothalamus (2, 26, 41, 54), histaminergic neurons in the tuberomammillary nucleus (TMN, 32), and noradrenergic neurons in the locus coeruleus (LC) (33).

Work reported here and previously published studies indicate that A2AR-mediated activation of preoptic sleep regulatory neurons is an additional mechanism through which adenosine can promote sleep. ICV and SS-rBF infusion of A2AR agonists promote sleep (10, 43-45), increase Fos expression in VLPO neurons and decrease Fos-IR in the TMN (46). Microinfusion of A2AR agonist into the lateral preoptic area, including the VLPO, promotes sleep (29). Bath application of adenosine in vitro reduces firing of some VLPO neurons via a direct A1 effects, but excites other VLPO neurons via effects on A2AR (9).

The MnPN and VLPO are sources of descending GABAergic projections to several wake-promoting regions in the posterior hypothalamus and brainstem. These include the TMN (47), dorsal raphe nucleus (DRN) and the LC (47, 49, 57). A subset of MnPN and VLPO neurons that project to the DRN and the adjacent ventrolateral periaqueductal gray, express Fos-IR during sleep (16, 59). Projections from the VLPO and MnPN to the hypocretin neuronal field in the perifornical lateral hypothalamus have also been described (60) and a subset of these
projection neurons exhibit sleep-related Fos-IR (58). The activation and inactivation of MnPN neurons have been shown to suppress and activate, respectively, wake-active neurons in the lateral hypothalamus (50). Inactivation of MnPN increases Fos expression in hypocretin neurons of hypothalamus and in serotonergic neurons of dorsal raphe (24). Activation of MnPN and VLPO GABAergic neurons by A2AR agonists, therefore, would be expected to result in suppression of multiple wake-promoting neuronal systems, leading to increased sleep. Antagonism of A2AR in the MnPN and VLPO would be expected to have the opposite effects on wake-promoting neuronal activity leading to diminished propensity for sleep following sleep deprivation, as we have described here.

Increased adenosinergic signaling occurring as a consequence of sustained wakefulness, and involving A1R-mediated inhibition of arousal systems, is hypothesized to be a critical component of sleep homeostasis (6, 7, 36). The contribution of A2AR-mediated excitation of preoptic sleep regulatory neurons to changes in homeostatic sleep drive is not known. ICV infusion of A2AR antagonist during sleep deprivation did result in reduced amounts of recovery sleep and reduced Fos-IR in MnPN and VLPO GABAergic neurons. Recovery sleep in ZM-241385-treated rats was characterized by prolonged latency to sleep onset and reduced EEG delta activity in nonREM sleep, suggestive of diminished homeostatic sleep pressure compared to equally sleep deprived vehicle-treated rats. However, it remains unclear if ZM-241385 effects on recovery sleep are the result of disfacilitation of MnPN and VLPO sleep regulatory neurons, or reflect activation of arousal systems via A2AR-responsive circuits involving the ventral striatum (25).

A comprehensive in vitro study of the responses of GABAergic VLPO neurons to adenosine identified two functional cell types (9). Type 1 cells are inhibited by serotonin and
adenosine A₁-R agonists, but are unresponsive to A₂ₐR agonists. Type 2 cells are excited by serotonin and excited by A₂ₐR agonists. The latter were hypothesized to be functionally important for sleep induction during period of high homeostatic sleep pressure by virtue of their excitatory response to adenosine (9). We infer that the subset of GAD-IR neurons expressing Fos-IR in response to A₂ₐR agonist in the present study, corresponds to Type 2 neurons characterized in vitro. The existence of two functional GABAergic cell types is supported by in vivo electrophysiological findings that some sleep-active neurons in the lateral preoptic area exhibit increased discharge in response to sleep deprivation and others do not (1).

The alerting drug, caffeine, is a mixed A₁ and A₂ₐR antagonist. While both A₁ and A₂ₐ receptors appear to play a role in sleep regulation (see above), the alerting effects of caffeine are more dependent on A₂ₐR mechanisms. It was demonstrated that caffeine increased wakefulness in both wild-type mice and A₁ receptor knockout mice, but had no wake-promoting effects in A₂ₐR knockout mice (17). A recent study demonstrated that selective lesions of A₂ₐR-expressing neurons in the nucleus accumbens (NAC) of mice, and knockdown of A₂ₐR in the NAC of rats with focal RNA interference, significantly attenuate the wake-promoting effects of caffeine (25). This suggests that A₂ₐR expressing neurons in the NAC have sleep-promoting properties, and that caffeine blocks A₂ₐRs in this site. It is possible that ICV and SS-rBF infusion of A₂ₐR agonists and antagonists in the current study could have targeted these NAC neurons, with observed changes in Fos expression in the MnPN and VLPO being a downstream consequence. However, SS-rBF drug administration might be expected to more directly affect MnPN and VLPO sleep-active GABAergic neurons, which lie in immediate proximity to the subarachnoid space where drugs were injected.
Although, the density of the $A_{2A}$R is reported to be low in the preoptic area (POA) in comparison to the striatum, nucleus accumbens and olfactory tubercle (8), evidence at the system and cellular levels (see above) is consistent with the existence of functional $A_{2A}$R on sleep regulatory neurons in the preoptic area. However, the experimental approach used in the current study cannot determine if centrally administered $A_{2A}$R agonist and antagonist directly targeted MnPN and VLPO neurons. Although the distribution of $A_{2A}$R in MnPN and VLPO is unknown, based on our current findings, one would expect that $A_{2A}$ receptors are differentially expressed in sleep-active, GABAergic versus nonGABAergic neurons in these nuclei.

**Perspectives and Significance**

Our study demonstrates that the ICV and SS-rBF administration of an $A_{2A}$R agonist at doses capable of inducing sleep, increase c-Fos-IR in GABAergic neurons in subregions of the preoptic hypothalamus implicated in sleep regulation, namely, the MnPN and the VLPO. In contrast, ICV infusion of adenosine $A_{2A}$R antagonist suppresses Fos-IR in MnPN and VLPO GABAergic neurons and suppresses recovery sleep following sleep deprivation. These findings are consistent with a hypothesis that $A_{2A}$R mediated activation of preoptic area sleep-regulatory neurons contributes to the adenosinergic regulation of sleep. Further studies are needed to quantify the co-localization of $A_{2A}$R on the sleep-active GABAergic population of the MnPN and VLPO neurons. The focal deletion of $A_{2A}$R in MnPN and VLPO using Cre/lox-P approaches may further help determine the relative contributions of $A_{2A}$R signaling in the preoptic area to sleep regulation.
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Disclosures

No conflicts of interest are declared by the authors.
Table 1. Effects of ICV administration of CGS-21680 on Fos-expression in GABAergic neurons of the MnPN and VLPO.

<table>
<thead>
<tr>
<th>Area</th>
<th>Treatment (n=7)</th>
<th># GAD-IR Neurons</th>
<th># Fos-IR Neurons</th>
<th># Dual GAD/Fos-IR Neurons</th>
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</thead>
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<td>MnPN Rostral</td>
<td>Vehicle</td>
<td>89.0±4.7</td>
<td>28.3±4.4</td>
<td>11.4±1.2</td>
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<td></td>
<td>8 nmol CGS</td>
<td>86.6±6.4</td>
<td>29.9±5.7</td>
<td>16.3±2.9</td>
</tr>
<tr>
<td></td>
<td>24 nmol CGS</td>
<td>85.1±3.2</td>
<td>30.4±2.7</td>
<td>19.7±1.5**</td>
</tr>
<tr>
<td></td>
<td>ANOVA</td>
<td>F(2,18) = 0.15</td>
<td>p = 0.85</td>
<td>F(2,18) = 0.057</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F(2,18) = 4.27</td>
</tr>
<tr>
<td>MnPN Caudal</td>
<td>Vehicle</td>
<td>74.6±1.7</td>
<td>23.3±4.78</td>
<td>6.0±0.99</td>
</tr>
<tr>
<td></td>
<td>8 nmol CGS</td>
<td>70.4±2.5</td>
<td>23.8±2.5</td>
<td>6.9±0.7</td>
</tr>
<tr>
<td></td>
<td>24 nmol CGS</td>
<td>67.3±4.5</td>
<td>24.9±3.7</td>
<td>14.1±1.9**#</td>
</tr>
<tr>
<td></td>
<td>ANOVA</td>
<td>F(2,18) = 1.32</td>
<td>p = 0.29</td>
<td>F(2,18) = 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F(2,18) = 11.16</td>
</tr>
<tr>
<td>VLPO Core</td>
<td>Vehicle</td>
<td>44.1±2.3</td>
<td>7.4±0.96</td>
<td>6.7±1.25</td>
</tr>
<tr>
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<td>8 nmol CGS</td>
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<td>8.6±1.37</td>
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</tr>
<tr>
<td></td>
<td>24 nmol CGS</td>
<td>35.7±2.9*</td>
<td>9.3±1.21</td>
<td>11.0±1.11*</td>
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<tr>
<td></td>
<td>ANOVA</td>
<td>F(2,18) = 3.70</td>
<td>p = 0.04</td>
<td>F(2,18) = 0.71</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>F(2,18) = 3.85</td>
</tr>
<tr>
<td>VLPO Extended</td>
<td>Vehicle</td>
<td>97.6±4.3</td>
<td>11.2±1.7</td>
<td>12.9±1.96</td>
</tr>
<tr>
<td></td>
<td>8 nmol CGS</td>
<td>90.2±5.4</td>
<td>14.1±0.66</td>
<td>17.3±2.3</td>
</tr>
<tr>
<td></td>
<td>24 nmol CGS</td>
<td>82.8±4.8</td>
<td>14.1±2.3</td>
<td>24.6±0.8**#</td>
</tr>
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<td></td>
<td>ANOVA</td>
<td>F(2,18) = 2.27</td>
<td>p = 0.13</td>
<td>F(2,18) = 1.18</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>F(2,18) = 0.51</td>
</tr>
</tbody>
</table>

All values are expressed as Mean ± SEM. *, significantly different to vehicle; #, to 8 nmol CGS-21680; *, #, p<0.05, **, ##, p<0.01 level of significance (Holm-Sidak test).
Table 2. Effects of SS-rBF administration of CGS-21680 on Fos expression in GABAergic neurons of the MnPN and VLPO.

<table>
<thead>
<tr>
<th>Area</th>
<th>Sleep-Wake Condition</th>
<th>Treatment (n=7)</th>
<th>#GAD-IR Neurons</th>
<th># Fos-IR Neurons</th>
<th># Dual GAD/Fos-IR Neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rostral MnPN</td>
<td>UD</td>
<td>Vehicle</td>
<td>92.6±3.5</td>
<td>17.4±3.2</td>
<td>6.7±1.97</td>
</tr>
<tr>
<td></td>
<td>8 nmol CGS</td>
<td></td>
<td>84.3±3.1</td>
<td>18.6±2.6</td>
<td>12.9±2.0</td>
</tr>
<tr>
<td></td>
<td>t-test</td>
<td></td>
<td>t_{(12)} = 1.75</td>
<td>t_{(12)} = -0.30</td>
<td>t_{(12)} = -2.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p = 0.10</td>
<td>p = 0.76</td>
<td>p &lt;0.05</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>Vehicle</td>
<td>94.8±2.3</td>
<td>20.2±1.98</td>
<td>7.1±1.12</td>
</tr>
<tr>
<td></td>
<td>8 nmol CGS</td>
<td></td>
<td>90.0±4.4</td>
<td>21.9±3.2</td>
<td>11.0±1.1</td>
</tr>
<tr>
<td></td>
<td>t-test</td>
<td></td>
<td>t_{(12)} = 0.96</td>
<td>t_{(12)} = -0.42</td>
<td>t_{(12)} = -2.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p = 0.35</td>
<td>p = 0.67</td>
<td>p &lt;0.05</td>
</tr>
<tr>
<td>Caudal MnPN</td>
<td>UD</td>
<td>Vehicle</td>
<td>73.5±0.8</td>
<td>12.4±2.7</td>
<td>3.7±0.5</td>
</tr>
<tr>
<td></td>
<td>8 nmol CGS</td>
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<td>69.1±2.9</td>
<td>11.8±1.9</td>
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</tr>
<tr>
<td></td>
<td>t-test</td>
<td></td>
<td>t_{(12)} = 1.40</td>
<td>t_{(12)} = 0.20</td>
<td>t_{(12)} = -2.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p = 0.18</td>
<td>p = 0.84</td>
<td>p &lt;0.05</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>Vehicle</td>
<td>69.1±2.9</td>
<td>11.8±1.9</td>
<td>5.4±0.96</td>
</tr>
<tr>
<td></td>
<td>8 nmol CGS</td>
<td></td>
<td>76.2±1.5</td>
<td>18.0±1.8</td>
<td>9.5±0.76</td>
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<tr>
<td></td>
<td>t-test</td>
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<td>t_{(12)} = 1.11</td>
<td>t_{(12)} = 0.95</td>
<td>t_{(12)} = -3.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p = 0.28</td>
<td>p = 0.35</td>
<td>p &lt;0.01</td>
</tr>
<tr>
<td>Core VLPO</td>
<td>UD</td>
<td>Vehicle</td>
<td>44.4±3.0</td>
<td>7.6±1.0</td>
<td>7.2±1.6</td>
</tr>
<tr>
<td></td>
<td>8 nmol CGS</td>
<td></td>
<td>41.9±3.2</td>
<td>9.1±1.6</td>
<td>11.5±1.0</td>
</tr>
<tr>
<td></td>
<td>t-test</td>
<td></td>
<td>t_{(12)} = 0.56</td>
<td>t_{(12)} = -0.80</td>
<td>t_{(12)} = -2.25</td>
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<tr>
<td></td>
<td></td>
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<td>p = 0.58</td>
<td>p = 0.43</td>
<td>p &lt;0.05</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>Vehicle</td>
<td>46.5±2.4</td>
<td>8.8±1.4</td>
<td>6.8±0.5</td>
</tr>
<tr>
<td></td>
<td>8 nmol CGS</td>
<td></td>
<td>43.0±2.8</td>
<td>10.9±2.3</td>
<td>10.1±0.87</td>
</tr>
<tr>
<td></td>
<td>t-test</td>
<td></td>
<td>t_{(12)} = 0.94</td>
<td>t_{(12)} = -0.79</td>
<td>t_{(12)} = -3.28</td>
</tr>
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<td></td>
<td>p = 0.36</td>
<td>p = 0.44</td>
<td>p &lt;0.01</td>
</tr>
<tr>
<td>Extended VLPO</td>
<td>UD</td>
<td>Vehicle</td>
<td>101.1±9.3</td>
<td>12.1±2.0</td>
<td>10.7±1.2</td>
</tr>
<tr>
<td></td>
<td>8 nmol CGS</td>
<td></td>
<td>86.3±4.2</td>
<td>15.9±1.6</td>
<td>24.0±1.4</td>
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<td></td>
<td>t-test</td>
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<td>t_{(12)} = -1.49</td>
<td>t = -7.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p = 0.17</td>
<td>p = 0.16</td>
<td>p &lt;0.01</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>Vehicle</td>
<td>101.2±4.7</td>
<td>12.9±1.9</td>
<td>10.3±1.8</td>
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<td>8 nmol CGS</td>
<td></td>
<td>86.0±5.56</td>
<td>16.6±2.36</td>
<td>19.5±1.5</td>
</tr>
<tr>
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<td>t-test</td>
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<td>t_{(12)} = 2.08</td>
<td>t_{(12)} = -1.20</td>
<td>t = -3.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p = 0.059</td>
<td>p = 0.25</td>
<td>p &lt;0.01</td>
</tr>
</tbody>
</table>

All values are expressed as Mean ± SEM (t-test). UD indicates rats that were permitted undisturbed sleep during 2 hours post injection. SD indicates rats subjected to 2 hours of sleep deprivation post injection.
Table 3. Effects of ICV infusion of ZM-241385 during sleep deprivation on Fos expression in GABAergic neurons of the MnPN and VLPO during 2 h of recovery sleep.

<table>
<thead>
<tr>
<th>Area</th>
<th>Treatment (n=6)</th>
<th>#GAD-IR Neurons</th>
<th># Fos- IR Neurons</th>
<th># Dual GAD/Fos-IR Neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rostral MnPN</td>
<td>Vehicle</td>
<td>85.7±1.8</td>
<td>27.5±2.3</td>
<td>14.9±1.2</td>
</tr>
<tr>
<td></td>
<td>5 nmol ZM</td>
<td>88.9±3.1</td>
<td>24.4±2.4</td>
<td>10.4±2.1*</td>
</tr>
<tr>
<td></td>
<td>25 nmol ZM</td>
<td>90.7±2.2</td>
<td>22.7±2.8</td>
<td>8.3±1.3**</td>
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<tr>
<td></td>
<td>ANOVA (Holm-Sidak test)</td>
<td>$F(2,15) = 1.05$</td>
<td>$F(2,15) = 0.97$</td>
<td>$F(2,15) = 7.79$</td>
</tr>
<tr>
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<td></td>
<td>$p = 0.37$</td>
<td>$p = 0.40$</td>
<td>$p &lt;0.01$</td>
</tr>
<tr>
<td>Caudal MnPN</td>
<td>Vehicle</td>
<td>70.1±3.3</td>
<td>17.5±2.3</td>
<td>8.5±0.8</td>
</tr>
<tr>
<td></td>
<td>5 nmol ZM</td>
<td>71.2±3.1</td>
<td>16.9±2.0</td>
<td>6.1±0.7*</td>
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<tr>
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<td>25 nmol ZM</td>
<td>75.2±2.8</td>
<td>14.1±1.9</td>
<td>5.2±0.2**</td>
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<td>ANOVA (Holm-Sidak test)</td>
<td>$F(2,15) = 0.90$</td>
<td>$F(2,15) = 0.71$</td>
<td>$F(2,15) = 7.00$</td>
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<tr>
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<td></td>
<td>$p = 0.42$</td>
<td>$p = 0.50$</td>
<td>$p &lt;0.01$</td>
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<tr>
<td>Core VLPO</td>
<td>Vehicle</td>
<td>38.9±2.7</td>
<td>7.1±1.2</td>
<td>9.5±1.2</td>
</tr>
<tr>
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<td>5 nmol ZM</td>
<td>40.5±1.9</td>
<td>7.1±1.2</td>
<td>6.6±0.7*</td>
</tr>
<tr>
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<td>25 nmol ZM</td>
<td>42.8±2.9</td>
<td>4.1±0.9</td>
<td>5.8±0.6**</td>
</tr>
<tr>
<td></td>
<td>ANOVA (Holm-Sidak test)</td>
<td>$F(2,15) = 0.70$</td>
<td>$F(2,15) = 2.35$</td>
<td>$F(2,15) = 4.86$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p = 0.51$</td>
<td>$p = 0.12$</td>
<td>$p &lt;0.05$</td>
</tr>
<tr>
<td>Extended VLPO</td>
<td>Vehicle</td>
<td>80.3±6.2</td>
<td>13.5±2.4</td>
<td>18.6±2.7**</td>
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<tr>
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<td>5 nmol ZM</td>
<td>87.1±2.7</td>
<td>13.4±2.4</td>
<td>10.9±1.9**</td>
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<tr>
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<td>25 nmol ZM</td>
<td>91.5±2.5</td>
<td>9.3±1.5</td>
<td>9.0±0.5**</td>
</tr>
<tr>
<td></td>
<td>ANOVA (Holm-Sidak test)</td>
<td>$F(2,15) = 1.90$</td>
<td>$F(2,15) = 1.26$</td>
<td>$F(2,15) = 7.19$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p = 0.18$</td>
<td>$p = 0.31$</td>
<td>$p &lt;0.01$</td>
</tr>
</tbody>
</table>

All values are expressed as Mean ± SEM. *, p<0.05, **, p<0.01 level of significance (Holm-Sidak test).
FIGURE LEGENDS

Figure-1. Effects of CGS-21680 on sleep-wakefulness.
Effects of microinjections of vehicle and CGS-21680 (8 nmol or 24 nmol) on % time spent in waking, nonREM sleep and REM sleep, when administered ICV (A) or into the SS-rBF (B). Figure-1A shows mean ± S.E.M data (n=7/group) for the 2 h post-injection recording period. CGS-21680 induced a dose dependent increase in % time spent in nonREM sleep (F(2,18) = 4.94; p = 0.019) and REM (F(2,18) = 8.61; p = 0.002) sleep whereas it resulted in a significant reduction in % time spent in waking (F(2,18) = 10.34; p = 0.001). Figure-1B shows mean ± S.E.M data (n=7/group) for the 2 h post-injection recording period. In SS-rBF, a lower dose of CGS-21680 (8 nmol) significantly increased % time spent in nonREM sleep (t = -4.88; p = <0.001) as well as REM sleep (t = -3.675; p = 0.003) and decreased % time spent in waking (t = 5.734; p = <0.001). * significantly different from vehicle, p<0.05; # significantly different from 8 nmol, p<0.05.

Figure-2. Effects of CGS-21680 on EEG delta power and nonREM and REM-latencies.
Effects of vehicle and CGS-21680 after ICV (8 nmol or 24 nmol) and SS-rBF (8 nmol) microinjection on nonREM delta power (A), nonREM sleep latency (B), and REM sleep latency (C). Both ICV and SS-rBF injections of CGS-21680 increased nonREM delta power (ICV: F(2,18) = 3.555; p = 0.05; SS-rBF: t = -2.39; p = 0.034) and decreased latencies for both nonREM sleep (ICV: F(2,18) = 7.14; p = 0.005; SS-rBF: t = 3.90; p = 0.002) and REM sleep (ICV: F(2,18) = 3.24; p = 0.063; SS-rBF: t = 2.82; p = 0.015). The nonREM sleep delta power is expressed as the %change from baseline waking values. * significantly different from vehicle, p <0.05.
Figure 3. Locations of the microinjection sites and counting boxes.

Photomicrographs of (A) a coronal section showing cannula tract into lateral ventricle (20X magnification), tissue stained for c-Fos and GAD and (B) a coronal section showing cannula tract into the SS-rBF (20X magnification), Nissle stained. (C) Photomicrograph (1000X magnification showing neuronal staining for Fos and GAD in MnPN. (D) Photomicrograph (1000X magnification showing neuronal staining for Fos and GAD in VLPO. Photomicrographs (40X magnification), showing the locations of counting boxes for the (E) rostral median preoptic nucleus, (F) caudal median preoptic nucleus and (G) ventrolateral preoptic area. AC, anterior commissure; CC, corpus callosum; LV, lateral ventricle; 3V, third ventricle; 1, core VLPO; 2 and 3 are extended VLPO.

Figure 4. Effects of CGS-21680 on Fos-IR in MnPN and VLPO GABAergic neurons.

Effects of CGS-21680 on the percentage of GAD+ neurons expressing Fos-IR (%GAD+ Neurons Double-Labeled) in the MnPN (A&B) and VLPO (C&D) after ICV or SS-rBF injections in undisturbed (UD) animals and after SS-rBF injections in animals that were sleep deprived (SD) for 2 h post drug or vehicle administration. In UD animals, both ICV and SS-rBF injections of CGS-21680 increased the percentage of GAD+ neurons expressing Fos-IR in all of the POA sub-regions examined; rostral MnPN (ICV: $F_{(2,18)} = 5.66$, $p = 0.012$; SS-rBF: $t = -2.35$, $p = 0.037$), caudal MnPN (ICV: $F_{(2,18)} = 11.75$, $p = <0.001$; SS-rBF: $t = -3.37$, $p = 0.006$), core VLPO (ICV: $F_{(2,18)} = 6.69$, $p = 0.007$; SS-rBF: $t = -2.76$, $p = 0.017$), and extended VLPO (ICV: $F_{(2,18)} = 17.63$, $p = <0.001$; SS-rBF: $t = -6.78$, $p = <0.001$). Like UD animals, CGS-21680 injection into the SS-rBF in SD animals also increased Fos-IR in GAD+ neurons in all of the POA-sub-regions examined (rostral MnPN: $t = -2.98$; $p = 0.011$; caudal MnPN: $t = -3.19$; $p =
0.008; core VLPO: t = -3.83; p = 0.002; extended VLPO: t = -5.40; p = <0.001). * significantly
different from vehicle, p<0.05;  # significantly different from 8 nmol, p <0.05.

**Figure 5. Sleep-wake profiles of animals during sleep deprivation.**

A. Average time (in minutes) spent awake and in nonREM sleep during 2h sleep deprivation
following SS-rBF administration of the A1R agonist, CGS21680, or vehicle. There were no
significant between group differences (time awake t=0.78, NS; time in nonREM sleep t=-0.72
NS). REM sleep was completely suppressed in all rats during sleep deprivation.  B. Average
time spent awake and in nonREM sleep during 3h sleep deprivation in groups of rats that
received ICV infusion of vehicle or A2AR antagonist, ZM241385 (5 or 25 nmol) or vehicle
during the final 2h of the sleep deprivation period. Although there was a significant overall
effect of drug/vehicle treatment on time spent in nonREM sleep during sleep deprivation
(F(2,15)=4.73, p<0.01), the maximum difference in mean time spent asleep across groups of 4.0
min would not be expected to have a significant functional impact on subsequent recovery sleep.

**Figure-6. Effects of ZM-241385 on sleep-wakefulness during the recovery period.**

A. Effects of ICV infusions of vehicle or ZM-241385 (5 nmol or 25 nmol) on % time spent in
waking (F(2,15) = 19.48; p = <0.001), nonREM sleep (F(2,15) = 7.99; p<0.01) and REM sleep
(F(2,15) = 18.56; p = <0.001). The data are shown as mean ± S.E.M (n=6/group) for the 2 h post-
infusion recording period when rats were permitted undisturbed recovery sleep. ZM-241385
induced a dose dependent reduction in % time spent in nonREM sleep and REM sleep whereas it
significantly increased % time spent in waking.  B. EEG delta power in nonREM sleep was
significantly decreased during the first hour of recovery sleep in ZM241385-treated rats
compared to vehicle controls (F(2,15) = 7.96, p<0.01)).  * significantly different from vehicle,
p<0.05;  # significantly different from 5 nmol, p <0.05.
Figure 7. Effects of ZM-241385 on Fos-IR in the POA GABAergic neurons.

Effects of ICV infusion of ZM-241385 (5 nmol or 25 nmol) vs. vehicle on the percentage of GAD+ neurons expressing Fos-IR in the POA sub-regions studied; rostral MnPN (F(2,15) = 8.95; p = 0.003), caudal MnPN (F(2,15) = 8.14; p = 0.004), core VLPO (F(2,15) = 7.14; p = 0.006), and extended VLPO (F(2,15) = 13.50; p = <0.001). ZM-241385 significantly decreased the percentage of GAD+ neurons expressing Fos-IR in all of the POA sub-regions examined. Significantly different from vehicle * p <0.05.
REFERENCES


45. **Satoh S, Matsumura H, Suzuki T Hayaishi O.** Promotion of sleep mediated by the A₂A-adenosine receptor and possible involvement of this receptor in the sleep induced by prostaglandin D2 in rats. *Proc Natl Acad Sci USA* 93: 5980–5984, 1996.


A. ICV injection of CGS-21680

- Vehicle (n=7)
- 8 nmol CGS-21680 (n=7)
- 24 nmol CGS-21680 (n=7)

B. SS-rBF injection of CGS-21680

- Percent time (Mean ± SEM)
A. Delta Power

B. nonREM Latency

C. REM Latency

**Legend:**
- Vehicle (n=7)
- 8 nmol CGS-21680 (n=7)
- 24 nmol CGS-21680 (n=7)
A. Rostral MnPN

B. Caudal MnPN

C. VLPO Core

D. VLPO Extended

% GAD+ Neurons Double-Labeled (Mean ± SEM)

Vehicle (n=7)
8 nmol CGS-21680 (n=7)
24 nmol CGS-21680 (n=7)
A. Sleep Deprivation and CGS-21680

B. Sleep Deprivation and ZM-241385
A. Sleep-Wake during Recovery Period

- Waking
- nonREM
- REM

B. Delta Power in Recovery Sleep

- First Hour
- Second Hour
A. Rostral MnPN

B. Caudal MnPN

C. VLPO Core

D. VLPO Extended

% GAD+ Neurons Double-Labeled (Mean ± SEM)

Vehicle (n=6)
5 nmol ZM-241385 (n=6)
25 nmol ZM-241385 (n=6)