Behavioral/Systems/Cognitive

Arousal Effect of Caffeine Depends on Adenosine A_{2A} Receptors in the Shell of the Nucleus Accumbens

Michael Lazarus,[1]* Hai-Ying Shen,[2,3]* Yoan Cherasse,[1]* Wei-Min Qu,[4] Zhi-Li Huang,[1,4] Caroline E. Bass,[5] Raphaelle Winsky-Sommerer,[6] Kazue Semba,[7] Bertil B. Fredholm,[8] Detlev Boison,[3] Osamu Hayaishi,[1] Yoshihiro Urade,[1] and Jiang-Fan Chen[2]

[1]Department of Molecular Behavioral Biology, Osaka Bioscience Institute, Suita, Osaka 565-0874, Japan, [2]Department of Neurology, Boston University School of Medicine, Boston, Massachusetts 02118, [3]R. S. Dow Neurobiology Laboratories, Legacy Research Institute, Portland, Oregon 97232, [4]Department of Pharmacology, Fudan University Shanghai Medical College, Shanghai 200032, China, [5]Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157, [6]Faculty of Health and Medical Sciences, University of Surrey, Guildford, Surrey GU2 7XH, United Kingdom, [7]Department of Anatomy and Neurobiology, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia B3H 1X5, Canada, and [8]Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm SE-171 77, Sweden

Caffeine, the most widely used psychoactive compound, is an adenosine receptor antagonist. It promotes wakefulness by blocking adenosine A_{2A} receptors (A_{2A}Rs) in the brain, but the specific neurons on which caffeine acts to produce arousal have not been identified. Using selective gene deletion strategies based on the Cre/loxP technology in mice and focal RNA interference to silence the expression of A_{2A}Rs in rats by local infection with adeno-associated virus carrying short-hairpin RNA, we report that the A_{2A}Rs in the shell region of the nucleus accumbens (NAc) are responsible for the effect of caffeine on wakefulness. Caffeine-induced arousal was not affected in rats when A_{2A}Rs were focally removed from the NAc core or other A_{2A}R-positive areas of the basal ganglia. Our observations suggest that caffeine promotes arousal by activating pathways that traditionally have been associated with motivational and motor responses in the brain.

Introduction

Caffeine is the most consumed psychoactive compound in the world. It is readily available through dietary products, such as coffee, tea, soft drinks, and chocolate treats, but is also added to nonprescription medications, such as pain-relievers and cold remedies. Regardless of the source, the worldwide average caffeine consumption has been estimated to be just under 80 mg/d, although the levels of intake in countries such as Sweden and Finland are in the range of 400 mg of caffeine per day (Fredholm et al., 1999).

Received Dec. 23, 2010; revised April 20, 2011; accepted April 20, 2011.

Author contributions: M.L., Z.-L.H., B.B.F., D.B., O.H., Y.U., and J.-F.C. designed research; M.L., H.-Y.S., and Y.C. performed research; C.E.B. and D.B. contributed unpublished reagents/analytic tools; M.L., H.-Y.S., Y.C., W.-M.Q., and R.W.-S. analyzed data; M.L., K.S., B.B.F., Y.U., and J.-F.C. wrote the paper.

*M.L., H.-Y.S., and Y.C. contributed equally to this work.

The authors declare no competing financial interests.

This work was supported by Japan Society for the Promotion of Science Grant 21300133, a grant from the Ministry of Education, Culture, Sports, Science, and Technology, a Health and Labor Science Research grant from the Ministry of Health, Labour and Welfare, a Research Grant for promoting Technological Seeds from the Japan Science and Technology Agency, the Takeda Science Foundation, the Sankyo Foundation, the Program of Basic and Applied Researches for Innovations in Bio-Oriented Industry of Japan, Ono Pharmaceutical Co. Ltd., Takeda Pharmaceutical Co. LTD., Osaka City, National Natural Science Foundation of China Grants 30625021 and 30821002, Ministry of Science and Technology Grants 2009CB5220004, 2011CB711000, and 2009ZX09303-006, Shanghai Leading Academic Discipline Project B119, NIH Grants NS048995, DA019362, DA024763, and MH083973, and Department of Defense Grant W81XWH-07-0012. We thank N. Matsumoto, E. Ko-Mitamura, M. Masaki, M. Nakamura, and X.-H. Xu for their technical assistance.

Correspondence should be addressed to either of the following: Yoshihiro Urade, Department of Molecular Behavioral Biology, Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565-0874, Japan, E-mail: uradey@obi.or.jp; or Jiang-Fan Chen, Department of Neurology, Boston University School of Medicine, 715 Albany Street, C329, Boston, MA 02118, E-mail: chenjf@bu.edu.

DOI:10.1523/JNEUROSCI.6730-10.2011

 0270-6474/11/3110067-09$15.00/0

Caffeine is widely used to promote wakefulness and to counteract fatigue. Caffeine binds with very similar affinity to adenosine A_1 (A_1Rs) and A_{2A} (A_{2A}Rs) receptors, and, at doses commonly consumed by humans, adenosine actions at both receptors are antagonized. Adenosine is an inhibitory neuromodulator involved in sleep–wake regulation (Porkka-Heiskanen et al., 1997; Huang et al., 2011). Using global genetic knock-outs of A_1Rs and A_{2A}Rs, in which the receptor is deleted from the entire animal, we demonstrated previously that the A_{2A}R, but not the A_1R, mediates the arousal effect of caffeine (Huang et al., 2005). However, the neurons with A_{2A}Rs on which caffeine acts to produce wakefulness have not yet been identified.

A_{2A}Rs are densely expressed on striatopallidal neurons in the indirect pathway of the basal ganglia (BG), in which dopamine D_2 receptors (D_2Rs) are coexpressed with the A_{2A}Rs and contribute to the control of locomotor activity, motivation, and addiction, all activities that require wakefulness (Rosin et al., 1998; Svenningsson et al., 1999a). The striatopallidal neurons also facilitate movement by operating in parallel with dopamine D_1 receptor (D_1R)-bearing striatonigral neurons in the direct pathway of the BG. Abilities to maintain arousal are compromised under low dopamine conditions, such as Parkinson's disease (Arnulf et al., 2002; Qu et al., 2010), but the extent to which A_{2A}Rs in the BG contribute to the regulation of wakefulness is not known and the role of A_{2A}Rs in other brain regions is unclear.

In the present study, we used site-specific gene deletion strategies based on the Cre/loxP technology in mice and also silenced focally the expression of A_{2A}Rs in rats by using stereotaxic microinjections of adeno-associated virus (AAV) carrying short-

hairpin RNA (shRNA). We found that the A_{2A}Rs in the shell region of the nucleus accumbens (NAc) are responsible for the effect of caffeine on wakefulness.

Materials and Methods

Genetic mouse models. Animals were handled according to the NIH *Guide for the Care and Use of Laboratory Animals* and in accordance with the protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the Boston University School of Medicine, the Legacy Research Institute IACUC, and the Animal Research Committee at the Osaka Bioscience Institute. All mice (weighing 24–28 g, 11–13 weeks old) and male Sprague Dawley rats (weighing 150–180 g, 6 weeks old; Shizuoka Laboratory Animal Center) used in the present study were housed at a constant temperature (24 ± 0.5°C) with a relative humidity of 60 ± 2% on an automatically controlled 12 h light/dark cycle (light on at 7:00 A.M.). Three genetic mouse lines on a C57BL/6 background were used in the present study: (1) global A_{2A}R knock-out mice (A_{2A}R KO) (Chen et al., 1999), (2) basal ganglia–A_{2A}R knock-out mice (BG–A_{2A}R KO), exclusively lacking BG A_{2A}Rs (Shen et al., 2008), and (3) a mouse line with a loxP-site-inserted A_{2A}R gene that is amenable to conditional disruption by the injection of Cre recombinase-expressing AAV.

Vigilance state assessment using electroencephalogram/electromyogram/locomotor activity recordings. Assessment of vigilance states was performed on adult male conditional A_{2A}R KO mice and their respective WT littermates ($n = 4$–5, per genotype and drug dose). Under anesthesia using 1.5% isoflurane in N_2O/O_2 (2:1), mice were implanted with electroencephalogram (EEG) and electromyogram (EMG) electrodes for polysomnographic recordings. To monitor EEG signals, two stainless steel EEG recording screws (Plastics One) were implanted epidurally over the frontal cortical area (1 mm anterior to bregma, 1.5 mm lateral to the midline) and over the parietal area (2 mm posterior to bregma, 3 mm lateral to midline) of the right hemisphere. EMG activity was monitored by stainless steel, Teflon-coated wires (0.2 mm in diameter; Plastics One) bilaterally placed into both trapezius muscles. Finally, the electrode assembly was anchored and fixed to the skull with Super6-Bond (Sun Medical Co.) and dental cement. After a 10 d recovery period, the mice were placed in experimental cages for a 4 d habituation/acclimatization period with connection of counterbalanced recording leads.

All mice that were subjected to EEG recordings received vehicle and drug treatment on 2 consecutive days. On day 1, mice were treated with vehicle (saline, intraperitoneally) at 9:00 A.M., and the 24 h recordings performed on day 1 were used as baseline data. On day 2, mice were treated with caffeine (intraperitoneally, in a volume of 10 ml/kg body weight), and EEG/EMG signals were recorded for 24 h. The EEG/EMG signals were amplified and filtered (EEG, 0.5–30 Hz; EMG, 20–200 Hz), then digitized at a sampling rate of 128 Hz, and recorded using SLEEPSIGN software (Kohtoh et al., 2008). In addition, locomotor activity (LMA) was recorded with an infrared photocell sensor (Biotex). The vigilance states were scored offline by 10 s epochs into three stages, including waking, rapid-eye movement (REM) sleep, and non-REM (NREM) sleep, according to standard criteria (Mizoguchi et al., 2001). As a final step, defined vigilance stages were examined visually and corrected when necessary.

Assessment of activity and inactivity. Assessment of LMA and inactivity was performed in adult male A_{2A}R KO mice, BG–A_{2A}R KO mice, and their respective WT littermates ($n = 8$, per genotype and drug dose). At 9:00 A.M., all animals received an intraperitoneal injection of either vehicle or caffeine at one of the following doses: 2, 10, or 30 mg/kg. Inactivity/activity was used to assess sleep and wakefulness based on a previous report (Pack et al., 2007) after caffeine treatment. LMA was recorded in standard polypropylene cages with seven infrared photocell beams (San Diego Instrument) in 50 s bins during the two experimental days. Inactivity and activity were defined based on LMA as follows: time spent in inactivity (no beam break/50 s) was used as an approximation of sleep; each period of activity was subdivided into high (at least two beam breaks/50 s, assessing ambulation) versus low (one beam break/50 s, assessing rest activity with fine movements) LMA. These three levels of activity were used to analyze the motor stimulant effects of caffeine as opposed to the arousal effects of caffeine, which were assessed using polygraphic recordings as described above.

Table 1. Coordinates for bilateral injections of AAV–Cre or AAV–mCherry in rats and mice according to Paxinos and Watson (2001, 2007)

	Coordinates (mm)		
Area	Anterior to bregma	Lateral to midline	Below dural surface
Rat			
OLT	1.8	1.5	8
	1.3	1.3	8
	0.8	1.3	8
NAc	1.8	1.5	7
	1.3	1.2	7
	0.8	1	6.8
CPu	1.8	2.0	4.5
	1.3	2.5	4
	0.8	2.5	4.5
GP	−0.4	2.2	7
	−0.9	2.8	6
Mouse			
NAc	1	0.75	4.2
	1.4	0.8	3.9
SI/HDB	0	1.4	4.6

Each animal received two or three bilateral injections using different sets of coordinates. A_{2A}R-positive areas in the BG: CPu, NAc, OLT, and GP. Arousal-related areas in the basal forebrain: SI and HDB.

Generation of AAV vectors. For the generation of the AAV–shRNA–mCherry vector plasmids, a U6–shA_{2A}R cassette was amplified by PCR from the psiSTRIKE–hMGFP plasmid (Promega) containing the rat A_{2A} receptor shRNA (target sequence 1913) (Chen et al., 2004) or a control shRNA (shCTRL) sequence (GTCAGGCTATCGCGTATCG) and was inserted into the MluI site of the pAAV–hrGFP plasmid (Stratagene). Subsequently, the hrGFP gene was replaced by the gene encoding mCherry. For the generation of the AAV–Cre plasmid, the hrGFP in the pAAV–hrGFP plasmid was replaced by the Cre recombinase coding sequence derived by PCR from the pBS185 plasmid (Sauer and Henderson, 1990). The AAVs of serotype rh10 were generated by tripartite transfection (AAV–rep2/caprh10 expression plasmid, adenovirus helper plasmid, and AAV–vector plasmid) into HEK293A cells and purified by iodixanol density step-gradient centrifugation, as described previously (Zolotukhin et al., 1999). The virus distributed in the 40% density step was concentrated and dialyzed against PBS with a centrifugal concentrator (molecular weight cutoff, 100 kDa; Sartorius) and then titered by quantitative PCR.

Stereotaxic AAV injection and placement of EEG/EMG electrodes. Surgeries for AAV injections were conducted under pentobarbital anesthesia (50 mg/kg, i.p.). Using aseptic techniques, 6-week-old rats were injected stereotaxically into the NAc and other BG nuclei with recombinant AAV–shA_{2A}R or AAV–shCTRL (250 nl/injection, 6×10^{12} particles/ml) with a glass micropipette and an air pressure injector system (Chamberlin et al., 1998). Also, 8- to 10-week-old conditional A_{2A}R KO mice were injected with AAV–Cre or AAV–mCherry. Table 1 summarizes coordinates used for bilateral injections into selected BG nuclei of rats or conditional A_{2A}R KO mice, according to the atlases of Paxinos and Watson (2001, 2007). At 3 weeks after the AAV injection, rats underwent surgery for implantation of electrodes for EEG and EMG recordings as described previously (Matsumura et al., 1994), whereas EEG/EMG electrodes in the conditional KO mice were implanted as described above. Postoperatively, animals were housed individually for 8–10 d. The caffeine treatment was performed as described above; intraperitoneal injections were made at 9:00 A.M. (mice) or 10:00 A.M. (rats). In addition, at least 1 week after the caffeine treatment, each animal received an injection of vehicle or modafinil on a 2 d schedule as described above. Modafinil (Sigma-Aldrich) was dissolved in saline containing 10% DMSO and 2% (w/v) cremophor immediately before use and administered intraperitoneally at 9:00 A.M. on the experimental day at a dose of 45 mg/kg.

Immunohistochemistry. After all of the above procedures, animals were deeply anesthetized with an overdose of chloral hydrate (500 mg/kg, i.p.) and perfused through the left ventricle of the heart with saline, followed by neutral buffered 10% Formalin. Brains were removed and placed in 10% sucrose in PBS overnight at 4°C to reduce freezing artifacts. The

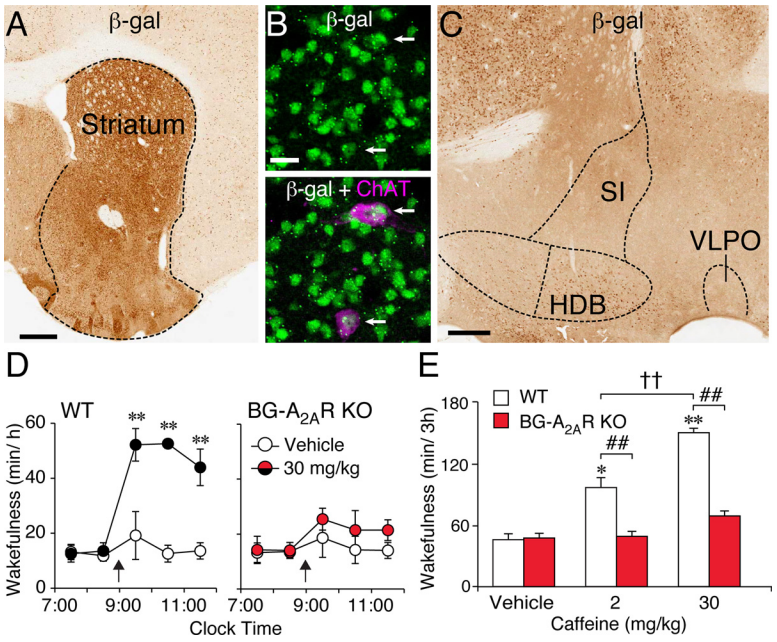

Figure 1. Arousal effect of caffeine was abolished in BG–A_{2A}R KO mice. ***A–C***, Typical sections from the Rosa26–Dlx5/6–Cre reporter mouse were stained with mouse polyclonal antibodies against β-gal to visualize Cre-expressing neurons indirectly. ***A***, Robust expression of β-gal is seen in the striatum of the reporter mouse. ***B***, At a single-cell level, double immunofluorescence for β-gal (green) and ChAT (magenta) on an adjacent section to ***A*** shows that cholinergic interneurons in the striatum also express β-gal. The arrows in the top and bottom of ***B*** indicate neurons with dual immunolabeling for β-gal and ChAT. ***C***, In the classical arousal/sleep-related cell groups of the basal forebrain and anterior hypothalamus, i.e., the nucleus of the HDB, SI, or VLPO, only moderate immunoreactivity for β-gal is detected in the HDB. β-gal immunolabeling is absent in neurons of the SI and VLPO. Scale bars: ***A***, 500 μm; ***B***, 20 μm; ***C***, 250 μm. ***D***, ***E***, The BG–A_{2A}R KO mice and WT littermates were treated with vehicle or caffeine (2 or 30 mg/kg, i.p.). Time course (***D***) and total time (***E***) of wakefulness during the first 3 h after caffeine injection (arrows) were assessed with EEG/EMG recordings. Data are presented as the mean ± SEM ($n = 4–5$). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with vehicle treatment within corresponding genotype. $^{\#\#}p < 0.01$ compared with corresponding WT littermates. $^{\dagger\dagger}p < 0.01$ compared between caffeine doses.

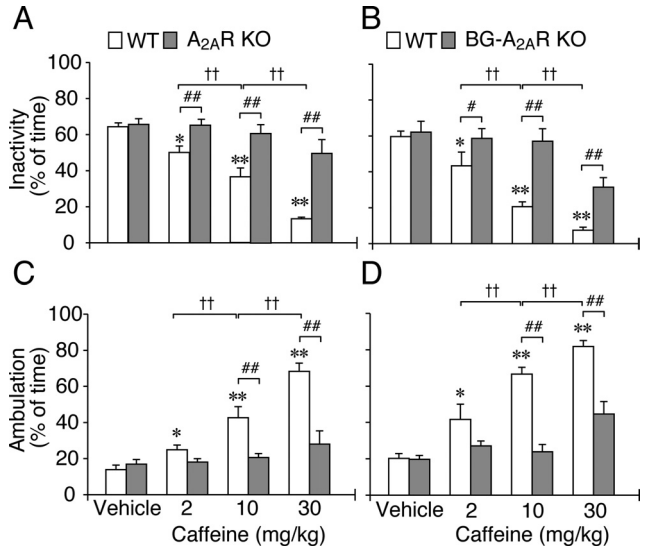

Figure 2. Locomotion in A_{2A}R KO and BG–A_{2A}R KO mice after caffeine treatment. Rest–activity assessment was performed based on the amount of LMA in 50 s bins for 3 h after caffeine (2, 10, or 30 mg/kg) treatment. Time spent in inactivity (***A***, ***B***) or high LMA (ambulation; ***C***, ***D***) is presented as percentage of total time (3 h). Data are presented as mean ± SEM ($n = 8$, per genotype and drug dose rest–activity assessment). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with vehicle treatment within corresponding genotypes. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with corresponding WT littermates. $^{\dagger\dagger}p < 0.01$ compared between caffeine doses.

brains were then frozen on dry ice and sectioned at 30 μm (mice) or 40 μm (rats) on a freezing microtome. Immunohistochemistry was performed on free-floating sections as described previously (Estabrooke et al., 2001). In brief, sections were rinsed in PBS, incubated in 3% hydrogen peroxide in PBS for 30 min at room temperature, and then sequentially at room temperature in 3% normal donkey serum and 0.25% Triton X-100 in PBS (PBT) for 1 h and primary antibody diluted in PBT with 0.02% sodium azide overnight. Primary antibodies included rabbit anti-Cre (1:10,000; EMD Biosciences), rabbit anti-mCherry (1:10,000; Clontech), mouse anti-A_{2A}R (1:2000; Millipore), goat anti-A_{2A}R (1:1000; Santa Cruz Biotechnology), chicken anti-β-galactosidase (β-gal) (1:4000; Abcam), and mouse anti-neuronal-specific nuclear protein (NeuN) (1:2000; Millipore). After incubation with the primary antisera overnight, sections were rinsed and incubated for 2 h in biotinylated anti-rabbit, anti-goat, anti-chicken, or anti-mouse secondary antiserum (Jackson ImmunoResearch) at a dilution of 1:1000. Immunoreactions for D_2R with a rabbit anti-D_2R antibody (1:500; Millipore) were conducted over two nights at 4°C and one night at room temperature. All tissue sections were then treated with avidin–biotin complex (1:1000; Vectastain ABC Elite kit; Vector Laboratories) for 1 h, and immunoreactive cells were visualized by reaction with 3,3′-diaminobenzidine and 0.1% hydrogen peroxide. Tissue sections mounted on glass slides were scanned with Aperio ScanScope, and digital photomicrographs were analyzed with Aperio ImageScope software version 10. The region of A_{2A}R knockdown by shA_{2A}R was identified by the expression of mCherry and confirmed by the absence of A_{2A}R immunoreactivity, whereupon the area of loss of A_{2A}Rs in the NAc was quantified on photomicrographs of sections containing the rostral, central, and caudal NAc. Digital photomicrographs were adjusted for optimal display for the output levels of the contained color values and then imported into NIH ImageJ 1.42 software for area measurements of mCherry expression in the NAc versus total nucleus extension. Double immunofluorescence staining for β-gal and choline acetyltransferase (ChAT) was also performed. Sections were incubated overnight at room temperature in a mixture of anti-β-gal and goat anti-ChAT (1:200; Millipore) primary antibodies in PBT with donkey normal serum. On the next day, sections were incubated for 2 h in a mixture of biotinylated anti-chicken and Alexa Fluor-594-conjugated anti-goat secondary antibodies (Invitrogen) at a dilution of 1:500. After several washes, sections were incubated for 1 h in Alexa Fluor-488-conjugated streptavidin (Invitrogen) at a dilution of 1:500. Fluorescence microscopy with tissue sections mounted on glass slides was performed with a Carl Zeiss laser scanning confocal microscope.

Statistical analysis. The data were presented as the mean ± SEM. Statistical comparisons between two groups were performed by using the unpaired Student's *t* test. Comparisons among combined multiple parameters (genotype, experimental conditions, and more than two groups) were performed by one-way ANOVA, followed by Bonferroni's *post hoc* comparisons.

Results

Deletion of A_{2A}Rs in the basal ganglia of mice abolishes the arousal effect of caffeine

We first examined sleep–wake profiles in our previously developed BG–A_{2A}R KO mice based on the Cre/loxP technology (Shen

et al., 2008). The existence of A_{2A}Rs in arousal-related cell groups surrounding the striatum, such as the nucleus of the horizontal limb of the diagonal band of Broca (HDB), the substantia inominata (SI), or the ventrolateral preoptic area (VLPO), remains elusive (Svenningsson et al., 1997; Rosin et al., 1998). However, to assess Cre-dependent knock-out of A_{2A}Rs in these adjacent areas, we cross-bred the Dlx5/6–Cre transgenic mouse, which was used to create the BG–A_{2A}R KO mouse, with a Rosa26 reporter line (Soriano, 1999) expressing β-gal only in the presence of Cre recombinase (Rosa26–Dlx5/6–Cre). The limit of this method may, however, hinge on the barely existing expression of A_{2A}Rs in various regions of the brain. As shown in Figure 1*A*, the striatum, including the olfactory tubercle (OLT), caudate–putamen (CPu), and NAc, showed robust β-gal staining. The vast majority of neurons in the striatum are GABAergic medium-sized spiny output neurons, but double immunofluorescence staining for β-gal and ChAT (Fig. 1*B*) revealed that the A_{2A}R knock-out in the striatum occurred also in the cholinergic interneurons. Outside of the striatum, sparse β-gal expression was detected in the HDB, whereas β-gal staining was absent in the SI and the VLPO (Fig. 1*C*). In addition, only scattered cells with β-gal immunostaining were observed in the septum, cerebral cortex, thalamic nuclei, and hippocampus (Fig. 1*A* or data not shown). Brain sections from Cre-negative mice of the Rosa26–Dlx5/6–Cre line did not show any β-gal staining (data not shown).

We then recorded EEG and EMG for 2 consecutive days in the BG–A_{2A}R KO mice and their wild-type littermates (Fig. 1*D*,*E*). On day 1, the mice were treated with vehicle (intraperitoneally) at 9:00 A.M. in the early phase of the light (inactive) period, and the recordings made on that day served as the baseline data. The animals were then treated with caffeine 24 h later (either 2 or 30 mg/kg, i.p.). The vigilance states were classified offline into three stages: waking, REM sleep, and NREM sleep. Caffeine dose-dependently increased wakefulness in control WT mice 2-fold and 3.2-fold after the 2 and 30 mg/kg doses, respectively (Fig. 1*D*,*E*). This arousal effect of caffeine was almost completely eliminated in the BG–A_{2A}R KO mice.

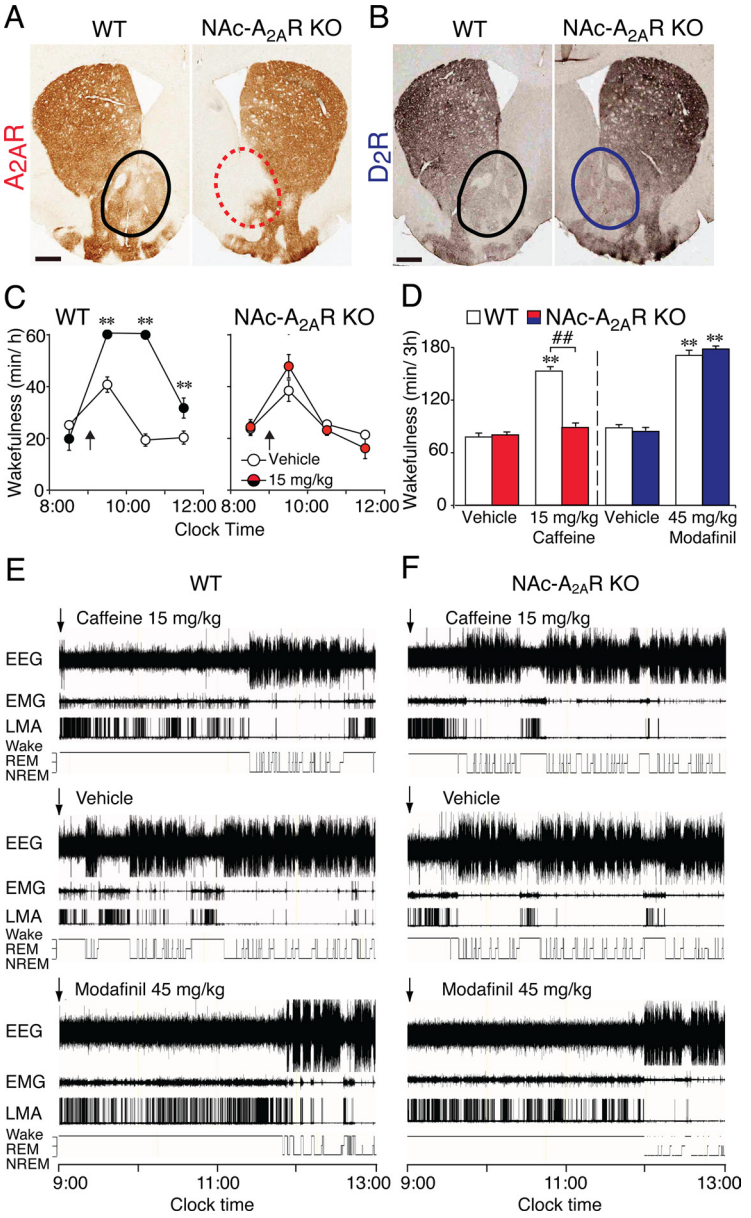

Figure 3. Arousal effect of caffeine was abolished in NAc–A_{2A}R KO mice. ***A***, ***B***, Typical sections of conditional A_{2A}R KO mice after injection with mCherry-expressing AAVs (WT; left photomicrograph) and AAV carrying Cre recombinase (NAc–A_{2A}R KO; right photomicrograph) were stained with a goat polyclonal antibody against A_{2A}R (Santa Cruz Biotechnology) to visualize the presence (black circle) or loss (red dashed circle) of A_{2A}Rs in the NAc. Immunostaining with a rabbit polyclonal antibody against D_2R (Millipore) confirms the integrity of the NAc (black and blue circles) in the WT and NAc–A_{2A}R KO mice (***B***). ***C***, ***D***, The NAc–A_{2A}R KO and WT mice were treated with caffeine (15 mg/kg, i.p.) or modafinil (45 mg/kg, i.p.). The time course (***C***) and total time (***D***) of wakefulness for the first 3 h after caffeine treatment were assessed from EEG/EMG recordings, as was modafinil-induced wakefulness for 3 h (***D***, right). Arrows in ***C*** indicate the time of caffeine injection. ***E***, ***F***, Typical examples of EEG, EMG, LMA, and hypnograms after administration of caffeine (15 mg/kg, i.p., top panels), vehicle for caffeine administration (middle panels), or modafinil (45 mg/kg, i.p., bottom panels) in a WT (***E***) and NAc–A_{2A}R KO (***F***) mouse. Arrows in ***E*** and ***F*** indicate the time of injection. Data are presented as the mean ± SEM ($n = 4–5$). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with vehicle treatment within corresponding AAV injection. $^{\#\#}p < 0.01$ compared with AAV treatment. Scale bars: ***A***, ***B***, 500 μm.

Deletion of A_{2A}Rs in the basal ganglia of mice abolishes caffeine-induced locomotor activity

We measured activity in global A_{2A}R KO (Chen et al., 1999) and BG–A_{2A}R KO mice to determine whether caffeine induces in these mice the motor pattern that is typical for an animal during caffeine-induced wakefulness. We injected intraperitoneally male A_{2A}R KO and BG–A_{2A}R KO mice with vehicle (saline) or caffeine in a physiologically relevant range of 2–30 mg/kg at 9:00 A.M. and monitored the behavior of the mice in a field of infrared photocell beams to assess inactivity versus low and high levels of activity. Caffeine dose dependently decreased time spent in inactivity in the control WT mice but not in A_{2A}R KO and BG–A_{2A}R

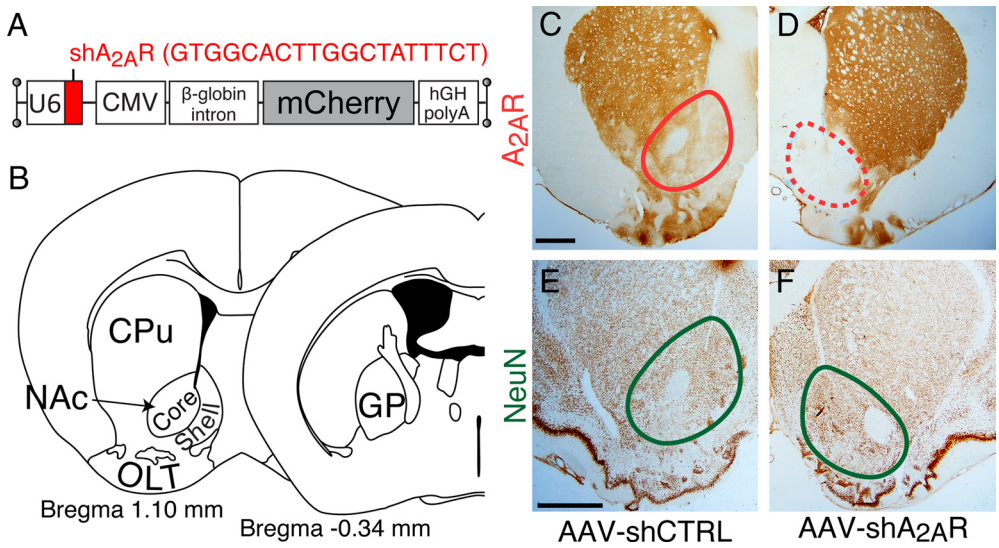

Figure 4. Site-specific deletion of A_{2A}Rs in rats by focal RNA interference. ***A***, Generation of AAV vectors that contained $shA_{2A}R$ and the red fluorescent protein mCherry as a reporter gene. ***B***, AAV–$shA_{2A}R$ vectors were stereotaxically injected into the $A_{2A}R$-positive core and shell of the NAc, as well as into the OLT, CPu, and GP. ***C***, ***D***, Typical sections from rats injected bilaterally with AAV carrying shCTRL or $shA_{2A}R$ into the NAc were stained with mouse monoclonal antibody against $A_{2A}R$. Immunoreactivity for $A_{2A}R$ is depleted selectively in the NAc of the AAV–$shA_{2A}R$-treated rats (***D***, dashed red circle), but it is unaffected in the AAV–shCTRL-treated rats (***C***, red circle). ***E***, ***F***, NeuN staining confirms the integrity of NAc neurons (green circles) in AAV–shCTRL-injected (***E***) and AAV-$shA_{2A}R$-injected (***F***) rats. Scale bars: ***C***, ***E***, 500 μm (also apply to ***D***, ***F***, respectively).

KO mice (Fig. 2*A*,*B*). Interestingly, although the time spent in low levels of locomotor activity (fine movement or quiet wakefulness) was not altered by caffeine in the $A_{2A}R$ KO and BG–$A_{2A}R$ KO mice (data not shown), the waking period characterized by high LMA (ambulation) was dose dependently increased by caffeine only in the control WT mice of both $A_{2A}R$ KO genotypes (global vs BG) (Fig. 2*C*,*D*). Thus, caffeine acts at A_{2A}Rs in the BG to promote wakefulness and associated locomotion.

Selective deletion of A_{2A}Rs in the NAc of mice eliminates the arousal effect of caffeine

The $A_{2A}R$ agonist CGS21680 (2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine), which induces sleep, also produces c-Fos expression in the NAc (Scammell et al., 2001). We therefore tested whether the arousal effect of caffeine depended on A_{2A}Rs in the NAc by using a mouse strain with a loxP-modified $A_{2A}R$ gene conditionally deletable by Cre recombinase. An AAV vector that contained the gene for Cre recombinase under the control of the cytomegalovirus (CMV) promoter was stereotaxically injected bilaterally into the NAc of loxP-modified $A_{2A}R$ mice to generate NAc–$A_{2A}R$ KO. At 3 weeks after the injection of AAV–Cre, immunohistochemistry for $A_{2A}R$ confirmed the loss of A_{2A}Rs in the NAc (Fig. 3*A*, right photomicrograph), whereas $A_{2A}R$ expression was unchanged in the control group of loxP-modified WT mice injected with the red fluorescent protein mCherry-expressing AAV (Fig. 3*A*, left photomicrograph). Microinjections of AAV vectors do not induce inflammation at the injection site, and tissue injury is minimal, as after the injection of saline (Lazarus et al., 2007). A_{2A}Rs are known to be coexpressed with D_2Rs on neurons of the NAc (Svenningsson et al., 1997; Durieux et al., 2009), and the unaltered D_2R staining confirmed the integrity of the NAc of AAV–mCherry- and AAV–Cre-injected mice except for the absence of the A_{2A}Rs (Fig. 3*A*,*B*).

Next, we injected both mouse groups with caffeine (15 mg/kg, i.p.) and recorded their EEG and EMG (Fig. 3*C*,*D*). Typically, the effect of caffeine on wakefulness was strongly attenuated in the NAc–$A_{2A}R$ KO mice generated by the AAV–Cre injection compared with the control group injected with AAV–mCherry (Fig. 3*C*). The time spent in waking in the control mice was increased twofold during a 3 h period after the 15 mg/kg dose of caffeine but was indistinguishable from the vehicle injection in mice with a deletion of A_{2A}Rs in the NAc (Fig. 3*D*, left). In addition, modafinils (45 mg/kg, i.p.), a wakefulness-inducing compound that primarily requires D_2R (Qu et al., 2008), induced strong arousal in the NAc–$A_{2A}R$ KO and control mice, causing almost complete insomnia during a 3 h period after injection (Fig. 3*D*, right). Typical examples of EEG, EMG, LMA, and hypnograms are shown in Figure 3, *E* and *F*, after the administration of caffeine (15 mg/kg, i.p.; top panels) or modafinil (45 mg/kg, i.p.; bottom panels) in a WT and NAc–$A_{2A}R$ KO mouse. The vehicle control is only shown for the caffeine administration (middle panels), because the vehicle response in the modafinil and caffeine experiment was similar. Caffeine increased wakefulness in the WT mouse but not in the mouse with a deletion of A_{2A}Rs in the NAc (Fig. 3*F*, top panel), whereas modafinil induced long-lasting suppression of sleep in both the NAc–$A_{2A}R$ KO and control mice. Because the possibility of a knock-out of A_{2A}Rs in the HDB of the BG–$A_{2A}R$ KO mice cannot be entirely excluded (Fig. 1*C*), we also injected AAV–Cre bilaterally into the basal forebrain (BF) region, including the HDB and SI, of loxP-modified $A_{2A}R$ mice and found that caffeine (15 mg/kg, i.p.) induced wakefulness (155 ± 7 min/3 h, $n = 4$) at similar levels as in WT mice (153 ± 3 min/3 h) (Fig. 1*D*). These experiments indicate that A_{2A}Rs in the NAc were specifically required for the caffeine-induced arousal and that dopamine D_2R functions of these neurons were not affected by the deletion of the A_{2A}Rs in the same neurons.

Site-specific knockdown of A_{2A}Rs in the NAc of rats blocks caffeine-induced arousal

We next aimed to validate our findings by using a knockdown of A_{2A}Rs and to define the extent to which the $A_{2A}R$-positive neurons in the core and shell regions of the NAc were required for the arousal effect of caffeine. Because rats are more suitable for ana-

tomical work than mice, we used rats to dissect the contribution of A_{2A}R-positive neurons in the core and shell of the NAc, as well as in several other BG regions [the OLT, CPu, and globus pallidus (GP)] by stereotaxically injecting AAV vectors that contained short-hairpin interfering RNA specific for A_{2A}R ($shA_{2A}R$) and the reporter gene mCherry (Fig. 4*A*,*B*). The A_{2A}R shRNA sequence was derived from a previously validated small-interfering RNA target for A_{2A}R (Chen et al., 2004). At 4 weeks after the injection, A_{2A}Rs were completely eliminated at the site of injection (Fig. 4*D*). A_{2A}R expression was not attenuated when injections were made with shCTRL with no homology to any known sequences in the rat genome (Fig. 4*C*). NeuN staining confirmed the absence of neuronal toxicity at the injection site in the NAc of both AAV–shCTRL- and $shA_{2A}R$-injected rats (Fig. 4*E*,*F*).

We then examined the effects of an intraperitoneal injection of caffeine (15 mg/kg) in rats that had been bilaterally injected with AAV–shCTRL or AAV–$shA_{2A}R$ into the NAc (Fig. 5). EEG/EMG recordings made during a 3 h period after caffeine and vehicle injections were analyzed to obtain the total duration of wakefulness after each injection, and the difference in total amount of wakefulness after caffeine versus vehicle injection was defined as caffeine-induced wakefulness. We also measured the area of reporter expression as an indicator for the loss of A_{2A} receptors, using tissue sections of the rostral, central, and caudal NAc that were immunostained for the reporter. A Pearson's correlation of $r = 0.8$ ($p < 0.01$) between the reporter immunoreactivity and the caffeine-induced wakefulness (Fig. 5*A*) showed that the disruption of A_{2A}Rs in the NAc was proportional to the loss of the arousal effect of caffeine. In contrast, the caffeine-induced arousal was not affected in rats when AAV–$shA_{2A}R$ was bilaterally injected into other A_{2A}R-positive areas of the BG, including the CPu, OLT, and GP, or in AAV–shCTRL-treated or AAV-untreated rats (Fig. 5*B*).

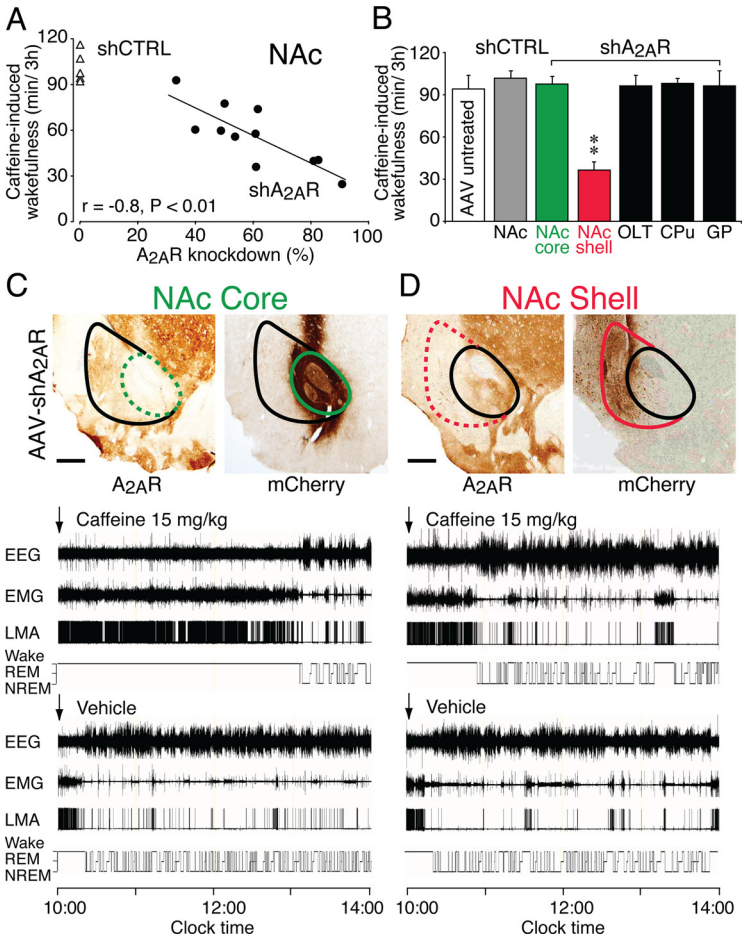

Figure 5. Arousal effect of caffeine was abolished in rats with site-specific deletion of A_{2A}Rs in the shell of the NAc. ***A***, The loss of the arousal response to caffeine (15 mg/kg, i.p.) in rats that had received AAV–$shA_{2A}R$ injections (black circles) into the NAc correlated closely with the knockdown of A_{2A}Rs in the NAc, whereas there was very little variation in caffeine-induced wakefulness in control animals injected with shCTRL (white triangles). ***B***, Caffeine-induced wakefulness for 3 h in AAV-untreated rats and in rats that received injection of AAV–shCTRL into the NAc or injection of AAV–$shA_{2A}R$ into the NAc shell and core, OLT, CPu, and GP. Caffeine was given intraperitoneally at 15 mg/kg. ***C***, ***D***, Typical sections from two rats injected bilaterally with AAV carrying $shA_{2A}R$ into the shell or core of the NAc that were stained with mouse monoclonal antibody against $A_{2A}R$ showed dominant depletion of A_{2A}Rs either in the shell (***D***, right photomicrograph) or the core (***C***, left photomicrograph) of the NAc. Adjacent sections to ***C*** and ***D*** were stained with rabbit polyclonal antibody against mCherry (Clontech) to confirm the extent to which neural cells in the NAc were transfected with AAV–$shA_{2A}R$ (***C***, ***D***, right photomicrographs). The red circles in the photomicrographs in ***D*** outline the entire NAc, including both the core and shell region, whereas the green circles in the photomicrographs in ***C*** delineate the core of the NAc. The polysomnographic recordings in ***C*** and ***D*** show typical examples of EEG, EMG, LMA, and hypnograms after administration of caffeine at a dose of 15 mg/kg (top polysomnographic panel) or vehicle (bottom polysomnographic panel) in two rats with AAV–$shA_{2A}R$ infections of the shell (***D***) or the core (***C***) of the NAc. Data are presented as mean ± SEM ($n = 5$–6 per AAV-treatment). $^{**}p < 0.01$ compared with AAV-untreated or AAV–shCTRL-injected rats, assessed by one-way ANOVA. Scale bars: ***C***, ***D***, 300 μm.

All injections into the NAc were aimed at the border area between the core and shell of the NAc (Fig. 5*D*), but occasionally injections fell more laterally. In several such cases, only neurons of the NAc core but not those of the shell portion were infected with AAV–$shA_{2A}R$ (Fig. 5*C*), and those rats showed a normal response to caffeine (Fig. 5*B*, green column). Figure 5, *C* and *D*, shows typical examples of EEG, EMG, LMA, and hypnograms after the administration of caffeine at a dose of 15 mg/kg (top polysomnographic panels) or vehicle (bottom polysomnographic panels) in two rats with AAV–$shA_{2A}R$ infections of the shell (Fig. 5*D*) or the core (Fig. 5*C*) of the NAc. The A_{2A}R depletion in the NAc shell attenuated the effect of caffeine on wakefulness (Fig. 5*D*, top polysomnographic panel), whereas the rat with a loss of A_{2A}Rs only in the core portion of the NAc showed a normal response to caffeine (Fig. 5*C*, top polysomnographic panel). These results indicate that A_{2A}R-positive neurons in the shell of the NAc were crucial for caffeine to induce wakefulness (Fig. 5*B*, red column).

Discussion

Our results, using a combination of different gene ablation strategies based on the Cre/loxP technology and RNA interference in two different species, clearly demonstrate that the expression of A_{2A}Rs by neurons in the shell of the NAc is essential for the caffeine-induced arousal. For caffeine to be effective as an A_{2A}R antagonist, excitatory A_{2A}Rs on NAc shell neurons must be tonically activated by endogenous adenosine.

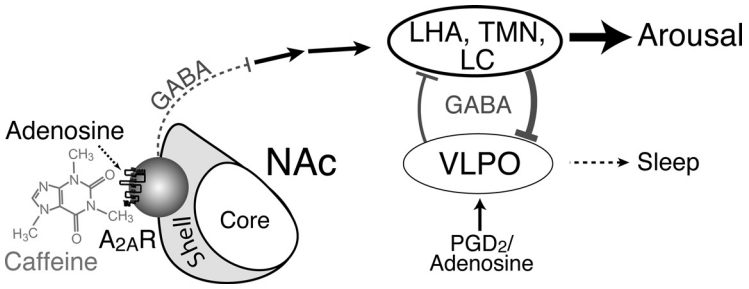

Figure 6. A proposed wake-regulatory role of the A_{2A}R-expressing neurons in the shell of the NAc that accounts for the wake-promoting effect of caffeine. Endogenous somnogens, such as adenosine and prostaglandin D_2 (PGD_2), promote sleep by activating sleep-promoting neurons of the VLPO (Saper et al., 2005, 2010; Urade, 2011), which, in a putative flip–flop arrangement, inhibit the arousal-promoting regions, including the LHA, TMN, and LC in the brainstem. Adenosine acting at A_{2A}Rs on medium spiny neurons in the shell of the NAc is hypothesized to exert inhibitory effects on the arousal systems via indirect (GABAergic and glutamatergic) pathways. Caffeine blocks the A_{2A}Rs in the NAc shell, thereby removing the restraint on the arousal systems to promote wakefulness.

Such tonic activation likely occurs because A_{2A}Rs are abundantly expressed in the NAc shell and, even under the most basal conditions, a finite level of adenosine is detected in the extracellular space (Svenningsson et al., 1999a,b). Thus, adenosine activates A_{2A}Rs on medium spiny projection neurons in the NAc shell and contributes to restrain the arousal system. As a consequence, caffeine clearly overrides the "adenosine brake" and promotes wakefulness. Therefore, based on a similarity between mouse and man, the area of the human brain in which caffeine acts to counteract fatigue, the shell of the NAc, is just about the astonishingly small size of a pea.

The depletion of A_{2A}Rs in the NAc shell diminished the caffeine-induced wakefulness but did not change the amount of wakefulness after the vehicle injection (Fig. 3*C*,*D*), indicating that the inhibition of A_{2A}Rs in the NAc shell is crucial for caffeine-induced, but not the basal, wakefulness. Therefore, the adenosine A_{2A}R system in the NAc shell is considered to function as an accessory nucleus for regulation of the main sleep center in the VLPO, which is activated by prostaglandin D_2 (Scammell et al., 1998) and adenosine acting via A_{2A}R (Scammell et al., 2001). The neural network accounting for the arousal effect of caffeine on A_{2A}R-expressing neurons in the NAc shell is summarized in Figure 6, in which blockade of the massive GABAergic output of NAc shell neurons activates classical arousal centers, such as the lateral hypothalamus (LHA), the tuberomammillary hypothalamic nucleus (TMN), and the locus ceruleus (LC), via direct or indirect projections from the NAc shell. Those arousal centers are reciprocally regulated by the primary sleep-promoting neurons in the VLPO via GABAergic inhibitory projections (Saper et al., 2005, 2010).

The inability of caffeine (15 mg/kg) to induce any arousal effect in mice with A_{2A}R gene deletions in the NAc shell (Figs. 3, 5) indicates that the blockade of A_{2A}Rs in the rest of the brain is not sufficient at this dose to promote arousal. However, this observation does not rule out the possibility that A_{2A}Rs in other brain regions may also contribute to caffeine-induced wakefulness. Selective reinsertion of A_{2A}Rs in the NAc shell of A_{2A}R KO mice would be necessary to show that A_{2A}Rs in the NAc shell are sufficient to produce caffeine arousal. In fact, caffeine at 30 mg/kg decreased inactivity and increased LMA compared with the vehicle treatment for the BG–A_{2A}R KO mice but not for the global A_{2A}R KO mice (Fig. 2), suggesting that caffeine at high concentrations has psychomotor effects at A_{2A}Rs outside the BG. This minor wake-promoting effect in BG–A_{2A}R KO mice with 30 mg/kg caffeine (Fig. 1*D*,*E*) may be attributable to the blockade of A_{2A}Rs in the leptomeninges near the VLPO, because these receptors are responsible for activation of sleep-promoting neurons in the VLPO (Scammell et al., 2001).

Adenosine clearly acts as an endogenous somnogen that regulates the homeostatic sleep drive (Urade and Hayaishi, 2010). There are multiple pathways through which sleep and wakefulness can be regulated. Because A_{2A}Rs on medium spiny neurons of the NAc are colocalized with D_2Rs, which are essential in the maintenance of wakefulness (Qu et al., 2008, 2010), caffeine acting on neurons in the shell of the NAc may modulate neural substrates through which dopamine produces arousal. Adenosine acting via A_1R has also been shown to induce sleep by inhibiting the cholinergic region of the BF (Basheer et al., 2004). For example, the unilateral infusion of the BF with an A_1R-selective antagonist increased waking and decreased sleep (Strecker et al., 2000). Single-unit recording of BF neurons in conjunction with *in vivo* microdialysis of an A_1R-selective agonist decreased, and an A_1R antagonist increased, the discharge activity of the neurons in the BF (Alam et al., 1999). In addition, the activation of A_1Rs expressed in the TMN inhibits the histaminergic system and promotes NREM sleep (Oishi et al., 2008). Although caffeine is an antagonist for both A_1R and A_{2A}R, it increased wakefulness in A_1R KO and WT mice but not in A_{2A}R KO mice (Huang et al., 2005), and, therefore, A_1Rs are clearly not required for the effect of caffeine on wakefulness.

Instead of acting at the classical sleep–wake-regulatory neurons, such as the cholinergic BF neurons and the sleep-promoting preoptic neurons, caffeine appears to induce arousal by activating, at least initially, many neuronal pathways that have traditionally been associated with locomotion and motivational behaviors. The NAc shell has long been thought to activate, mainly through indirect pathways via the ventral pallidum and substantia innominata, midbrain–pontine areas that are involved in exploratory locomotion (Mogenson et al., 1983; Groenewegen and Trimble, 2007). In addition, reciprocal connections between the NAc shell and the ventral tegmental area (Zahm and Heimer, 1993), a site of dopamine neurons involved in motivation, reward, and motor control, promote arousal driven by motivation (Sesack and Grace, 2010).

The NAc shell is also well positioned to recruit the cortex, in particular the medial prefrontal cortex (mPFC), into sleep-regulatory circuits. The mPFC is a key executive interface between cognition and emotion but is also uniquely sensitive to sleep and sleep need (Muzur et al., 2002) and might promote sleep (Koenigs et al., 2010). In addition to its modulatory reentrant projections to the NAc shell, the mPFC could provide a top-down modulation through its direct descending projections to sleep–wake-regulatory systems in the hypothalamus (e.g., the TMN containing histamine and the LHA containing orexins) and the brainstem, including the LC containing noradrenaline (Hurley et al., 1991; Saper et al., 2005). Many of these cortical and subcortical areas also directly or indirectly receive NAc shell outputs (Zahm and Heimer, 1993; Yoshida et al., 2006; Sano and Yokoi, 2007; Sesack and Grace, 2010) and produce strong c-Fos expression, a marker for neuronal activation, in response to systemic caffeine (Deurveilher et al., 2006). The critical role of the

NAc shell and its A_{2A}Rs in caffeine-induced arousal suggests that this unique transition area between the striatum and the stress and anxiety systems within the extended amygdala may play a regulatory role for sleep mechanisms.

Different from amphetamine, A_{2A}R antagonists (including caffeine) enhance motor and arousal activities but have minimal addictive potential (Fredholm et al., 1999). This difference is probably attributable to the unique cellular localization of the A_{2A}R in the D_2R-bearing neurons of the indirect pathway but not in the D_1R-bearing striatonigral neurons, in which the psychostimulants amphetamine and cocaine predominantly act and which constitute the major therapeutic target site implicated in drug addiction and dependence. Caffeine may, however, influence the intake or actions of dependence-producing drugs, because the blockade of A_{2A}R can synergize with agents that activate D_1R pathways (Le Moine et al., 1997).

References

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

Arnulf I, Konofal E, Merino-Andreu M, Houeto JL, Mesnage V, Welter ML, Lacomblez L, Golmard JL, Derenne JP, Agid Y (2002) Parkinson's disease and sleepiness: an integral part of PD. Neurology 58:1019–1024.

Basheer R, Strecker RE, Thakkar MM, McCarley RW (2004) Adenosine and sleep-wake regulation. Prog Neurobiol 73:379–396.

Chamberlin NL, Du B, de Lacalle S, Saper CB (1998) Recombinant adeno-associated virus vector: use for transgene expression and anterograde tract tracing in the CNS. Brain Res 793:169–175.

Chen JF, Huang Z, Ma J, Zhu J, Moratalla R, Standaert D, Moskowitz MA, Fink JS, Schwarzschild MA (1999) A_{2A} adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. J Neurosci 19:9192–9200.

Chen Y, Epperson S, Makhsudova L, Ito B, Suarez J, Dillmann W, Villarreal F (2004) Functional effects of enhancing or silencing adenosine A_{2B} receptors in cardiac fibroblasts. Am J Physiol Heart Circ Physiol 287:H2478–H2486.

Deurveilher S, Lo H, Murphy JA, Burns J, Semba K (2006) Differential c-Fos immunoreactivity in arousal-promoting cell groups following systemic administration of caffeine in rats. J Comp Neurol 498:667–689.

Durieux PF, Bearzatto B, Guiducci S, Buch T, Waisman A, Zoli M, Schiffmann SN, de Kerchove d'Exaerde A (2009) D_2R striatopallidal neurons inhibit both locomotor and drug reward processes. Nat Neurosci 12:393–395.

Estabrooke IV, McCarthy MT, Ko E, Chou TC, Chemelli RM, Yanagisawa M, Saper CB, Scammell TE (2001) Fos expression in orexin neurons varies with behavioral state. J Neurosci 21:1656–1662.

Fredholm BB, Bättig K, Holmén J, Nehlig A, Zvartau EE (1999) Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol Rev 51:83–133.

Groenewegen HJ, Trimble M (2007) The ventral striatum as an interface between the limbic and motor systems. CNS Spectr 12:887–892.

Huang ZL, Qu WM, Eguchi N, Chen JF, Schwarzschild MA, Fredholm BB, Urade Y, Hayaishi O (2005) Adenosine A_{2A}, but not A_1, receptors mediate the arousal effect of caffeine. Nat Neurosci 8:858–859.

Huang ZL, Urade Y, Hayaishi O (2011) The role of adenosine in the regulation of sleep. Curr Top Med Chem 11:1047–1057.

Hurley KM, Herbert H, Moga MM, Saper CB (1991) Efferent projections of the infralimbic cortex of the rat. J Comp Neurol 308:249–276.

Koenigs M, Holliday J, Solomon J, Grafman J (2010) Left dorsomedial frontal brain damage is associated with insomnia. J Neurosci 30:16041–16043.

Kohtoh S, Taguchi Y, Matsumoto N, Wada M, Huang ZL, Urade Y (2008) Algorithm for sleep scoring in experimental animals based on fast Fourier transform power spectrum analysis of the electroencephalogram. Sleep Biol Rhythms 6:163–171.

Lazarus M, Yoshida K, Coppari R, Bass CE, Mochizuki T, Lowell BB, Saper CB (2007) EP_3 prostaglandin receptors in the median preoptic nucleus are critical for fever responses. Nat Neurosci 10:1131–1133.

Le Moine C, Svenningsson P, Fredholm BB, Bloch B (1997) Dopamine-adenosine interactions in the striatum and the globus pallidus: inhibition of striatopallidal neurons through either D_2 or A_{2A} receptors enhances D_1 receptor-mediated effects on c-fos expression. J Neurosci 17:8038–8048.

Matsumura H, Nakajima T, Osaka T, Satoh S, Kawase K, Kubo E, Kantha SS, Kasahara K, Hayaishi O (1994) Prostaglandin D_2-sensitive, sleep-promoting zone defined in the ventral surface of the rostral basal forebrain. Proc Natl Acad Sci U S A 91:11998–12002.

Mizoguchi A, Eguchi N, Kimura K, Kiyohara Y, Qu WM, Huang ZL, Mochizuki T, Lazarus M, Kobayashi T, Kaneko T, Narumiya S, Urade Y, Hayaishi O (2001) Dominant localization of prostaglandin D receptors on arachnoid trabecular cells in mouse basal forebrain and their involvement in the regulation of non-rapid eye movement sleep. Proc Natl Acad Sci U S A 98:11674–11679.

Mogenson GJ, Swanson LW, Wu M (1983) Neural projections from nucleus accumbens to globus pallidus, substantia innominata, and lateral preoptic-lateral hypothalamic area: an anatomical and electrophysiological investigation in the rat. J Neurosci 3:189–202.

Muzur A, Pace-Schott EF, Hobson JA (2002) The prefrontal cortex in sleep. Trends Cogn Sci 6:475–481.

Oishi Y, Huang ZL, Fredholm BB, Urade Y, Hayaishi O (2008) Adenosine in the tuberomammillary nucleus inhibits the histaminergic system via A_1 receptors and promotes non-rapid eye movement sleep. Proc Natl Acad Sci U S A 105:19992–19997.

Pack AI, Galante RJ, Maislin G, Cater J, Metaxas D, Lu S, Zhang L, Von Smith R, Kay T, Lian J, Svenson K, Peters LL (2007) Novel method for high-throughput phenotyping of sleep in mice. Physiol Genomics 28:232–238.

Paxinos G, Franklin KBJ (2001) The mouse brain in stereotaxic coordinates. San Diego: Academic.

Paxinos G, Watson C (2007) The rat brain in stereotaxic coordinates. Boston: Elsevier.

Porkka-Heiskanen T, Strecker RE, Thakkar M, Bjorkum AA, Greene RW, McCarley RW (1997) Adenosine: a mediator of the sleep-inducing effects of prolonged wakefulness. Science 276:1265–1268.

Qu WM, Huang ZL, Xu XH, Matsumoto N, Urade Y (2008) Dopaminergic D_1 and D_2 receptors are essential for the arousal effect of modafinil. J Neurosci 28:8462–8469.

Qu WM, Xu XH, Yan MM, Wang YQ, Urade Y, Huang ZL (2010) Essential role of dopamine D_2 receptor in the maintenance of wakefulness, but not in homeostatic regulation of sleep, in mice. J Neurosci 30:4382–4389.

Rosin DL, Robeva A, Woodard RL, Guyenet PG, Linden J (1998) Immunohistochemical localization of adenosine A_{2A} receptors in the rat central nervous system. J Comp Neurol 401:163–186.

Sano H, Yokoi M (2007) Striatal medium spiny neurons terminate in a distinct region in the lateral hypothalamic area and do not directly innervate orexin/hypocretin- or melanin-concentrating hormone-containing neurons. J Neurosci 27:6948–6955.

Saper CB, Scammell TE, Lu J (2005) Hypothalamic regulation of sleep and circadian rhythms. Nature 437:1257–1263.

Saper CB, Fuller PM, Pedersen NP, Lu J, Scammell TE (2010) Sleep state switching. Neuron 68:1023–1042.

Sauer B, Henderson N (1990) Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase. New Biol 2:441–449.

Scammell T, Gerashchenko D, Urade Y, Onoe H, Saper C, Hayaishi O (1998) Activation of ventrolateral preoptic neurons by the somnogen prostaglandin D_2. Proc Natl Acad Sci U S A 95:7754–7759.

Scammell TE, Gerashchenko DY, Mochizuki T, McCarthy MT, Estabrooke IV, Sears CA, Saper CB, Urade Y, Hayaishi O (2001) An adenosine A_{2A} agonist increases sleep and induces Fos in ventrolateral preoptic neurons. Neuroscience 107:653–663.

Sesack SR, Grace AA (2010) Cortico-basal ganglia reward network: microcircuitry. Neuropsychopharmacology 35:27–47.

Shen HY, Coelho JE, Ohtsuka N, Canas PM, Day YJ, Huang QY, Rebola N, Yu L, Boison D, Cunha RA, Linden J, Tsien JZ, Chen JF (2008) A critical role of the adenosine A_{2A} receptor in extrastriatal neurons in modulating psychomotor activity as revealed by opposite phenotypes of striatum and forebrain A_{2A} receptor knock-outs. J Neurosci 28:2970–2975.

Soriano P (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet 21:70–71.

Strecker RE, Morairty S, Thakkar MM, Porkka-Heiskanen T, Basheer R, Dauphin LJ, Rainnie DG, Portas CM, Greene RW, McCarley RW (2000) Adenosinergic modulation of basal forebrain and preoptic/anterior hypothalamic neuronal activity in the control of behavioral state. Behav Brain Res 115:183–204.

Svenningsson P, Le Moine C, Kull B, Sunahara R, Bloch B, Fredholm BB (1997) Cellular expression of adenosine A_{2A} receptor messenger RNA in the rat central nervous system with special reference to dopamine innervated areas. Neuroscience 80:1171–1185.

Svenningsson P, Le Moine C, Fisone G, Fredholm BB (1999a) Distribution, biochemistry and function of striatal adenosine A_{2A} receptors. Prog Neurobiol 59:355–396.

Svenningsson P, Fourreau L, Bloch B, Fredholm BB, Gonon F, Le Moine C (1999b) Opposite tonic modulation of dopamine and adenosine on c-fos gene expression in striatopallidal neurons. Neuroscience 89:827–837.

Urade Y (2011) Prostaglandin D_2 and adenosine as endogenous somnogens. Sleep Biol Rhythms 9:10–17.

Urade Y, Hayaishi O (2010) Crucial role of prostaglandin D_2 and adenosine in sleep regulation: experimental evidence from pharmacological approaches to gene-knockout mice. Future Neurol 5:363–376.

Yoshida K, McCormack S, España RA, Crocker A, Scammell TE (2006) Afferents to the orexin neurons of the rat brain. J Comp Neurol 494:845–861.

Zahm DS, Heimer L (1993) Specificity in the efferent projections of the nucleus accumbens in the rat: comparison of the rostral pole projection patterns with those of the core and shell. J Comp Neurol 327:220–232.

Zolotukhin S, Byrne BJ, Mason E, Zolotukhin I, Potter M, Chesnut K, Summerford C, Samulski RJ, Muzyczka N (1999) Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene Ther 6:973–985.