Behavioral/Systems/Cognitive

Essential Role of Dopamine D_2 Receptor in the Maintenance of Wakefulness, But Not in Homeostatic Regulation of Sleep, in Mice

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Dopamine (DA) and its D_2 receptor (R) are involved in cognition, reward processing, and drug addiction. However, their roles in sleep–wake regulation remain unclear. Herein we investigated the role of D_2R in sleep–wake regulation by using D_2R knock-out (KO) mice and pharmacological manipulation. Compared with WT mice, D_2R KO mice exhibited a significant decrease in wakefulness, with a concomitant increase in non-rapid eye movement (non-REM, NREM) and REM sleep and a drastic decrease in the low-frequency (0.75–2 Hz) electroencephalogram delta power of NREM sleep, especially during the first 4 h after lights off. The KO mice had decreased mean episode duration and increased episode numbers of wake and NREM sleep, many stage transitions between wakefulness and NREM sleep during the dark period, suggesting the instability of the wake stage in these D_2R KO mice. When the KO mice were subjected to a cage change or an intraperitoneal saline injection, the latency to sleep in the KO mice decreased to half of the level for WT mice. The D_2R antagonist raclopride mimicked these effects in WT mice. When GBR12909, a dopamine transport inhibitor, was administered intraperitoneally, it induced wakefulness in WT mice in a dose-dependent manner, but its arousal effect was attenuated to one-third in the D_2R KO mice. However, these 2 genotypes showed an identical response in terms of sleep rebound after 2, 4, and 6 h of sleep deprivation. These results indicate that D_2R plays an essential role in the maintenance of wakefulness, but not in homeostatic regulation of NREM sleep.

Introduction

Dopamine (DA) is critically involved in regulating processes responsible for the generation of complex movements and emotions, cognition, reward processing, and drug addiction (Nicola et al., 2000). In contrast, the role assigned to DA in sleep–wake regulation has been relatively limited, because the activity of DA neurons in the ventral tegmental area and substantia nigra pars compacta is not significantly modulated by the sleep–wake cycle (Miller et al., 1983); and, furthermore, neurotoxic lesions of the ventral tegmental area do not decrease behavioral wakefulness (Lai et al., 1999). However, the results of *in vivo* microdialysis studies indicate that during the light period, when rats typically sleep, extracellular DA levels are lower than during the dark period in the medial prefrontal cortex and the nucleus accumbens (Léna et al., 2005). Moreover, DA-specific reuptake blockers can promote wakefulness in normal and narcoleptic animals (Nishino et al., 1998). Mice with deletion of the DA transporter (DAT) gene show increased wakefulness and reduced non-rapid eye movement (non-REM, NREM) sleep (Wisor et al., 2001). In addition, patients with Parkinson's disease, who exhibit consistent dopaminergic lesions and inconsistent alterations in other monoamines, experience severe excessive daytime sleepiness (Arnulf et al., 2002). c-Fos protein is expressed in 50% of the DA neurons in the ventral periaqueductal gray matter during natural wakefulness or wakefulness induced by environmental stimulation, but not during sleep (Lu et al., 2006). These observations suggest potential wake-promoting actions of DA neurotransmission.

DA receptors are subdivided into D_1-like (D_1 and D_5) and D_2-like (D_2, D_3, and D_4) receptors (Gingrich and Caron, 1993). The D_1 receptor (D_1R) and D_2R are the most widely and abundantly expressed receptors for DA in the brain, whereas D_3R, D_4R, and D_5R have lower abundance and a very restricted localization (Kobayashi et al., 2004). We previously showed that D_1R and D_2R are essential for the arousal effect of the wake-promoting drug modafinil, with D_2R being the receptor of primary importance (Qu et al., 2008). To clarify the roles of D_2R in sleep–wake regulation, researchers have often used the pharmacological approach. Systemic administration of a D_2R antagonist increases NREM sleep and the electroencephalogram (EEG) power density in the low-frequency bands (Monti et al., 1988;

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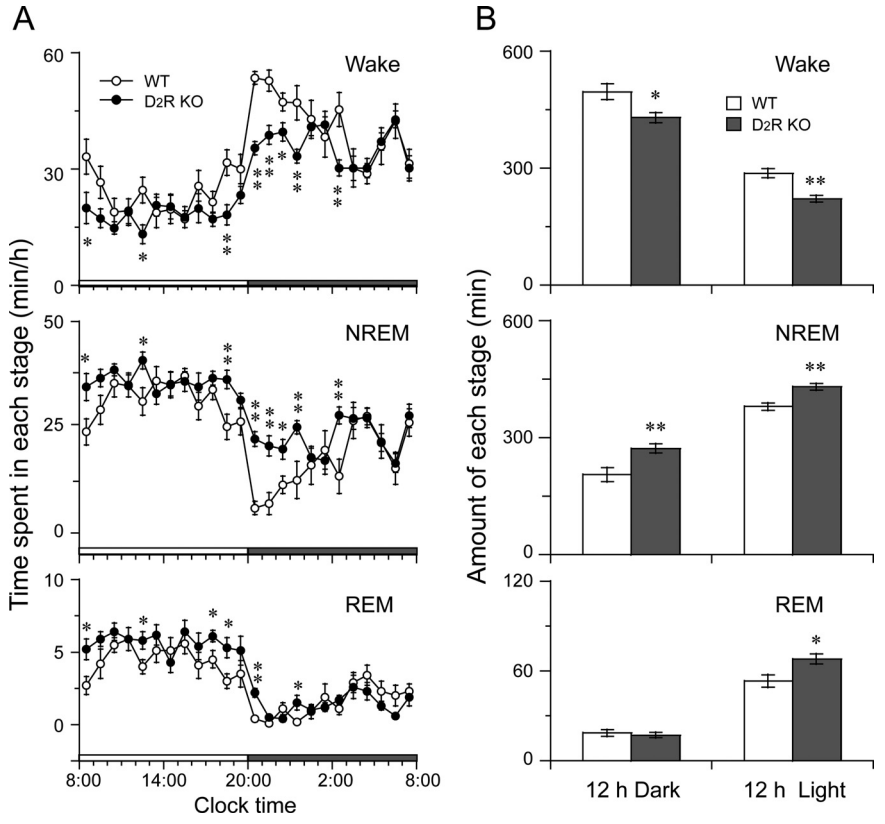

Figure 1. Sleep–wake profiles of WT and D_2R KO mice under baseline conditions. ***A***, Time course changes in wakefulness, NREM, and REM sleep. Each circle represents the hourly mean amount of each stage. Open and closed circles stand for the WT and D_2R KO profiles, respectively. The horizontal open and filled bars on the χ-axes indicate the 12 h light and 12 h dark periods, respectively. ***B***, Total time spent in wakefulness, NREM, and REM sleep during the 12 h dark and 12 h light phases. Open and filled bars show the profiles for the WT and D_2R KO, respectively. Values are the means ± SEM ($n = 10$). *$p < 0.05$, **$p < 0.01$, compared with corresponding WT littermate value, assessed by two-way ANOVA followed by the PLSD test.

Ongini et al., 1993; Sebban et al., 1999). Under conditions of low arousal, the D_2R agonist quinpirole promotes wakefulness after an intracerebroventricular administration (Isaac and Berridge, 2003). However, the systemic injection of a D_2R agonist induces biphasic effects, such that low doses reduce wakefulness, whereas large doses have the opposite effect (Monti et al., 1988). Therefore, pharmacological tools used to determine the role of D_2R in sleep–wake regulation show limited selectivity and/or incomplete blockade. Mice with a mutation disrupting the expression of the D_2R might provide insight into the contribution of this receptor to sleep–wake regulation.

In the present study, we characterized sleep–wake profiles of D_2R KO mice under baseline conditions and after an intraperitoneal injection of saline, environmental change, sleep deprivation (SD), and increased DA transmission with a DAT inhibitor. Compared with WT mice, the D_2R KO mice exhibited decreased wakefulness, increased stage transition between wakefulness and NREM sleep under baseline conditions, shortened latency to NREM sleep after a cage change or injection of saline, an attenuated arousal effect after inhibition of DAT, but the same sleep rebound after SD. These findings indicate that D_2R plays an important role in arousal maintenance but not in homeostatic regulation of sleep.

Materials and Methods

Animals. Male D_2R KO and their WT mice of the inbred C57BL/6 strain were generated from heterozygotes (Yamaguchi et al., 1996), weighing 20–26 g (11–13 weeks old), were maintained at Oriental Bioservice and used in these experiments. As reported by Yamaguchi et al. (1996), to generate mutant mice which express neither isoform of the D_2R, the targeting vector was constructed to have the *neo* gene disrupt exon 2 containing the ATG start codon, with HSV-*tk* at the 3′ end of the construct for negative selection. Lack of D_2R activity in the D_2R KO mice was confirmed by *in situ* hybridization, ligand binding autoradiography and so on. PCR with the animal tail DNA was used to determine their genotypes. The animals were housed in an insulated sound-proof recording room maintained at an ambient temperature of 22 ± 0.5°C with a relative humidity (60 ± 2%) on an automatically controlled 12 h light/12 h dark cycle (light on at 8:00 A.M.) and had free access to food and water. Experimental protocols were approved by the Animal Care Committee of Osaka Bioscience Institute. Every effort was made to minimize the number of animals used and any pain and discomfort experienced by the animals.

Polygraphic recordings and vigilance state analysis. Under pentobarbital anesthesia (50 mg/kg, i.p.), mice were chronically implanted with EEG and electromyogram (EMG) electrodes for polysomnographic recordings. The implant consisted of 2 stainless steel screws (1 mm diameter) inserted through the skull into the cortex (anteroposterior, +1.0 mm and left-right, −1.5 mm from bregma or lambda) according to the atlas of Franklin and Paxinos (1997) and served as EEG electrodes. Two insulated stainless steel, Teflon-coated wires bilaterally placed into both trapezius muscles served as EMG electrodes. All electrodes were attached to a microconnector and fixed to the skull with dental cement.

The recordings of EEG and EMG were performed by means of a slip ring, designed so that behavioral movement of the mice would not be restricted. After a 10 d recovery period, the mice were housed individually in transparent barrels and habituated to the recording cable for 3–4 d before polygraphic recordings. For the study of spontaneous sleep–wakefulness cycles, each animal was recorded for 24 h, beginning at 8:00 A.M., the onset of the light period. The animals then entered the pharmacological phase of the study, in which sleep–wakefulness parameters were recorded for 48 h. The data collected during the first 24 h also served as baseline comparison data for the second experimental day.

First, cortical EEG and EMG signals were amplified and filtered (EEG, 0.5–30 Hz; EMG, 20–200 Hz) and then digitized at a sampling rate of 128 Hz and recorded by using SLEEPSIGN (Kissei Comtec) as described earlier (Huang et al., 2005; Qu et al., 2006). When completed, polygraphic recordings were automatically scored off-line by 4 s epochs as wakefulness, REM, and NREM sleep by SLEEPSIGN according to standard criteria (Huang et al., 2001, 2005; Kohtoh et al., 2008). As a final step, defined sleep–wake stages were examined visually, and corrected, if necessary.

Pharmacological treatments. Raclopride (Sigma), a selective D_2R antagonist, was dissolved in sterile saline. GBR12909 (Sigma), a DAT inhibitor, was dissolved in 10% DMSO. Either one was administered intraperitoneally at 10:00 A.M. For baseline data, mice were injected intraperitoneally with vehicle only.

Sleep deprivation. At least 4 d after adaptation in recording chambers, mice were recorded for 2 consecutive days for EEG and EMG. The first day served as the baseline day; and on the second day the animals were subjected to total sleep deprivation for 2 h, 4 h or 6 h (from 6:00, 4:00, or 2:00 P.M., respectively, to 8:00 P.M.) by lightly tapping the cage via a soft tissue ball.

Statistical analysis. All results were expressed as means ± SEM. Time course changes in the amounts of sleep–wake were compared between WT and KO mice by using two-way, repeated measures ANOVA followed by the *post hoc* Fisher's probable least-squares difference (PLSD) test or two-tailed Student's *t* tests. Comparisons of sleep/wake amounts, number and duration of sleep/wake bouts in light/dark phases and first 4 h of darkness between WT and KO mice were made by use of the nonpaired, two-tailed Student's *t* test. In all cases, $p < 0.05$ was taken as the level of significance.

Results

Decreased wakefulness in D_2R KO mice in spontaneous sleep–wake cycles

Under baseline conditions, both WT and D_2R KO mice exhibited a clear circadian sleep–wake rhythm, with larger amounts of sleep during the light period than during the dark one (Fig. 1*A*). However, when their sleep–wake profiles were compared with those of WT littermates, the D_2R KO mice showed decreased wakefulness with increased NREM sleep, especially during the dark period (Fig. 1*A*). These effects were much apparent for the first 4 h of the dark period in D_2R KO mice, because wakefulness decreased by 30%, and NREM and REM sleep increased by 2.4- and 2.5-fold, respectively, compared with those for WT mice. During the 12 h light period, the amount of wakefulness deceased by 23%, with a concomitant increase in NREM and REM sleep by 1.1- and 1.3-fold, respectively, in the D_2R KO mice (Fig. 1*B*), compared with WT amounts. Similarly, during the 12 h dark period, the wakefulness decreased by 13%, with a 1.3-fold increase in NREM sleep in D_2R KO mice compared with that for the WT; however, the amount of REM sleep was almost the same between the 2 genotypes (Fig. 1*B*). These results indicate that the deficiency in D_2R decreased wakefulness.

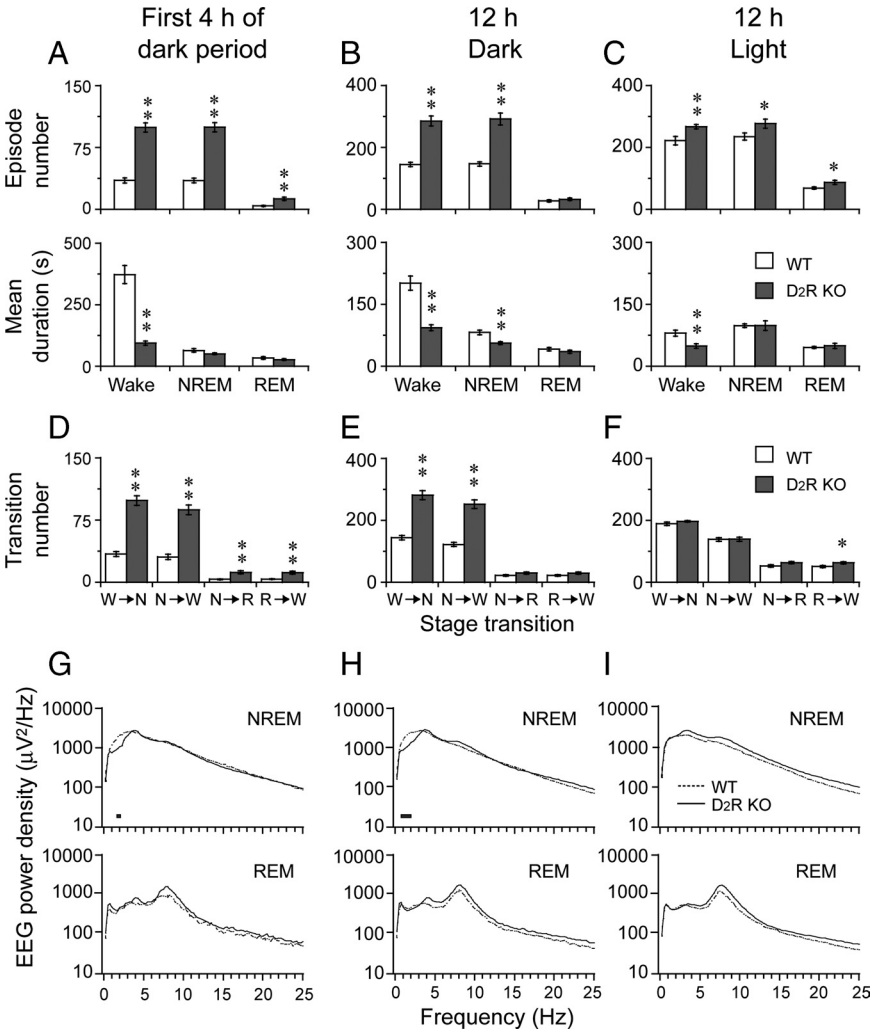

Figure 2. ***A–F***, Episode numbers, and mean durations (***A–C***) and stage transition (***D–F***) during the first 4 h of darkness, 12 h dark and 12 h light phases. Open and filled bars show the profiles for the WT and D_2R KO, respectively. ***G–I***, EEG power density during the first 4 h of darkness, 12 h dark period, and 12 h light period. The curves represent logarithmic mean values of absolute power densities [power density (μV^2/Hz) = power value (μV^2)/(frequency resolution 0.25 Hz × adjustment parameter of hanning window 1.5)]. The horizontal bars indicate where there is a statistical difference ($p < 0.05$). Values are the means ± SEM ($n = 10$). *$p < 0.05$, **$p < 0.01$ compared with the corresponding WT littermate value, assessed by two-way ANOVA followed by the PLSD test.

Shortened wake duration and increased episodes of wake and NREM sleep in D_2R KO mice

Remarkable changes in episode occurrence and mean duration were found during the first 4 h of the dark period (Fig. 2*A*), in which D_2R KO mice exhibited increased episode numbers of wakefulness, NREM, and REM sleep by 2.8-, 2.8-, and 3.1-fold, respectively, and the decreased mean duration of wakefulness by 74%; whereas their mean duration of NREM and REM sleep was the same as that for the WT mice. Similarly, during the 12 h dark period (Fig. 2*B*), the D_2R KO mice showed the increased episode numbers of wakefulness and NREM sleep by twofold, decreased mean durations of wakefulness and NREM sleep by 54% and 32%, respectively, and unchanged episode numbers and mean duration of REM sleep, when these were compared with those for WT mice. To a lesser extent, similar results were obtained during the 12 h light period (Fig. 2*C*), in which the D_2R KO mice exhibited the increased episode numbers of wakefulness, NREM, and REM sleep, decreased mean duration of wakefulness, and the same duration of NREM and REM sleep, compared with WT mice. These results indicate that D_2R KO mice showed a significant shortened wake duration and increased number of NREM sleep and wake episodes during the dark period, especially during the first 4 h after lights off.

Increased stage transition between wakefulness and NREM sleep in D_2R KO mice

The increase in the episode numbers of wakefulness and sleep in D_2R KO mice was associated with an increase in the stage transition between sleep and wakefulness. During the first 4 h of darkness (Fig. 2*D*) and the 12 h dark period (Fig. 2*E*), D_2R KO mice showed increased transitions from wakefulness (W) to NREM (N) by 2.9- and 2.1-fold, respectively, and from N to W by 2.9 and twofold, respectively, compared with WT mice. During the 12 h light period (Fig. 2*F*), the transitions from W to N, N to W, and N to R were almost identical between the 2 genotypes; but there was

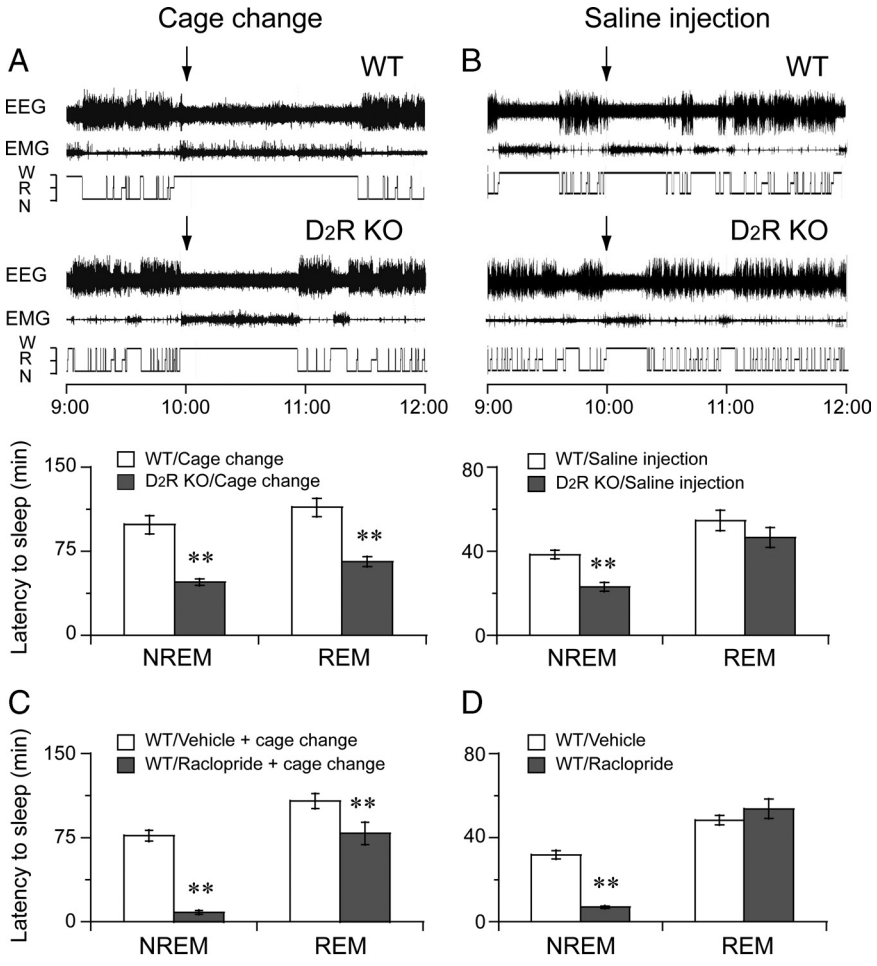

Figure 3. Latency to NREM and REM sleep after host cage change or saline injection intraperitoneally at 10:00 A.M. ***A, B***, Top and middle, Typical examples of polygraphic recordings and corresponding hypnograms illustrating the effects of cage change (***A***) or injection of saline (***B***) on WT and D_2R KO mice. Bottom, Average latency to NREM and REM sleep after cage changes (***A***) or injection of saline (***B***). Open and filled bars show the profiles for the WT and KO mice, respectively. ***C, D***, Average latency to NREM and REM sleep after administration of raclopride, a D_2R antagonist, to WT mice combined with a cage change (***C***) or not (***D***). Open and filled bars show the profiles for the vehicle and raclopride, respectively. Values are the means ± SEM ($n = 6 \sim 8$). $^{**}p < 0.01$ compared with the corresponding value for WT littermates, assessed by one-way ANOVA followed by the PLSD test.

a slight increase in the stage-transition number from R to W, by 1.2-fold, in the D_2R KO mice. The significant increase in the stage-transition number between W and N clearly indicates that D_2R KO mice had difficulty in maintaining wakefulness during the dark phase.

Altered EEG power density of NREM and REM sleep in D_2R KO mice

EEG power spectra analysis (Fig. 2*G–I*) revealed that the power density of NREM sleep was significant decreased in D_2R KO mice compared with that in WT mice over the frequency range of the delta activity from 1.5 to 2 and 0.75 to 2 Hz during the first 4 h of darkness and 12 h dark period, respectively, whereas the power density of NREM sleep in D_2R KO mice was the same as that in WT mice during the 12 h light period. However, there was no essential statistical difference in the EEG power density of REM sleep between the 2 genotypes during the first 4 h of darkness, 12 h dark period, or 12 h light period. These results clearly indicate that the sleep quality of the D_2R KO mice was lower than that of the WT mice, as judged from the lower content of the delta activity, a parameter of deep sleep.

Shortened latency to sleep after a cage change or a saline injection in D_2R KO mice or in D_2R antagonist-treated WT mice

To observe the ability to maintain the arousal state after stimulation, we subjected the 2 genotypes to a new-environment test or a saline injection at 10:00 A.M. and then examined their latencies to NREM and REM sleep, which are defined as the time from the cage change or the saline injection to the first appearance of an NREM or REM sleep episode lasting for at least 20 s. Typical examples of polygraphic recordings and the corresponding hypnograms are illustrated for a WT and a D_2R KO mouse after the environmental change (Fig. 3*A*) or the saline injection (Fig. 3*B*). The WT mouse maintained wakefulness for 110 min after the transfer of the mouse to a new cage and for 40 min after the saline injection (Fig. 3*A*, *B*, top), which were significantly longer than the 50 and 20 min, respectively, for the D_2R KO mouse (Fig. 3*A*, *B*, middle). The average latencies to NREM and REM sleep after the cage change were 48 and 66 min, respectively, for the D_2R KO mice, being significantly shorter than the 99 and 114 min, respectively, for the WT ones (Fig. 3*A*, bottom). Similar results were found after the saline injection, in which the latency to NREM sleep of 23 min for the D_2R KO mice was significantly shorter than the 39 min for the WT mice (Fig. 3*B*, bottom). However, the latency to REM sleep after the saline injection was almost identical between the 2 genotypes.

When raclopride, a selective D_2R antagonist, was administered intraperitoneally to WT mice 10 min before the cage change, it shortened their latencies to NREM and REM sleep by 89% and 27%, respectively, compared with the vehicle control (Fig. 3*C*). The intraperitoneal injection of raclopride to WT mice during the sleep period shortened the latency to NREM sleep by 78% but did not alter the latency to REM sleep, compared with the saline (vehicle) injection (Fig. 3*D*). The shortened sleep latency in D_2R KO mice and D_2R antagonist-injected WT mice clearly indicate that D_2R was necessary for arousal maintenance.

Attenuated arousal effect of a DAT inhibitor in D_2R KO mice

We then administered the DAT inhibitor GBR12909, which increases dopaminergic transmission in the brain, into WT and D_2R KO mice intraperitoneally at 10:00 A.M. to investigate the contribution of D_2Rs to the arousal effects of DA. Time course changes in wakefulness revealed that GBR12909 (20 mg/kg) induced complete wakefulness for 3 h and increased wakefulness for a further 3 h in WT mice (Fig. 4*A*). However, the arousal effect was remarkably shortened in D_2R KO mice for only 2 h compared with the vehicle control (Fig. 4*A*, *B*). The enhancement of wakefulness was concomitant with decreases in NREM and REM sleep in both genotypes (data not shown) and did not result in further

disruption of sleep architecture during the subsequent light and dark periods.

Calculated as the total amount of wakefulness for 6 h after the administration, GBR12909 significantly increased wakefulness at 5, 10, and 20 mg/kg, by 1.4-, 2.0-, and 2.4-fold, respectively, in WT mice (Fig. 4*C*) but only at doses of 10 and 20 mg/kg, by 1.2- and 1.4-fold, respectively, in the D_2R KO mice (Fig. 4*D*). However, when the EEG power activity of NREM sleep was analyzed for 10 h after GBR12909 administration, there was no essential statistical difference between the genotypes (data not shown). These findings indicate that GBR12909 significantly increased wakefulness in WT mice but only slightly in D_2R KO mice, indicating that D_2R mediated a major portion of the arousal effect by this DAT inhibitor.

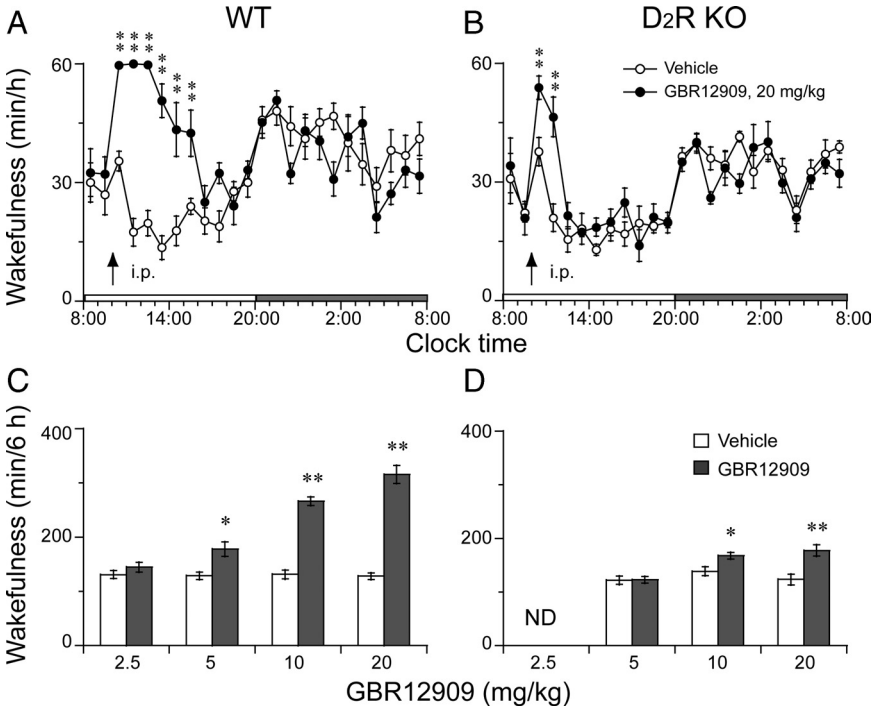

Figure 4. Effect of inhibition of dopamine transport on wakefulness. ***A, B***, Time course changes in wakefulness in WT (***A***) and D_2R KO (***B***) mice treated with the DAT inhibitor GBR12909. Each circle represents the hourly mean amount of wakefulness. Open and closed circles stand for the profiles of vehicle and GBR12909 treatments, respectively. The horizontal open and filled bars on the χ-axes indicate the 12 h light and 12 h dark periods, respectively. ***C, D***, Dose–response effect on total time spent in wakefulness for 6 h after administration of vehicle or GBR12909 to WT (***C***) and the KO (***D***) mice. Open and filled bars show the profiles of vehicle and GBR12909 treatments, respectively. Values are the means ± SEM ($n = 6–8$). $^*p < 0.05$, $^{**}p < 0.01$ compared with corresponding vehicle control, assessed by two-way ANOVA followed by the PLSD test.

Same sleep rebound after SD in D_2R KO mice as in WT mice

To determine the role of D_2R in the homeostatic regulation of sleep, we performed 2, 4, or 6 h SD from 6:00, 4:00, or 2:00 P.M. to 8:00 P.M. in both WT and D_2R KO mice, respectively, and compared their rebound sleep. Both genotypes exhibited a similar response of their NREM and REM sleep during the subsequent periods after 4 or 6 h SD (Fig. 5*A–E*). We calculated the total time spent in NREM and REM sleep for 6 h after SD, because the increase in NREM sleep was maintained for 6 h during the recovery period after 6 h SD in WT mice. The total amounts of NREM sleep increased from their respective baseline control by 1.5- and 1.7-fold after 4 or 6 h SD, respectively, in WT mice, and by 1.3- and 1.2-fold, respectively, in D_2R KO mice (Fig. 5*C*). The total amounts of REM sleep also increased by 1.1- and 2.9-fold after 4 or 6 h SD, respectively, in WT mice, and by 1.6- and 1.9-fold, respectively, in D_2R KO mice (Fig. 5*C*). When slow-wave activity (SWA) within NREM sleep, which encompasses the frequency band between 0.75 and 4 Hz, was compared between WT (Fig. 5*D*) and D_2R KO (Fig. 5*E*) mice, both genotypes showed essentially the same time course changes, in which the SWA increased for 2 h after the end of SD and then gradually declined to the baseline level. However, 2 h SD did not affect sleep rebound in both genotypes (data not shown). On the other hand, under the baseline condition, D_2R KO mice exhibited a significant decrease in SWA from 9:00 P.M. to 4:00 A.M. during the dark period, compared with WT mice, although there was no essential statistical difference during the light period between the genotypes (Fig. 5*F*). These results indicate that D_2R was not critically involved in the homeostatic regulation of NREM and REM sleep.

Discussion

D_2R KO mice exhibited a significant decrease in wakefulness concomitant with an increase in NREM and REM sleep under baseline conditions, which is consistent with an apparent reduction in the spontaneous locomotor activity previously reported to occur in these mice (Kelly et al., 1998; Doi et al., 2006). D_2R KO mice sleep longer than WT mice, exhibit NREM delta power lower than that of WT mice, and frequently enter sleep after short periods of wakefulness, indicating that D_2R KO mice have poor quality of NREM sleep. On the other hand, mice lacking various genes involved in sleep–wake regulation, such as those for orexin (Mochizuki et al., 2004), histamine H_1 receptor (Huang et al., 2006), adenosine A_1 or A_{2A} receptor (Stenberg et al., 2003; Huang et al., 2005), interleukin-6 (Morrow and Opp, 2005), and prostaglandin DP_1 receptor (Mizoguchi et al., 2001; Qu et al., 2006), have been generated and demonstrated to show no significant change in the amounts of sleep–wake stages. Sleep and wakefulness are regulated by multiple neuronal and humoral systems, and the loss of one system is efficiently compensated by the others. However, D_2R KO mice showed a rare phenotype, i.e., a remarkable decrease in wakefulness and an increase in sleep under baseline conditions.

Several abnormalities observed in D_2R KO mice, such as decreased wake duration, increased episode numbers for wake and sleep, and more transitions between wakefulness and NREM sleep, indicated that lack of D_2R resulted in the instability of wakefulness. Furthermore, when exposed to new environments or stimulation with the saline injection, D_2R KO mice could not remain awake as long as WT littermates. These findings reveal that D_2R KO mice had difficulty in maintaining wakefulness under baseline conditions or when exposed to new environments.

The previous study by Lu et al.(2006) showed a similar increase in sleep (~30% at maximum) with lesions of the ventral periaqueductal dopaminergic neurons, but this was noted in both the light and dark phase about equally. The major sleep increase predominantly during the dark phase in the D_2R KO mice is quite different, and suggests that the D_2R may play a

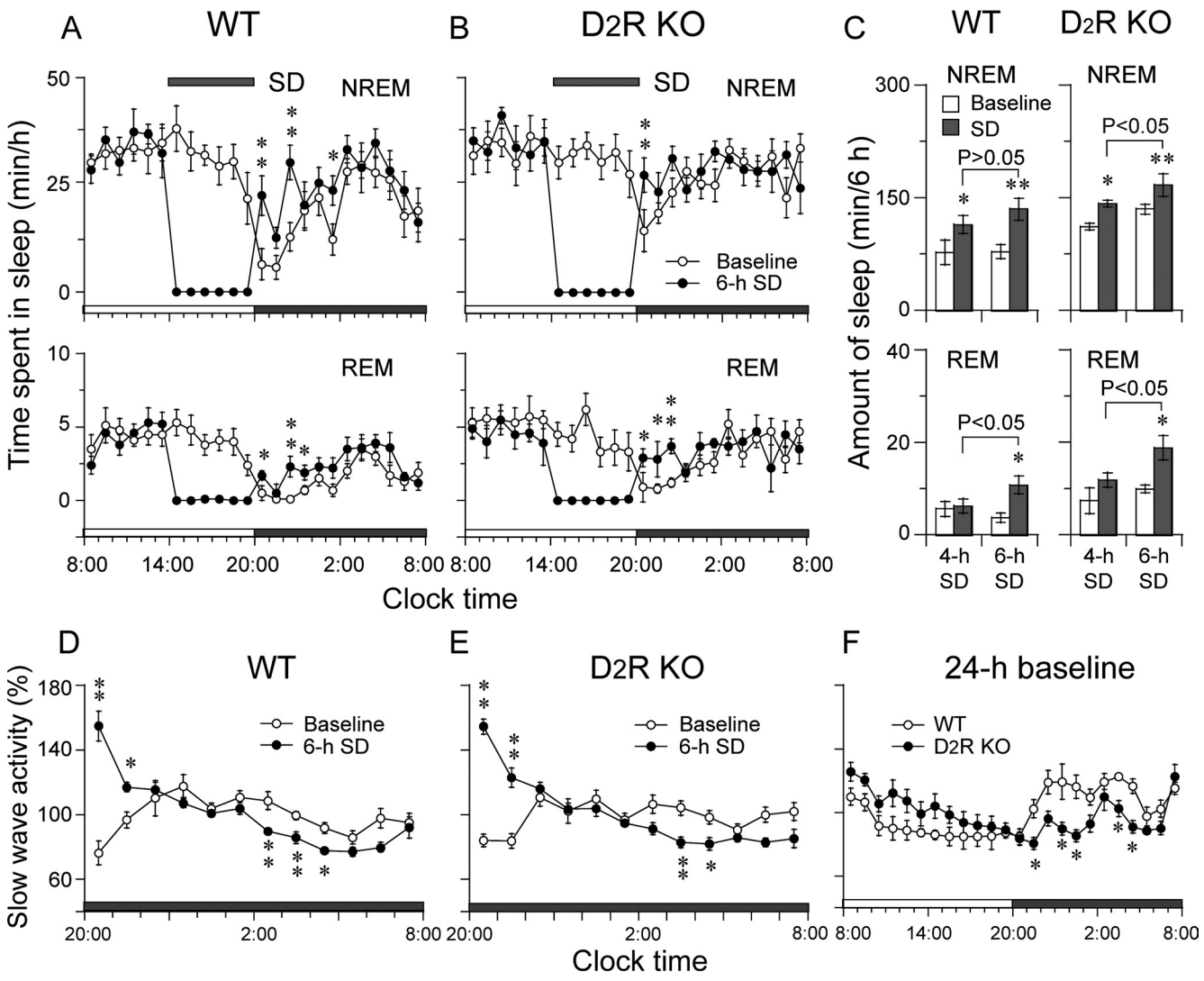

Figure 5. Sleep–wake profiles of WT and D_2R KO mice after SD. ***A, B***, Time course changes in NREM and REM sleep in WT (***A***) and D_2R KO (***B***) mice for 6 h SD (2:00 – 8:00 P.M.). Each circle represents the hourly mean amount of sleep. Open and closed circles stand for the baseline and SD profiles, respectively. The horizontal open and filled bars on the χ-axes indicate the 12 h light and 12 h dark periods, respectively. ***C***, Total amount of NREM and REM sleep for 6 h after 4 and 6 h SD. ***D–F,*** Slow-wave activity of NREM for 6 h after SD of WT (***D***) and D_2R KO (***E***) mice, and 24 h baseline (***F***). Each delta power of NREM sleep in the range of 0.75–4 Hz was first summated and then normalized as a percentage of the corresponding mean delta power of NREM sleep during 12 h (***D, E***) or 24 h baseline (***F***), respectively. Open and shaded columns or circles show the baseline and SD, respectively. Values are the means ± SEM ($n = 10$). $^*p < 0.05$, $^{**}p < 0.01$ compared with the corresponding baseline value or between the two genotypes, assessed by two-way ANOVA followed by the PLSD test.

particularly important role in maintaining wakefulness during the normal wake phase.

This has two implications. First, the use of D_2R agonist drugs has been found in patients with Parkinson's disease to cause sudden sleep attacks, but in humans these are mainly during the active (light) phase (Tan, 2003). Of course D_2R agonists are a two-edged sword, because they activate postsynaptic D_2R, but also reduce dopamine release via presynaptic D_2R (Monti et al., 1988; Ongini et al., 1993; Sebban et al., 1999), and thus may actually decrease dopamine availability at D_1R. Second, the flip-flop switch model of sleep regulation predicts that when either side of the sleep-arousal circuit is weakened (Saper et al., 2005; Lu et al., 2006), there should be an increase in the instability in both states (i.e., that we would see an increase in both wake to NREM transitions, but also in NREM to wake transition, a result that is counter-intuitive if one thinks of the D_2R effect as only being to promote arousal). Our results not only support that model, but also suggest that the dopamine system is an important part of the arousal side of the wake-sleep flip-flop switch.

Modafinil is the most potent wake-promoting medicine for enhancing the extracellular DA level in the nucleus accumbens and the prefrontal cortex, and for increasing wakefulness in rats (de Saint Hilaire et al., 2001; Murillo-Rodríguez et al., 2007). We recently reported that modafinil administered at 45, 90, and 180 mg/kg induced wakefulness for 3, 5, and 9 h, respectively, in WT mice, but only for 1, 3, and 6 h, respectively, in the D_2R KO mice (Qu et al., 2008). Similar results were found with GBR12909, a DAT inhibitor, given to the D_2R KO mice (Fig. 4). Therefore, D_2R plays an important role in wakefulness caused by an increased DA transmission.

It has been reported that mice lacking norepinephrine (Hunsley and Palmiter, 2003) or histamine (Parmentier et al., 2002; Hunsley and Palmiter, 2003) fall asleep rapidly after a mild stress, such as transfer to a new cage, demonstrating that these arousal neurotransmissions are necessary for responding to challenges of a new environment. DA neurons send their excitatory projections to the norepinephrinergic locus ceruleus, the histaminergic tuberomammillary nucleus, and other arousal regions (Monti and

Jantos, 2008), and thus the short bouts of wakefulness in D_2R KO mice may be a consequence of inadequate activation of these fundamental arousal regions due to the D_2R deficiency.

The new environment change test may be useful as an insomnia model to mimic the "first-night effect" transient insomnia. This effect is a well known phenomenon that is considered to result from a subject's lack of adaptation to an unfamiliar sleep environment. The D_2R antagonist raclopride significantly shortened the latency to sleep in WT mice. Also, we reported earlier that D_1/D_2 receptor-targeting L-stepholidine, an active ingredient of the Chinese herb *Stephonia*, significantly abridged sleep latency in mice (Qiu et al., 2009). These findings, together, indicate that D_2R plays an important role in the maintenance of wakefulness and that a D_2R antagonist may be useful to treat the "first-night effect" transient insomnia.

The target fields involved in the dopaminergic modulation of behavioral states remain unknown. The D_2R is the predominant D_2-like subtype in the brain and is expressed highly in the caudate–putamen, the nucleus accumbens, and the olfactory tubercle (Missale et al., 1998); where adenosine A_{2A} receptors are also densely expressed. There is increasing evidence that A_{2A}R plays an important role in sleep induction (Satoh et al., 1996; Hong et al., 2005; Huang et al., 2005). A_{2A}Rs abundantly expressed in the nucleus accumbens (Scammell et al., 2001) or the tuberculum olfactorium (Basheer et al., 2001) may be important for sleep induction. Here we demonstrated that D_2R is important for the maintenance of wakefulness in a mode different from A_{2A}R. Opposing effects of A_{2A}R and D_2R have also been shown at the levels of neurotransmitter release, receptor binding, and gene expression (Schiffmann et al., 2007). Therefore, A_{2A}R and D_2R are involved in sleep–wake regulation in a different and coordinate manner.

In conclusion, we found here that D_2R KO mice showed decreased wakefulness and increased stage transition between wakefulness and NREM sleep, and had difficulty in maintaining a long wake episode under baseline conditions. D_2R KO mice could not remain awake as long as their WT littermates, when exposed to a new environment, or after stimulation with a saline injection or a DAT inhibitor, the latter of which increases DA transmission in the brain. On the other hand, both genotypes respond to SD with an identical increase in sleep rebound and SWA during the subsequent period, suggesting that the homeostatic control of sleep in D_2R KO mice is not significantly affected. Thus we conclude that D_2R plays a critical role in the maintenance of wakefulness but not in the homeostatic regulation of sleep.

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