

How to Keep the Brain Awake? The Complex Molecular Pharmacogenetics of Wake Promotion

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Wake-promoting drugs are widely used to treat excessive daytime sleepiness. The neuronal pathways involved in wake promotion are multiple and often not well characterized. We tested *d*-amphetamine, modafinil, and YKP10A, a novel wake-promoting compound, in three inbred strains of mice. The wake duration induced by YKP10A and *d*-amphetamine depended similarly on genotype, whereas opposite strain differences were observed after modafinil. Electroencephalogram (EEG) analysis during drug-induced wakefulness revealed a transient ~2-Hz slowing of theta oscillations and an increase in beta-2 (20–35 Hz) activity only after YKP10A. Gamma activity (35–60 Hz) was induced by all drugs in a drug- and genotype-dependent manner. Brain transcriptome and clustering analyses indicated that the three drugs have both common and specific molecular signatures. The correlation between specific EEG and gene-expression signatures suggests that the neuronal pathways activated to stay awake vary among drugs and genetic background.

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INTRODUCTION

Excessive daytime sleepiness can be a serious and debilitating condition accompanying a wide range of sleep disorders such as narcolepsy, idiopathic hypersomnia, hypersomnia associated with psychiatric or neurological disorders, and breathing-related sleep disorders. Currently, modafinil is the most widely used wake-promoting drug to alleviate excessive daytime sleepiness, especially in the treatment of narcolepsy. In a substantial number of narcolepsy patients, modafinil is, however, not or only partially effective (Bastuji and Jouvet, 1988; Dauvilliers *et al*, 2002). As alternative wake-promoting compounds are not yet available, in ‘modafinil-resistant’ patients, amphetamines and amphetamine-like stimulants are prescribed despite the many known undesired side effects (Nishino and Mignot, 2005). Since the discovery of amphetamine’s stimulant action (Alles, 1933), alternatives are being developed acting on wake-promoting neurotransmission such as histamine and catecholamines (Barbier *et al*, 2004; Bonaventure *et al*, 2007; Boutrel and Koob, 2004; Parmen-tier *et al*, 2007).

In contrast to the pharmacology of sleep induction by GABAergic or GABA-modulating compounds, the mode of action of non-amphetaminic wake-promoting drugs remains poorly understood and might be achieved through a variety of pathways and mechanisms. YKP10A (aka R228060; [(R)–(β -amino-benzenepropyl) carbamate monohydrochloride]) is the hydrochloric acid salt of an optically pure phenylalanine derivative (see detailed information at: <http://www.wipo.int/pctdb/en/wo.jsp?wo=2006133393&IA=WO2006133393&DISPLA>), which has been developed for its potential antidepressant activity observed in animal models of depression (Gordon *et al*, 1998). YKP10A presents weak interactions at the dopamine transporter but no relevant interactions with serotonin or norepinephrine reuptake in receptor-binding studies (Gordon *et al*, 1998). Its antidepressant effect in humans is still under debate (Amsterdam *et al*, 2002). Among the most significant effects in human studies was insomnia (Amsterdam *et al*, 2002) and based on preliminary data, YKP10A was also shown to have wake-promoting action in rats (A Ahnaou and W Drinkenburg; unpublished observations). YKP10A’s mode of action is unclear but may affect dopamine neurotransmission as its clinical profile resembles that of the dopamine-reuptake inhibitors nomifensine and bupropion (Amsterdam *et al*, 2002) although YKP10A does not appear to greatly reduce dopamine reuptake (Gordon *et al*, 1998). Also, modafinil is believed to act through the dopaminergic system although its precise mode of action remains unclear (Ballon and Feifel, 2006; Minzenberg and

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Carter, 2008; Parmentier *et al*, 2007; Wisor and Eriksson, 2005). Amphetamine is known to increase catecholamine release and inhibit their reuptake (Nishino and Mignot, 2005).

Differences in genetic background are likely to underlie part of the variation in the response to modafinil mentioned above. Numerous drug studies both in mice and humans have demonstrated that genetic-background differences in drug efficacy and their side effects are more a rule than an exception. Genetic background is therefore a crucial variable when evaluating novel drugs. Examples of specific gene polymorphism or transgenic models associated with altered efficacy of stimulants are, for amphetamine, the dopamine transporter (Lott *et al*, 2005; Wisor *et al*, 2001), *homer1a* (Tappe and Kuner, 2006), casein kinase I- ϵ (Palmer *et al*, 2005), and brain-derived neurotrophic factor (BDNF) (Flanagin *et al*, 2006); for modafinil, catechol-O-methyltransferase (Dauvilliers *et al*, 2002) and the dopamine transporter (Wisor *et al*, 2001); and for caffeine, the adenosine A2a receptor (Alsene *et al*, 2003; Retey *et al*, 2007). The finding that casein kinase I- ϵ is involved in the response to amphetamine in mice (Palmer *et al*, 2005) was recently confirmed in humans (Veenstra-VanderWeele *et al*, 2006), demonstrating that the mouse model can be of direct relevance for predicting genetic-background effects in humans. Similar pharmacogenetic approaches in mice have been used to predict the response to antidepressant drugs in humans (Crowley *et al*, 2006).

Here we evaluate the wake-promoting efficacy of three stimulants in three inbred strains of mice. We have shown earlier that the compensatory sleep response and the changes in brain gene expression after enforced wakefulness greatly differ among these three genetic backgrounds (Franken *et al*, 2001, 1999; Maret *et al*, 2007). To gain insight into how wake-promoting compounds keep the brain awake, we emphasize first the spectral composition of the electroencephalogram (EEG) during drug-induced wakefulness, and second their effects on brain gene expression. Our results indicate that each stimulant leaves a genotype-dependant signature on both EEG and gene expression. Moreover, wakefulness induced by each drug might be qualitatively different as some induce slow theta oscillations whereas the others a high-frequency EEG activity, reminiscent of EEG activity during demanding cognitive tasks.

MATERIALS AND METHODS

Animals

Adult male mice from three inbred strains, DBA/2J (D2), C57BL/6J (B6), and AKR/J (AK), were used in this study. All mice (age: 10–11 weeks at the time of surgery; weight: 21–32 g) were purchased from Jackson Laboratory (Maine). Mice were kept individually in polycarbonate cages (31 cm \times 18 cm \times 18 cm) with food and water available *ad libitum*, and maintained on a 12-h light–dark cycle (lights on at 0900 hours) at an ambient temperature of 24.5–25.5°C. The protocols used were approved by the Veterinary Office of the Canton of Vaud, Switzerland.

Surgery

EEG and EMG electrodes were implanted under deep anesthesia with a mixture of ketamine and xylazine (intraperitoneal, 75 and 10 mg/kg, respectively, at a volume of 8 ml/kg). Two gold-plated miniature screws (diameter, 1.1 mm) served as EEG electrodes and were screwed into the cranium over the right cerebral hemisphere, in a frontoparietal position (according to Franken *et al*, 1998). Four additional anchor screws were implanted; one over the right hemisphere and three over the left hemisphere. Two semirigid gold wires served as EMG electrodes and were inserted between two neck muscles. The EEG and EMG electrodes were soldered to a connector and the anchor screws were cemented to the skull. Four to 8 days of recovery from surgery was allowed before animals were connected to the recording leads. A minimum of six adaptation days were scheduled before data collection.

Protocol

All mice used for EEG/EMG analyses were recorded continuously for 72 h using EMBLA™ hardware and Somnologica-3™ software, both developed by Flaga (Island). The first day served as baseline and was initiated at light onset (0900 hours); the second day was considered as the control condition with a saline injection; and the third day, a drug was administered. Sample sizes were the following: YKP10A: $n = 20, 18$, and 19 ; *d*-amphetamine: $n = 6, 8$, and 8 ; and modafinil $n = 7, 8$, and 8 , for D2, B6, and AK mice, respectively.

Determining Drug Efficacy

YKP10A (Johnson and Johnson, Beerse, Belgium) and *d*-amphetamine were dissolved in saline solution (NaCl 0.9%). Modafinil was not dissolved but suspended in saline solution by vortexing and injecting immediately to each mouse. Drug solutions were freshly prepared the same day before light onset. Shortly after light onset (0901 to 0917 hours), all drug and saline (serving as controls) solutions were administered by intraperitoneal injection at a volume of 5 ml/kg body weight.

B6 mice were used to determine a YKP10A dose that would induce a period of wakefulness in the same range as our standard sleep-deprivation protocol; that is 6 h (from 9:00 to 15:00). Five doses were administered: 25 ($n = 9$), 50 ($n = 9$), 100 ($n = 10$), 150 ($n = 6$), and 200 mg/kg ($n = 6$). To determine equally effective doses for *d*-amphetamine and modafinil, B6 mice received five (1 ($n = 3$), 2 ($n = 3$), 4 ($n = 4$), 5 ($n = 5$), and 8 mg/kg ($n = 5$)) and three doses (100 ($n = 5$), 200 ($n = 5$), and 300 mg/kg ($n = 6$)), respectively. Each mouse received one drug only (preceded by one saline injection). Sleep onset was defined as the time elapsed between the time of injection and the first sleep episode lasting at least 1 min and not interrupted by more than two 4-s epochs scored as wakefulness. On the basis of the dose-response curves thus obtained in B6 mice (Figure 1), we decided on a dose of 150 mg/kg for YKP10A, 6 mg/kg for *d*-amphetamine, and 150 mg/kg for modafinil, for all subsequent studies.

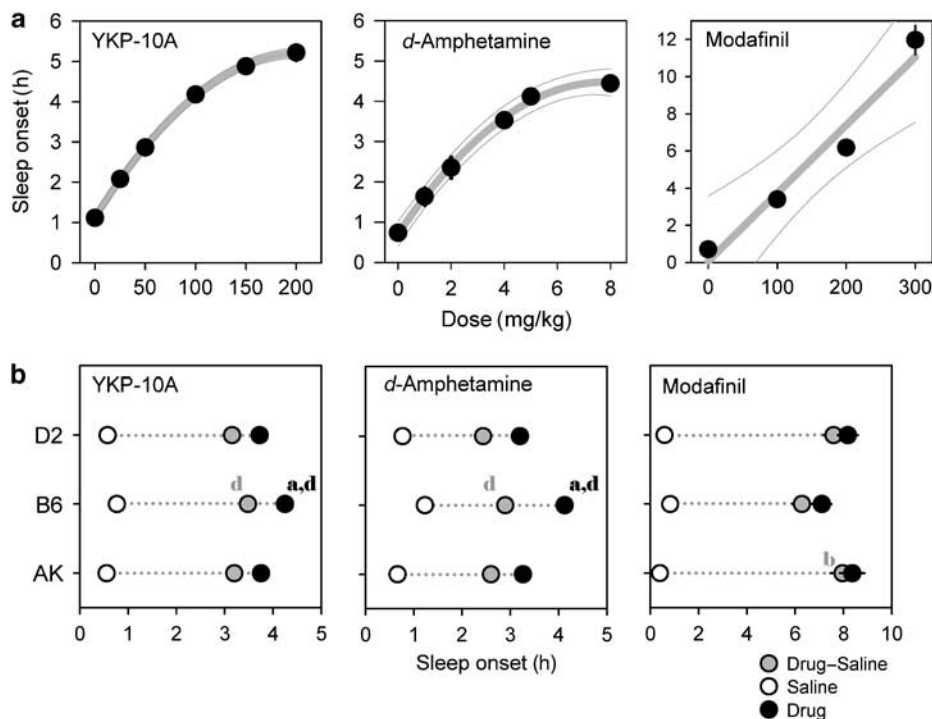


Figure 1 Effects of saline, YKP10A, *d*-amphetamine, and modafinil on sleep onset. (a) Dose–response relationship for the delay in sleep onset in B6 mice for three wake-promoting drugs. Symbols represent mean (± 1 SEM; $n = 16$ –40, 6–10, 3–5, and 5–6 for saline, YKP10A, *d*-amphetamine, and modafinil, respectively; see Materials and Methods for details) values for sleep onset after light onset (thick gray lines represent first- and second-order fitted curves; thinner lines delimit the 95% confidence intervals). (b) Strain distribution pattern of the effects of a single injection of YKP10A (150 mg/kg), *d*-amphetamine (6 mg/kg), and modafinil (150 mg/kg) in three inbred strains of mice (D2, B6, and AK). Symbols represent mean (± 1 SEM; YKP10A: $n = 20$, 18, and 19; *d*-amphetamine: $n = 6$, 8, and 8; modafinil: $n = 7$, 8, and 8; for D2, B6, and AK, respectively) post-injection sleep-onset times. Open symbols denote saline control injection, closed black symbols drug injection, and gray symbols represent the mean difference between the effect of drug and saline injection within individual mice. Characters in superscript (a, b, and d) indicate significant strain differences from AK (a), B6 (b), or D2 (d); $P < 0.05$; *t*-tests. Note that SEMs are smaller than symbol size in most cases and that the time axis for modafinil is twice those of the other drugs.

EEG Analyses

Analog EEG and EMG signals were digitized at 2000 Hz and subsequently stored at 200 Hz on hard disc. The EEG was subjected to a discrete Fourier transformation, yielding power spectra (range: 0.25–90 Hz; resolution: 0.25 Hz; window function: hamming) for consecutive 4-s epochs.

The animal's behavior was classified as REM sleep, NREM sleep, or wakefulness, for consecutive 4-s epochs, on the basis of the EEG and EMG signals according to the standard criteria. REM sleep is characterized by regular theta oscillations (5–9 Hz) in the EEG and a low-amplitude EMG. During NREM sleep, the EEG amplitude is higher and dominated by both delta (1–4 Hz) and theta frequency components, whereas the EMG activity remains low. Wakefulness is characterized by a high and variable EMG activity and a low-amplitude mixed-frequency EEG signal. The behavioral states were determined by the visual inspection of the EEG and EMG signals, which were displayed on a PC screen. Four-second epochs containing EEG artifacts were marked so that they could be excluded from EEG spectral analyses.

Sleep onset was determined in all animals recorded. For quantitative EEG analyses, some animals had to be discarded due to artifacts in the EEG (YKP10A: $n = 15$, 15, 15; *d*-amphetamine: $n = 6$, 6, 8; modafinil: $n = 6$, 8, 7; for D2, B6, and AK, respectively were used for all quantitative EEG analyses).

The spectral changes in the waking EEG after drug injection were determined for artifact-free wakefulness (mean = $96.3 \pm 0.67\%$ of recording time) occurring between the time of injection and sleep onset. EEG spectra were expressed individually as a percentage of the wakefulness spectra in the 12-h baseline light period (range: 0.75–90 Hz; resolution: 0.25 Hz; values between 48.25 and 51.75 Hz were excluded due to 50 Hz noise in some of the recordings). The time course of spectral EEG changes during the drug-induced wakefulness was assessed by dividing this period into 12 intervals (for YKP10A and *d*-amphetamine) or 24 intervals (for modafinil) to which an equal number of 4-s waking epochs contributed within individual mice (ie, percentiles).

To further quantify the spectral changes in the waking EEG, the following frequency ranges were defined: theta: 4.75–9.75 Hz, beta-2: 20.25–34.75 Hz, gamma: 35.25–59.75 Hz (excluding 48.25–51.75 Hz), and 'high-frequency': 60.25–80.0 Hz. Mean EEG power in these frequency bands was expressed as a percentage of the EEG power reached in that band during wakefulness in the 12-h baseline light period. To quantify changes in EMG, calculated as signal variance, values were similarly expressed relative to the baseline light period. Owing to movement artifacts in the EMG, especially during the *d*-amphetamine and modafinil conditions, the EMG of several mice could not be used (YKP10A: $n = 11$, 15, 9; *d*-amphetamine: $n = 3$, 2, 5; modafinil: $n = 3$, 5, 3; for D2, B6, and AK were used, respectively).

Marked changes in theta-peak frequency in the waking EEG were observed after YKP10A. The frequency at which EEG power density was maximal within the theta band was visually determined for each animal and interval. Theta power at peak frequency was calculated by averaging power in the 1 Hz range surrounding the individually determined theta peak (± 0.5 Hz). Theta power after drug administration was expressed as a percentage of that observed after saline injection.

Drug effects on recovery of sleep were assessed as follows. Recovery patterns for sleep time were summarized as accumulated differences from the saline condition for consecutive hours starting at light onset. REM sleep-onset latency was defined as the time elapsed between sleep onset and the first, uninterrupted REM sleep episode lasting at least 8 s. EEG delta power during NREM sleep was calculated by averaging power density in the frequency bins from 1 to 4 Hz. Values were individually normalized by expressing them as a percentage of the mean delta power over 4-s epochs scored as NREMS in the last 4 h of the baseline light period; that is the lowest average level reached during baseline. The time course of EEG delta power in NREM sleep was assessed by dividing the 12-h period into 12 (for the baseline and the saline light periods), 8 (for light post-YKP10A/amphetamine period), 5 (for light post-modafinil period), or 6 (for the dark periods) intervals to which an equal number of 4-s epoch scored as NREM sleep contributed within individual mice.

Microarray Analyses

YKP10A and modafinil (both at 150 mg/kg) and *d*-amphetamine (6 mg/kg) were administered at light onset. Control animals (same number for each drug) were injected with saline and sleep deprived by gentle handling for 5 h. To minimize the confounding effect of sleep deprivation by gentle handling, saline- and drug-injected animals were observed side-by-side and each time an intervention was necessary to keep saline-injected animals awake, the same intervention also being introduced in drug-injected animals. All animals were killed by cervical dislocation, the brain was rapidly removed, frozen on dry ice, and stored at -80°C . RNA from whole brain was isolated and purified with the RNeasy Maxi kit (Qiagen) and DNase treated. All RNA quantities were assessed with a NanoDrop ND-1000 spectrophotometer, and the quality of RNA was controlled on Agilent 2100 Bioanalyzer chips. Experiments were performed separately for each drug and therefore control animals were repeated for each drug treatment. Equal amounts of total RNA were pooled from three mice within each of the 18 experimental groups (three strains, three drugs, and three corresponding sleep deprivations; all in biological triplicate: $18 \times 3 = 54$ chips). Microarray was performed as described (Maret *et al*, 2007). Briefly, 3 μg was used for labeling. Twelve micrograms of amplified, biotinylated cRNA from each sample were fragmented and hybridized to GeneChip Mouse 430 2.0 arrays. RMA normalization was performed separately for each drug treatment (Irizarry *et al*, 2003). For each drug treatment and each strain (nine conditions), differentially expressed features compared with sleep-deprived control (three chips vs three chips) were identified using Limma (Smyth, 2004).

The moderated *t*-statistics calculated by Limma were combined in one matrix with rows (probe sets) ordered according to the variance of the moderated *t*-statistics across the nine comparisons. This procedure allows comparing the results across the three experiments. Two-way complete linkage hierarchical clustering was performed using Pearson correlation distance metric. Cluster significance was assessed using the R package Pvcust (Suzuki and Shimodaira, 2006).

c-FOS Immunohistochemistry

A preliminary neuroanatomic comparative experiment between the three stimulants and saline was realized only in B6 mice to analyze the c-FOS activation pattern in brain sections 2 h after YKP10A, modafinil (150 mg/kg for both), and *d*-amphetamine (6 mg/kg) injection at light onset. Control animals were injected with saline and sleep deprived by gentle handling for 2 h. Immunostaining experiment was performed according to Verret *et al*, (2005).

RESULTS

YKP10A Delays Sleep Onset Without Stereotypy or Increased Locomotion

We first assessed whether YKP10A could delay sleep onset without undesirable effects in C57BL/6J (B6) mice. During the initial dose-response experiments (Figure 1a), video and EEG recordings were carefully screened for abnormal behavior and EEG activity. Even at the highest dose (200 mg/kg), no signs of paroxysmal or otherwise abnormal EEG activity or stereotyped behavior were noted. Instead, mice were initially quiet while alert with eyes open responding to stimuli (mobile) and attentive without pronounced locomotor activity; this is in stark contrast to *d*-amphetamine, which, at 6 mg/kg, initiated hyperactivity with stereotypic movements such as licking and repeated attempts to climb the sides of the cage. After this initial effect of YKP10A, which lasted for 30–40 min (at 150 mg/kg), activity levels returned to the levels normally associated with wakefulness.

We then compared the wake-promoting effects of the three drugs in B6 mice to determine an equipotent dose. YKP10A, *d*-amphetamine, and modafinil all produced dose-dependent increases in the duration of EEG-defined wakefulness well beyond the waking induced by the saline control injections (Figure 1a). For both YKP10A and *d*-amphetamine, a maximal attainable response was reached within the range of concentrations tested, whereas this was not the case for modafinil. For subsequent experiments, we selected a close-to-maximum dose of 150 and 6 mg/kg, for YKP10A and *d*-amphetamine, respectively, anticipating a 4- to 5-h period of sustained wakefulness. On the basis of the dose-response relationship, a 150 mg/kg dose was chosen for modafinil, anticipating a comparable wake duration.

The efficacy of these three wake-promoting drugs was compared among three inbred strains of mice; that is B6, DBA/2J (D2), and AKR/J (AK), at the doses established above for B6. In the new set of B6 mice, we could reproduce the effects of 150 mg/kg of YKP10A on sleep onset and we successfully matched this effect with a 6 mg/kg dose of

d-amphetamine (Figure 1b). For modafinil, 150 mg/kg proved to be too high a dose, resulting in a ~7-h delay in sleep onset instead of the anticipated 4–5 h (compare Figure 1a and b).

YKP10A and *d*-amphetamine delayed sleep onset in a similar, genotype-dependent fashion (one-way ANOVA, factor 'genotype': $F_{2,54} = 11.8$ for YKP10A, $F_{2,19} = 28.7$ for

d-amphetamine; $P < 0.0001$ for both drugs). This difference was due to B6 mice staying awake longer compared with D2 and AK mice after both drugs (Figure 1b). A similar strain-distribution pattern in sleep-onset times was, however, observed after saline injections (Figure 1b). Controlling for this effect reduced the strain differences in sleep onset observed for both drugs, and B6 mice no longer differed

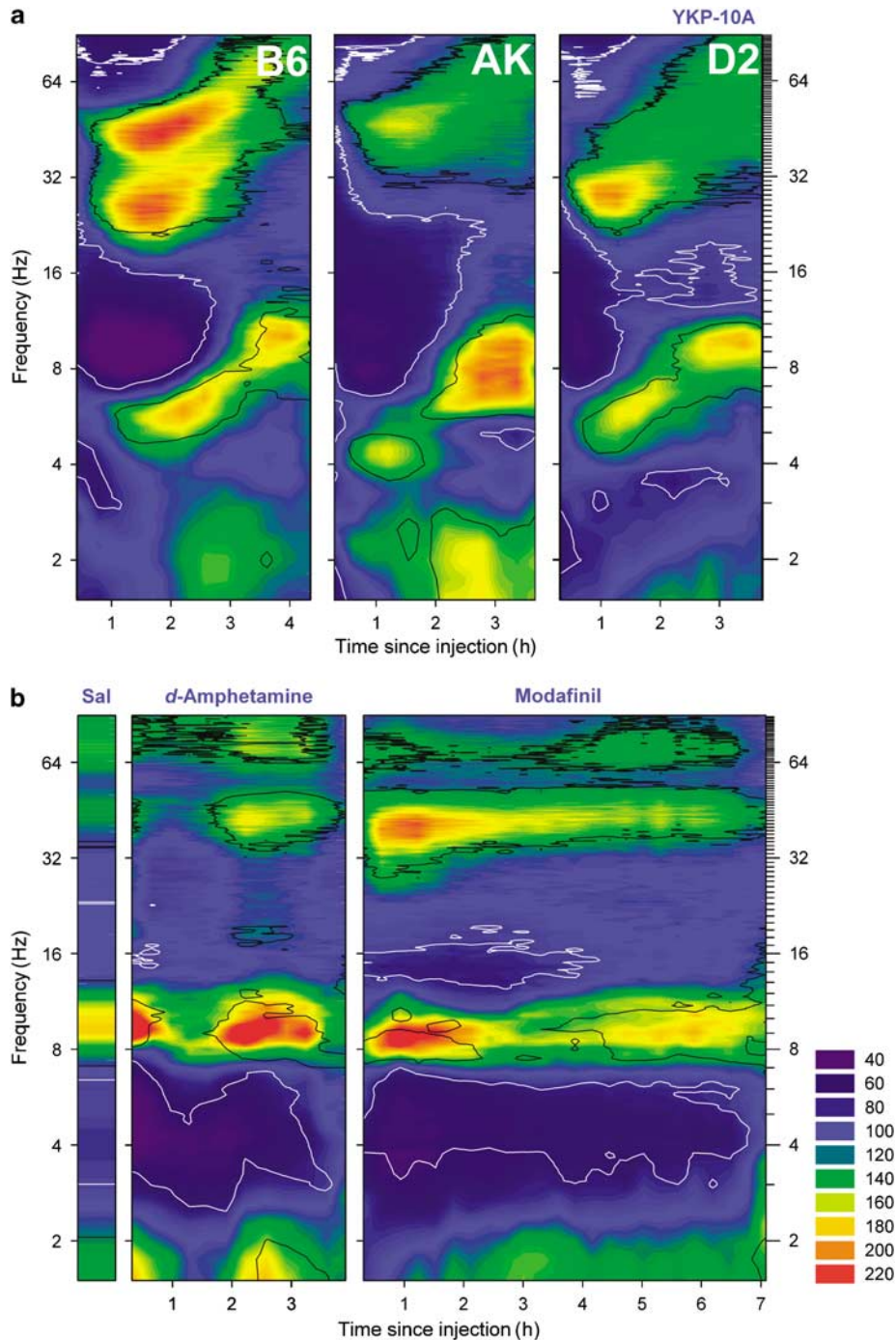


Figure 2 Time course of the effects of saline, YKP10A, *d*-amphetamine, and modafinil on the EEG spectral profiles during wakefulness between time of injection and sleep onset. Mean EEG spectra within 12 (or 24 for modafinil) intervals were expressed as a percentage of the mean spectral profile for the waking EEG in the baseline light period. Changes from baseline are plotted as a heat map, with darker colors marking reduced EEG power density compared with baseline, light-blue values similar to baseline (100%), and warmer colors (green red) increases. (a) EEG spectral changes induced by YKP10A in B6, AK, and D2 mice ($n = 15$ for each inbred mice). (b) EEG spectral changes induced by saline ($n = 29$), *d*-amphetamine ($n = 6$), and modafinil ($n = 8$) in B6 mice. Black and white contour lines delimit areas within which EEG power density was significantly decreased and increased, respectively ($P < 0.01$; paired *t*-tests).

from AK mice (see 'drug-saline difference' in Figure 1b). Although not significant, an opposite strain-distribution pattern was apparent for sleep-onset times after modafinil administration (one-way ANOVA; $F_{2,20} = 2.3$, $P = 0.12$; Figure 1b). For this drug, controlling for saline strain differences effectively increased the strain differences, and the drug-saline difference became significantly greater in AK mice compared with B6 mice (Figure 1b).

Drug-Induced EEG Changes During Wakefulness

To evaluate and compare the drug effects on the waking EEG among the three inbred strains we calculated EEG spectra over a wide frequency range (0.75–90 Hz) from the time of injection to sleep onset. Spectra were expressed relative to the EEG spectra of spontaneous wakefulness observed during the baseline light period (Supplementary Figure S1). Of the many significant drug and strain differences we observed, only the most salient differences will be discussed below.

Saline, *d*-amphetamine, and modafinil injections were all followed by an immediate increase in theta activity, significant in the 7–11 Hz range, compared with baseline (Figure 2). Increases up to threefold were observed for *d*-amphetamine and modafinil and were present for most of the time mice stayed awake. In contrast, after YKP10A administration, a profound, two- to threefold reduction in EEG activity in this frequency range was observed that lasted between 1.5 and 2.5 h (Figure 2). Besides this decrease that affected EEG power density in the 7–20 Hz range, no other changes were observed for the first two intervals (ie, ~40 min). One hour after YKP10A injection, an increase in theta activity appeared at the low end of the theta frequency range (4–7 Hz) in all three strains (Figure 2), and theta oscillations significantly slowed by ~2 Hz compared with saline (Figure 3a). At the same time, EEG activity in the beta-2 (20–35 Hz) and gamma (35–60 Hz) frequency bands markedly increased lasting for ~2 h (Figures 2 and 3b). Both the slowing of the theta oscillations and the increase in beta-2 activity were not observed after *d*-amphetamine or modafinil. Interestingly, the increase in beta-2 activity after YKP10A administration greatly depended on genetic background and was absent in AK mice. During the last 1–1.5 h of the waking period induced by YKP10A, the EEG spectra of the three strains resembled those observed after saline, *d*-amphetamine, and modafinil injections, with an elevated theta activity in the 7–11 Hz range and increases in EEG activity in the gamma range and even higher frequencies ('60–80 Hz'; Figures 2 and 3b).

The high-frequency EEG changes did not seem to be confounded by changes in muscle tone. In general, the time course of the changes in EMG differed from that in the beta-2, gamma, and '60–80 Hz' frequency bands. This is best illustrated for the YKP10A experiments for which we observed profound changes in all four variables (Figure 3). YKP10A administration resulted in an immediate and profound reduction in EMG during wakefulness that gradually returned to basal levels over the course of the first 2–3 h after injection (Figure 3c). This decrease in muscle tone coincided with the reduction in locomotor activity noted by video observations (see above). In contrast, after *d*-amphetamine, an immediate increase in EMG was observed, whereas after modafinil no change or a reduction in EMG levels could be seen.

Recovery of Sleep After Drug-Induced Wakefulness

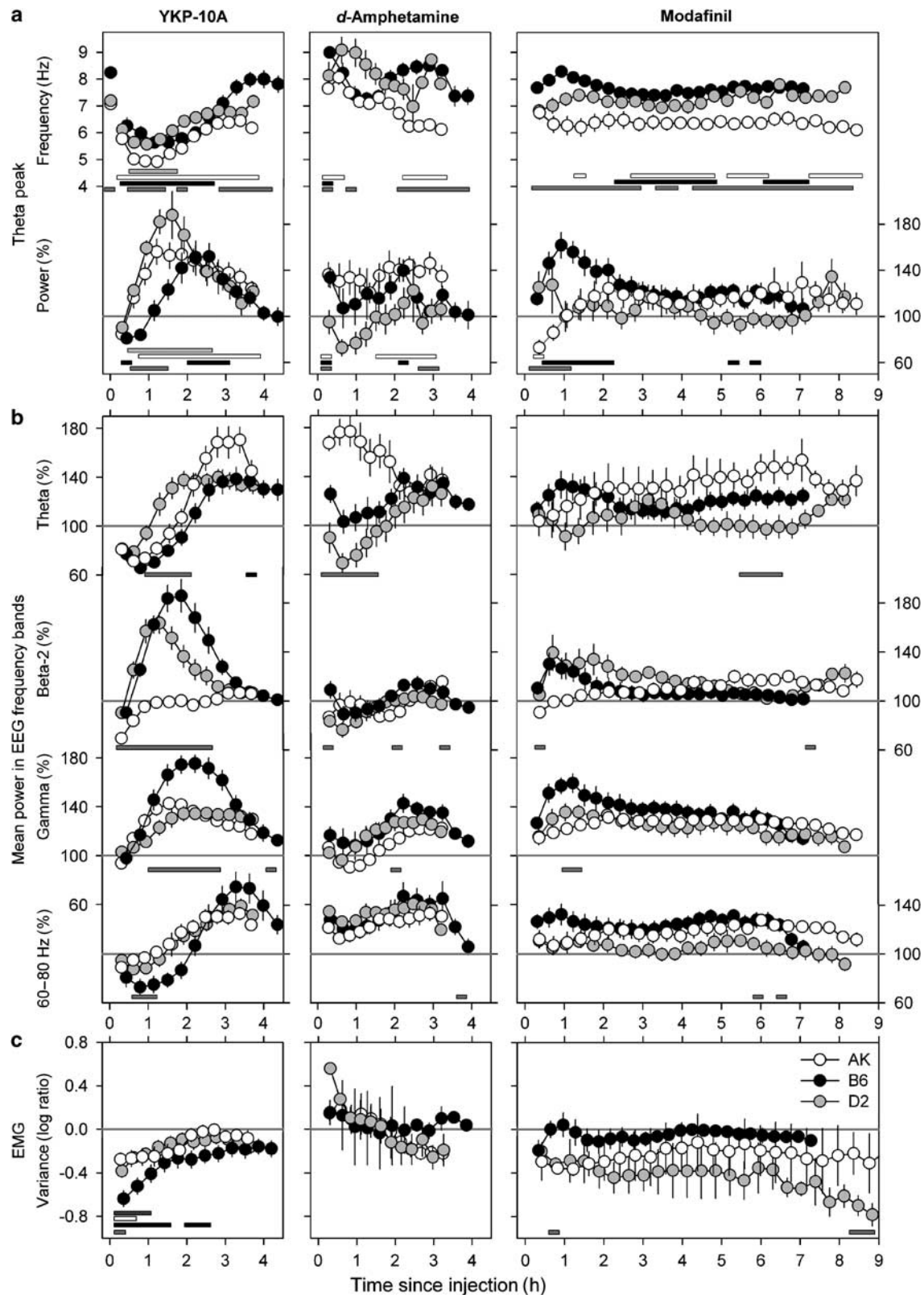
To evaluate a wake-promoting compound it is essential to determine its impact on subsequent sleep. We therefore analyzed drug effects on recovery of sleep time lost, on EEG delta power, an index of sleep need, and on REM sleep latency (Supplementary Figure S2). The dynamics of the recovery of sleep time lost after administering the three wake-promoting drugs was analyzed by accumulating the hour-by-hour differences in sleep time between the drug and saline conditions. Thus analyzed negative slopes of accumulation reflect a loss in sleep time and positive slopes a gain relative to the saline condition (Figure 4). The comparison of the recovery patterns among the drugs was confounded by the fact that with the modafinil dose used here, animals stayed awake longer than anticipated, allowing for direct comparisons between YKP10A and *d*-amphetamine only.

Recovery patterns differed between sleep states and among drugs and strains. In general, recovery of REM sleep time varied less with strain, and recovery seemed to be regulated 'better' as compared with that of NREM sleep time insofar that REM sleep deficits were smaller 24 h after drug injection (Figure 4). Thus, after YKP10A administration, REM sleep recovery was complete (ie, reaching 0 level) by hour 21 for all three strains, whereas for NREM sleep, this was true only for D2 mice; that is B6 and AK mice still showed a ~1-h deficit at hour 24 with respect to the saline condition (Figure 4). Even after modafinil, despite a larger sleep loss and a shorter recovery period, recovery of REM sleep was almost complete by hour 24, whereas for NREM sleep a 1.5- to 3.5-h deficit remained. The strain differences in NREM sleep recovery observed after modafinil can to a large extent be explained by the fact that recovery sleep

Figure 3 Time course of the effects of YKP10A, *d*-amphetamine, and modafinil on EEG theta-peak frequency and power (a), EEG power in selected EEG bands (b), and EMG variance during wakefulness between time of injection and sleep onset (c; see Materials and Methods and Figure 2 for details). Symbols represent mean (\pm SEM) values. (a) Theta-peak frequency (upper curve) after drug injection and after saline injection (first three dots plotted at time = 0 h). Mean EEG power at theta peak was expressed as percentage of the theta peak power obtained after saline condition (= 100%). Note that the frequency range over which theta power is calculated moves as theta-peak frequency changes. (b) Mean EEG power in theta (upper), beta-2 (second), gamma, and 'high-frequency' (bottom curve) bands was expressed as percentage of baseline as in Figure 2 (for a and b, saline: $n = 27, 29, 30$; YKP10A: $n = 15, 15, 15$; *d*-amphetamine: $n = 6, 6, 8$; modafinil: $n = 6, 8, 7$; for D2, B6, and AK, respectively). (c) EMG variance was expressed relative (log ratio) to baseline (= 0) (YKP10A: $n = 11, 15, 9$; *d*-amphetamine: $n = 3, 2, 5$; modafinil: $n = 3, 5, 3$; for D2, B6, and AK, respectively). Gray, white, and black bars at the bottom indicate intervals in which theta-peak frequency differed from saline and EMG differed from baseline in D2, AK, and B6 mice, respectively ($P < 0.01$; paired *t*-tests). Dark-gray bars mark intervals in which the drug effect differed according to genotype ($P < 0.01$; one-way ANOVA). Open symbols: AK, black symbols: B6, gray symbols D2 mice.

started ~1 h earlier in B6 mice (Figure 4, see Figure 1). Interestingly, *d*-amphetamine was followed by an overcompensation of REM sleep time in all three strains and an overcompensation of NREM sleep in D2 and AK mice but not in B6 mice.

For all three drugs, the compensation of NREM sleep occurred mostly during the dark period. During the light period, recovery patterns differed among strains. Both after YKP10A and *d*-amphetamine, no recovery of the NREM sleep time lost was observed in B6 mice, whereas REM sleep



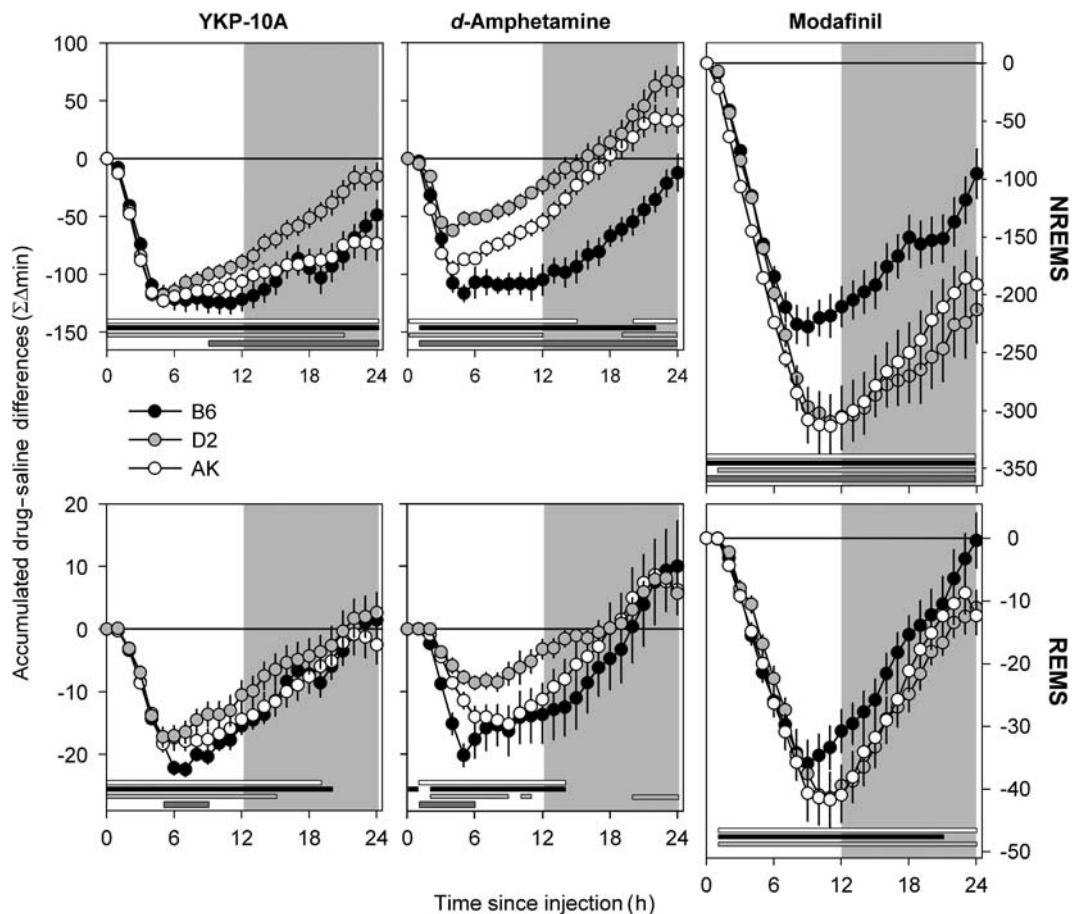


Figure 4 Time course of accumulated differences in sleep time between the drug (YKP10A, *d*-amphetamine, and modafinil) and saline conditions. NREMS (upper) and REMS values (lower panels; mean \pm 1 SEM; YKP10A: $n = 15, 15, 15$; *d*-amphetamine: $n = 6, 6, 8$; modafinil: $n = 6, 8, 7$; for D2, B6, and AK, respectively) were expressed relative to saline conditions (black horizontal line). White (AK), black (B6), and gray (D2) bars at the bottom indicate intervals in which the accumulated difference differed from saline ($P < 0.05$; paired *t*-tests). Dark-gray bars mark intervals in which the drug effect differed according to genotype ($P < 0.05$; one-way ANOVAs). Gray areas denote the dark period.

did increase during this period (Figure 4). During the 4–5 h of recovery time that remained during the light period after modafinil administration, none of the strains significantly reduced their NREM or REM sleep deficit; if at all, in D2 and AK mice, the NREM sleep deficit further increased during this period.

EEG Delta Power During NREM Sleep

EEG delta power is widely used as an index of sleep need. It is thus of interest to know whether the wakefulness induced by the three drugs affected EEG delta power in subsequent NREM sleep to the same extent. Irrespective of treatment and genotype, the level of EEG delta power showed its typical declining trend during the light periods when NREM sleep is prevalent and increased during the dark period when mice are mostly awake (Figure 5). Saline injections did not greatly impact EEG delta power although subtle changes were observed that are relevant for interpreting the drug effects (see below); in the first interval after the saline injection, EEG delta power was significantly increased relative to baseline in B6 mice and decreased in AK mice (Figure 5; statistics not shown). In addition, values in the

first two intervals of the dark period were lower than baseline for B6 and D2 mice.

As expected, drug-induced wakefulness was followed by an increase in EEG delta power. YKP10A induced a higher EEG delta power in all three strains during the first four intervals after sleep onset compared with the first four intervals after sleep onset in the saline condition (Figure 5). Also, after *d*-amphetamine, an initial increase over saline was observed, but this increase was less pronounced and limited to the first interval and significant only for AK and D2 mice. The lack of an increase in B6 mice could have been due to the fact that only for this strain EEG delta power was already significantly increased after the saline injection. The relative increase in EEG delta power immediately after sleep onset did not significantly differ between the YKP10A and *d*-amphetamine conditions but later on during the light period the relative values after *d*-amphetamine were lower than after YKP10A only for AK (intervals 3, 6–7 after sleep onset; minimum $F_{1,21} = 4.4$) and D2 (intervals 3–4; minimum $F_{1,19} = 5.7$, $P < 0.05$; *t*-tests). As EEG delta power decreases during NREM sleep, the lower relative values observed after *d*-amphetamine might be a consequence of the smaller deficit in NREM sleep time during the light period observed after *d*-amphetamine as compared with

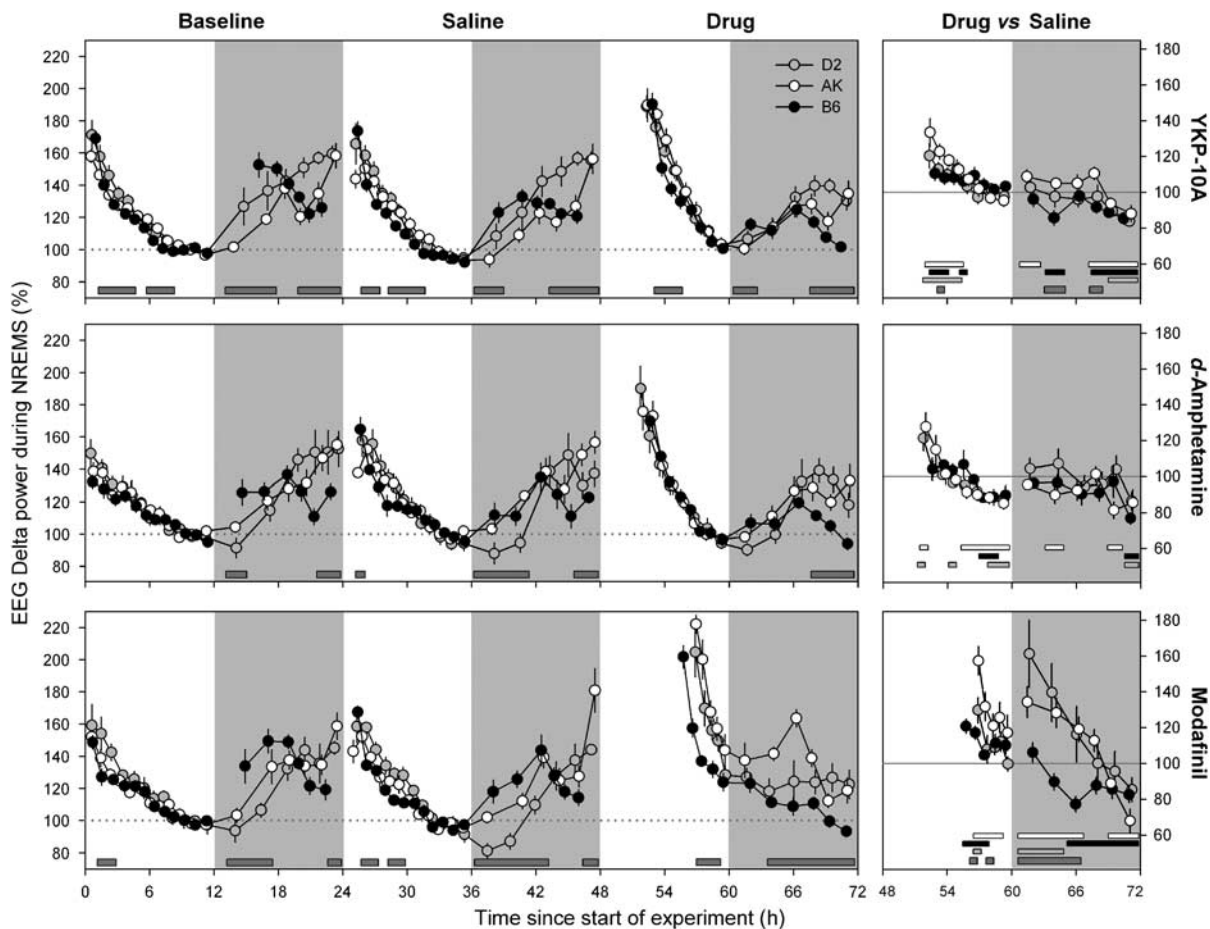


Figure 5 The effects of YKP10A, *d*-amphetamine, modafinil, and saline injections on the time course of EEG delta power during NREM sleep. Left panels: EEG delta power (mean \pm SEM; saline: $n = 27, 29, 30$; YKP10A: $n = 15, 15, 15$; *d*-amphetamine: $n = 6, 6, 8$; modafinil: $n = 6, 8, 7$; for D2, B6, and AK, respectively) was expressed relative to the last 4 h of the baseline light period ($= 100\%$). Dark-gray bars at the bottom of each panel mark intervals in which significant strain differences were observed ($P < 0.05$; one-way ANOVAs). Right panels: changes in EEG delta power after drug injection expressed as a percentage of the saline condition. In the light periods, corresponding intervals were compared with respect to sleep onset; in the dark period with respect to dark onset. Significant differences from saline are marked by colored bars (same color coding as in Figure 4; $P < 0.05$; paired *t*-tests). Dark-gray bars indicate intervals in which the drug–saline difference differed among strains ($P < 0.05$; one-way ANOVA).

YKP10A (see Figure 4). The same effect might underlie the decrease in EEG delta power below saline levels observed in the last two intervals of the dark period (Figure 5) because most of the NREM sleep time lost was compensated during this period (Figure 4). This decrease below saline levels was observed also for modafinil (Figure 5).

Modafinil caused a significant increase in EEG delta power above saline levels in all three strains (Figure 5). Consistent with the longer waking period induced by modafinil, levels reached in the first interval after sleep onset seemed higher compared with those observed after the other two drugs. Significant differences were, however, observed only for AK (modafinil vs YKP10A; $F_{1,20} = 4.2$ and *d*-amphetamine; $F_{1,13} = 12.1$) and for B6 (modafinil vs *d*-amphetamine; $F_{1,12} = 12.9$, $P < 0.05$; *t*-tests).

Drug Effects on Brain Gene Expression

The differential effects of the three wake-promoting drugs and three genotypes on brain mRNA levels were assessed by microarray analysis. It should be emphasized that the drug-induced changes in gene expression reported here were

contrasted to the levels reached in mice that were injected with saline and then kept awake by gentle handling for the same duration. This way, the profound changes in gene expression due to extended waking *per se* (Maret et al, 2007) are accounted for.

Only few of the $\sim 45\,000$ probe sets tested were found to be significantly affected by the drug condition (Supplementary Figure S3). The number of genes that changed expression after modafinil and YKP10A was higher for D2 compared with the other two strains. Especially, in B6 mice, very few genes changed (two and eight, respectively, vs 50 and 75 in D2 mice; Supplementary Figure S3). The opposite was true for *d*-amphetamine that changed two- to threefold fewer genes in D2 mice. In general, after YKP10A, the number of genes that expressed was twofold higher than for the other two drugs (Supplementary Figure S3). It should also be noted that only very few (or none for some conditions) probe sets were similarly affected by drug or genotype (Figure 3).

In B6 mice, *d*-amphetamine caused a significant increase in the expression of *Dio2*, *Crem*, *Homer1a*, and the heat-shock proteins *Hspa1a*, *-alb*, and *-b1* (Supplementary Table S1), confirming the earlier reports (Budziszewska

et al, 1996; Fujiyama *et al*, 2003; Sokolov *et al*, 2003). The expression of the same set of genes was found to be increased in mice kept awake by gentle handling alone (Maret *et al*, 2007), suggesting that amphetamine further potentiates some of the same pathways already activated by prolonged wakefulness. In AK and D2 mice treated with amphetamine, the same genes again ranked among the most significant but, excepting *Dio2*, the mRNA changes failed to pass the false discovery rate (FDR) set at 5% (Supplementary Tables S2 and S3). Of the remaining drugs, only modafinil gave significant results in D2 mice (Supplementary Table S4–S9).

The low number of genes identified, despite the large fold changes observed for some, suggests that our gene expression analysis lacks sufficient power. As a complementary approach we therefore undertook a gene signature approach using the genes with the highest variance across the nine conditions (three drugs \times three strains) as markers of the effect of a particular drug in a particular strain. To determine the number of genes required to differentiate the nine conditions, we performed a sequence of hierarchical clustering using the 2000, 1000, 500, and 200 most variable probe sets across the nine *t*-statistic values (Figure 6a). Both bootstrap and bias-corrected probabilities were computed to assess cluster-branch strength. We observed that the conditions clustered according to drug treatment first, implying that the effect of drug on gene expression is stronger than the effect of genetic background. YKP10A treatment produced a gene expression response resembling modafinil more than *d*-amphetamine. This is particularly clear for the 'YKP10A—AK' condition that clusters either with modafinil or with the other two YKP10A conditions (ie, B6 and D2) depending on the number of probe sets retained for clustering. For subsequent analysis, we decided to use the 500 most variable probe sets because (i) using 2000 genes, the classification of 'YKP10A—AK' condition did not give a strong cluster probability, (ii) using 1000 genes, the 'YKP10A—AK' condition was still classified with modafinil, and (iii) using 200 genes, the overall cluster probabilities started to deteriorate.

Gene (ie, probe sets) clustering identified five main patterns of change in gene expression (Figure 6b; Supplementary Table S10). Clusters 2 and 4 were dominated by the modafinil response (cluster 2: upregulation; cluster 4: downregulation). Cluster 2 included many metabolic genes *Sgk*, *Slc2a1*, *Ugt1a1*, *Sult1a1*, *Xdh*, *Pdk4*, and others such as *Fos* and *Cdkn1a*. Glycine receptor *Gla2* was present in cluster 4. Cluster 3 contained genes upregulated by *d*-amphetamine (*Arc*, *Crem*, *Dio2*, *Homer1a*, *Hspa1a*, *Hspa1b*, *Hspb1*, *Hsp110*, *Insl5*, *Npas4*, *Nr4a1*, and *Ppp1r3c*). Clusters 1 and 5 showed the most interesting patterns, with opposite effects of YKP10A and

d-amphetamine. Moreover, in cluster 1, a strain effect was noticeable: gene expression in AK mice treated with either *d*-amphetamine or YKP10A was affected less or in the opposite direction compared with B6 and D2. Among these genes, we found the enzymes *Adarb1* and *Gad2* (GABA synthesis) (cluster 1), the transcription factor *Dbp* (cluster 5), which is downregulated by sleep deprivation (Franken *et al*, 2007; Wisor *et al*, 2002), and amphetamine (Sokolov *et al*, 2003), the ion channels, and receptors *Cacna2d1*, *Gabrb2* (GABA-A) (cluster 1) and *Oprm1*, *Kcnn2*, *Ntrk2* (cluster 5), and the signaling molecules *Stxbp1*, *Adrbk1*, *Creb1* (cluster 1) and *Crebbp*, *Pten*, and *Vtla* (cluster 5).

Phenotype–Gene Expression Correlations

In an effort to link gene expression with physiology, we used the nine strain \times drug conditions and correlated gene expression-moderated *t*-statistic values with the most salient drug-dependent changes in the waking EEG; that is the relative changes in EEG power in the theta, beta-2, and gamma frequency bands during the time that showed the largest difference among drugs (ie, the first 2- to 2.5-h period after drug injection). We observed that cluster 1 genes tend to correlate negatively and cluster 5 genes positively with beta-2 activity (Figure 6c). Among the genes that correlated negatively, we found syntaxin-binding protein 1 (*Stxbp1*; $r = -0.80$) and adenosine deaminase, RNA-specific, B1 (*Adarb1*; $r = -0.82$), which is known to be involved in the editing of the pre-mRNA of glutamate receptor B subunit (Mittaz *et al*, 1997). Among those that correlated positively, we found neurocan (*ncan*; $r = 0.88$) and NMDA receptor-regulated 1-like (*Narg1l*; $r = 0.85$) (Supplementary Table S10). Only eight probe sets were significantly correlated with changes in EEG gamma activity. Among those, we found *Slc7a10* (also known as *Asc-1*; $r = 0.81$), which is involved in controlling hyperexcitability (Xie *et al*, 2005). Almost all significant probe sets were negatively correlated with theta activity and belong to cluster 5, such as *Narg1l* ($r = -0.91$), *Dnajb14* (*Hsp40*) ($r = -0.82$), and *Gsk3b* ($r = -0.81$), a cell-proliferation gene associated with antidepressant treatment response (Tsai *et al*, 2008). It should be noted that the EEG measurements for theta and beta-2 activity were negatively correlated ($r = -0.64$), and thus probe sets that correlated with one tended to show an opposite correlation with the other.

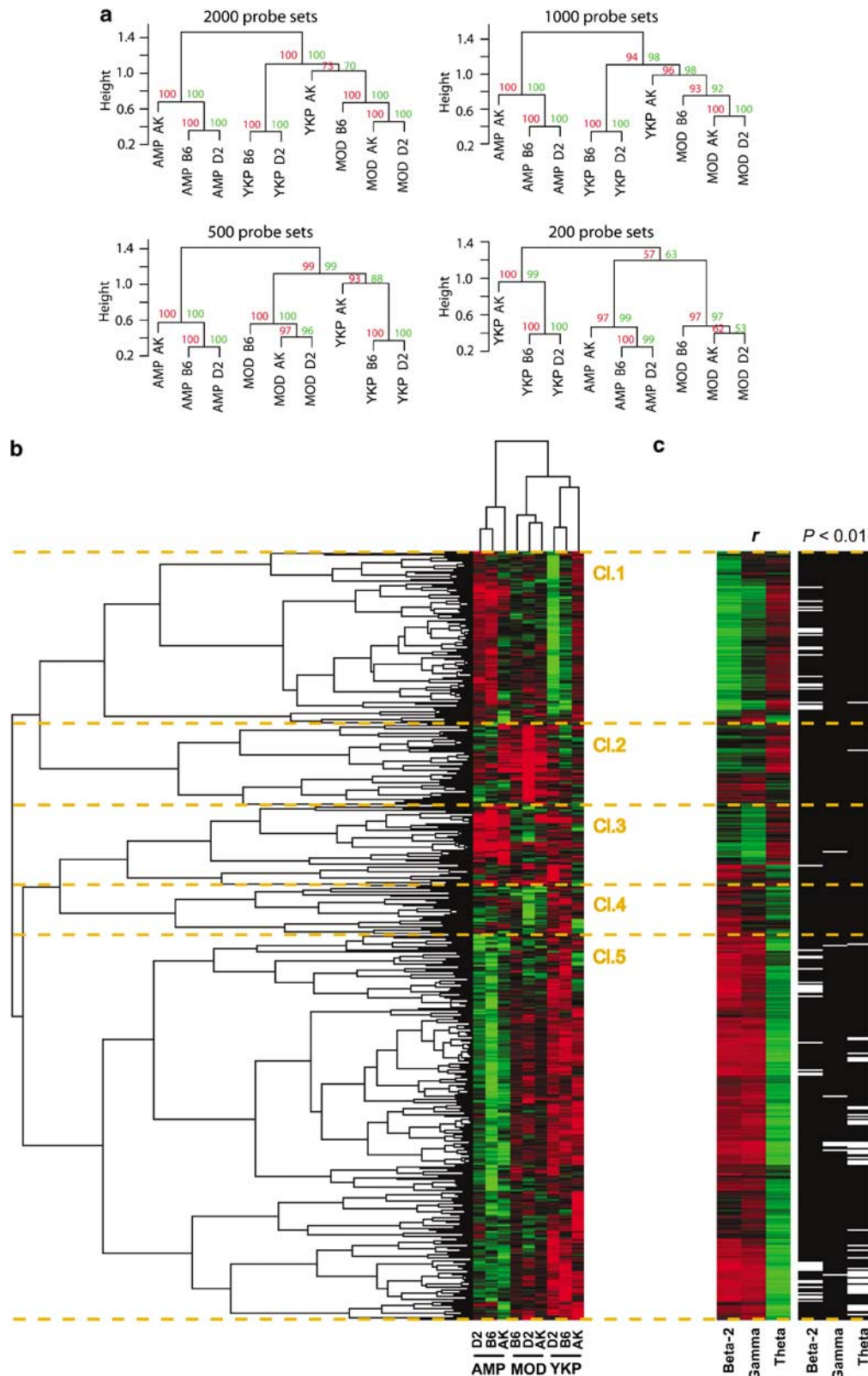
Drug-Induced c-FOS Activity

We examined c-FOS induction in the brain to assess whether among the three drugs common neuronal pathways were implicated in wake promotion. c-FOS immunoreactivity

Figure 6 Comparative transcriptome analysis indicates that YKP10A (YKP) is affected more by genetic background than amphetamine and modafinil ($n = 9$ for each drug and strain, $n = 9$ for each control and strain). (a) Hierarchical clustering of the nine drugs vs saline comparisons using the 2000, 1000, 500, or 200 most variable probe sets. Moderated *t*-statistic data were used for clustering analysis, as outlined in Materials and Methods. The bootstrap probability value (green) and the approximately unbiased probability values are indicated for each cluster. (b) Two-way hierarchical clustering of the 500 most variable probe sets and the nine drug vs saline conditions. Probe sets are grouped in five clusters (C1–5). Red: positive *t*-statistic; green: negative *t*-statistic. (c) Left panel: correlation coefficient (r) between the moderated *t*-statistic values for the 500 most-variable probe sets and EEG power in the frequency bands beta-2, gamma, and theta, using the nine strain \times drug conditions as samples. Red: positive correlation; green: negative correlation. Genes are ordered as in the clustering analysis. Right panel: genes with significant correlations (Pearson's correlation test, $P < 0.01$) with one of the three phenotypes indicated with white bands.

was determined in brain sections from B6 mice killed 2 h after drug administration. Amphetamine and modafinil induced c-FOS in similar brain regions but at different levels (Supplementary Figure S4). The cerebral cortex was activated more by amphetamine (especially clear in the

piriform, somatosensory, motor, and cingulate cortex), whereas other regions such as the dentate gyrus, dorsomedial hypothalamus, and amygdale were equally affected. The striatum was clearly activated more after modafinil administration. Moreover, also the hippocampal CA1 and CA3



regions seemed to be activated more. In the ventromedial hypothalamus, paraventricular thalamus, periaqueductal gray, substantia nigra, and lemniscus, c-FOS was induced by amphetamine, specifically while the vertical limb of the diagonal band, accumbens (shell), and pallidum were activated only after modafinil. As opposed to the two other stimulants, YKP10A did not induce c-FOS in the striatum, whereas more activated neurons were observed in the hippocampus (in the CA1 area, specifically; see Supplementary Figure S4). YKP10A and modafinil shared many areas of similar activation such as the lateral septal nucleus, the septohippocampal nucleus, the dorsal tenia tecta, the seminular nucleus, the island of calleja, the olfactory tubercle, and the arcuate nucleus. The subiculum, ventral tenia tecta, the nucleus of solitary tract gelatinous, dorsal endopiriform, and suprachiasmatic nucleus were activated by YKP10A, specifically.

DISCUSSION

We have compared the EEG activity and brain gene-expression 'signatures' of three wake-promoting compounds in inbred strains of mice for which the expression and regulation of sleep and wakefulness differed greatly (Franken *et al*, 2001). The specific EEG and brain gene-expression signatures were determined primarily by drug and then modified by genetic background. The c-FOS immunoreactivity results confirmed that drugs greatly differ in the structures they target, which was especially clear in the cerebral cortex, hippocampus, and striatum. Taken together, this broad range of findings underscores the notion that various pathways can be activated pharmacologically to maintain the brain awake. In addition, these EEG and gene-expressing findings also indicate that the wakefulness induced by the three compounds differed in quality as confirmed by behavioral observations. Finally, irrespective of the pathways activated to delay sleep onset and the type of wakefulness induced, all three compounds induced an increased sleep drive that, for *d*-amphetamine, even resulted in an overcompensation of sleep time.

Drug Effects on the Waking EEG

We demonstrated that the waking EEG profiles following *d*-amphetamine and modafinil administration were similar corroborating observations in the cat (Lin *et al*, 2000, but see also Chapotot *et al*, 2003). The most salient EEG changes concerned an increase in both power and frequency of theta oscillations and an increase in gamma activity. These EEG changes differed markedly from those induced by YKP10A, which was followed by a pronounced slowing of theta oscillations and a reduction in EEG power in the 7–20 Hz range, concomitant with an increase in gamma activity. Gamma activity is often associated with the presence of theta oscillations in rodents (Sainsbury, 1998), but YKP10A seems to dissociate these two types of EEG activities. A similar theta-slowness concomitant with an induction of gamma was observed after administering the muscarinic agonist oxotremorine in rats (Timofeeva and Gordon, 2001). The gradual and transitory slowing of theta oscillations that followed YKP10A might thus result from a

continuum of increasing activation of additive drives from the brainstem and from cholinergic sources as suggested by Vertes and Kocsis (1997).

YKP10A also induced a specific increase in beta-2 activity not observed with the other drugs. Pronounced increases in beta oscillations have also been observed after dopamine depletion in the rat (Sharott *et al*, 2005). This increase in beta-2 activity combined with YKP10A's clinical profile that resembled that of the drugs known to affect dopamine transmission and preliminary *in vitro* results (see Introduction) thus lends further support to the notion that this compound affects dopamine signaling. Activity in the beta frequency range seems also to be negatively correlated with voluntary movements in humans (Kuhn *et al*, 2004) consistent with the pronounced reduction in locomotor activity observed shortly after the injection in this study. YKP10A administration could not elicit beta-2 activity in AK mice, whereas the slowing of theta was also observed in this strain.

Although in most pharmacology studies, the waking EEG is quantified up to 30 Hz only, here we could demonstrate that all three drugs induced gamma and even higher '60–80 Hz' EEG activities. In freely moving rats, these high-frequency activities showed an association with waking and REM sleep (Franken *et al*, 1994) and spontaneous behaviors (Maloney *et al*, 1997). The '60–80 Hz' EEG activity might be related to the series of fast (60–115 Hz) hippocampal population spikes or 'high' gamma (Pais *et al*, 2003; Traub *et al*, 2001). These high-frequency components have been reported to be associated with the presentation of meaningful stimuli, with attentional processes and voluntary movements, and have been suggested to represent a correlate of cognition and synaptic plasticity (Canolty *et al*, 2006; Crone *et al*, 2006; Gray and Singer, 1989; Sanes and Donoghue, 1993; Tiitinen *et al*, 1993; Traub *et al*, 2005). The enhanced gamma activity observed during drug-induced wakefulness might thus reflect heightened cognitive performance and, at least for modafinil, an increase in working memory has been demonstrated in mice (Beracochea *et al*, 2001, 2002, 2003; Pierard *et al*, 2006) and rats (Miller *et al*, 2000; Ward *et al*, 2004). Other corroborating observations come from the increased c-FOS activity in the hippocampus of B6 mice, which was largest for the drug that also induced the largest increase in gamma activity; that is YKP10A. Gamma activity in the hippocampus is thought to coordinate CA3 and CA1 networks during memory task performance (Montgomery and Buzsaki, 2007) in which c-FOS reactivity was exclusively found.

Effects on Sleep Following Drug-Induced Waking

The waking induced by all three drugs was followed by a compensatory rebound in both duration and intensity (ie, EEG delta power), indicating that sleep homeostatic mechanisms were activated irrespective of the differences the drug induced in EEG activity or gene expression. Ideally, a wake-promoting compound should promote wakefulness without rebound hypersomnolence. In this respect, *d*-amphetamine's performance is especially poor as an 'overcompensation' of sleep time was observed for both REM and NREM sleep. A similar overcompensation has been already reported for REM sleep after *d*-methamphetamine

administration (Kitahama and Valatx, 1979). An over-compensation of NREM sleep has not been reported earlier and was due to the fact that recovery started immediately after sleep onset, whereas after YKP10A and modafinil, the recovery was delayed and started after dark onset only. Again a marked strain difference was observed; for B6 mice, the usual delay in recovery of NREM sleep time was also present under *d*-amphetamine conditions. Similar drug differences in the sleep recovery dynamics have been reported in rats in which the effects of amphetamine and modafinil were compared (Edgar and Seidel, 1997; Wisor *et al*, 2006). In contrast to our study, other studies in rats (Touret *et al*, 1995) and cats (Lin *et al*, 2000) did, however, not find evidence for rebound sleep after modafinil, which added to the interest in this drug as a wake-promoting agent.

Similarly, an increase in EEG delta power during NREM sleep, an index of sleep need and depth or intensity of NREM sleep, compared with the levels reached after saline, was observed for most conditions and strains. In mice, the EEG delta power rebound after modafinil administration did not differ from the increase observed after manual sleep deprivation of the same duration (Kopp *et al*, 2002), indicating that the sleep homeostatic mechanisms are similarly activated in both conditions. This was confirmed also in rats (Edgar and Seidel, 1997), whereas in that study, methylamphetamine did not elicit a significant rebound. The modafinil-*d*-amphetamine comparison in this study was confounded by the longer duration of waking induced by modafinil. The YKP10A-*d*-amphetamine comparison indicated, however, that also in our study amphetamine induces a less robust and shorter lasting increase in EEG delta power and, similar to the rat study, no significant increase was observed in B6 mice. Finally, also in humans, slow-wave sleep duration, that is the fraction of NREM sleep with high levels of EEG delta power, was significantly increased after modafinil and sleep deprivation, but not after amphetamine (Buguet *et al*, 1995). This different response after *d*-amphetamine could indicate that because EEG delta power increased to a lesser extent, the increased sleep need was coped with by sleeping longer (over-compensation).

The drug-dependent effects of wakefulness on subsequent EEG delta power are of interest in the context of sleep function. One prominent hypothesis on sleep function poses that sleep plays a role in synaptic homeostasis by counteracting ('down-scaling') the synaptic strengthening imposed by wakefulness. More specifically, down-scaling could be functionally related to the expression of EEG delta oscillations (reviewed by Tononi and Cirelli, 2006). Accordingly, EEG delta power is increased more after longer and/or more intense waking periods, such as periods of exploratory behavior, as was reported in rats (Huber *et al*, 2007). Given this, the behavioral quiescence and marked suppression of theta activity after YKP10A administration should have been followed by only a modest EEG delta increase, whereas the hyperactivity and pronounced theta activation following *d*-amphetamine should have been followed by a higher rebound. However, in this study, the opposite is found suggesting either that beta or gamma activity might be more relevant in inducing synaptic strength (as they were induced more by YKP10A) or that

d-amphetamine could interfere with the expression of EEG delta oscillations even after sleep onset.

Effect on Brain Gene Expression

Psychostimulants affect the expression of immediate early genes, such as *Fos*, *Arc*, and *Homer1a*, the most (Berke and Hyman, 2000; Yano and Steiner, 2007). We have shown earlier that the increase in the expression of the same genes ranks among the highest after extended wakefulness (Maret *et al*, 2007), raising the possibility that changes in gene expression reported for these compounds are mediated through the induced wakefulness and not specific to the drug. Although we quantified expression in whole-brain homogenates, the pronounced FOS reactivity in the striatum after modafinil administration seems consistent with a specific role of this structure in wake promotion and attention (Willuhn *et al*, 2003; Yano and Steiner, 2005a, b). The induction of c-FOS was, however, not observed after the other two compounds.

Whole-brain gene-expression profiling suffers many limitations, the most important being the lack of power to detect subtle but functionally significant changes in small brain regions (see discussion in Maret *et al*, 2007). However, sampling specific brain regions without established evidence for their involvement might also present the risk of missing valuable information from unsuspected brain regions. Moreover, Cirelli *et al* (2004) have reported similarities in gene expression as a function of vigilance states between the cortex (site of the EEG correlates) and the cerebellum (with no major contribution to sleep), suggesting more global than local effects of sleep and wakefulness on brain gene expression.

Our cluster analysis revealed several interesting genes or gene pathways characterizing each stimulant. Cluster 2 contained transcripts that were mostly increased by modafinil. Several of those are implicated in metabolism (see Results), supporting the role of modafinil in activating brain metabolism (Pierard *et al*, 1995). Another pathway identified concerned genes implicated in apoptosis (ie, *Ccnd3*, *Cdkn1a*, *Ddit4*, *Emilin2*, *Fos*, *Klf4*, *Lcn2*, *Mgp*, *Nfkbia*, *S100a8*, *S100a9*, *Sgk1*, *Slc2a1*, *Tgm2*, *Txnip*, *Xdh*, and *Zbtb16*; *in-silico* pathway analysis; Ingenuity Systems Inc.: $P = 3.8 \times 10^{-8}$). One representative apoptotic gene is *Cdkn1a* (P21), a regulator of cell-cycle progression and related to stress response (Rodriguez and Meuth, 2006). Cluster 5, characterized mostly by genes overexpressed by YKP10A, included six genes (ie, *Crebbp*, *Gsk3b*, *Ncan*, *Ntrk2*, *Ptprd*, and *Vldlr*) that were positively correlated with beta-2 and/or gamma activity, and negatively correlated with theta. These genes are implicated in long-term potentiation (LTP; Ingenuity Systems Inc.: $P = 6.3 \times 10^{-4}$). *Ntrk2*, the receptor that binds BDNF, is well known to be implicated in hippocampal LTP and synaptic plasticity (Gartner *et al*, 2006; Nagappan *et al*, 2008; Santi *et al*, 2006; Tanaka *et al*, 2008) and EEG delta power during recovery from sleep deprivation (Faraguna *et al*, 2008; Hairston *et al*, 2004). Together with the pronounced induction in EEG gamma activity and increase in hippocampal FOS, the changes in the expression of these genes indicate that YKP10A might modulate memory processes and cognitive performance. Stimulant-induced gene expression may also have

detrimental impacts on behavior and/or EEG activity. The Na⁺-independent alanine-serine-cysteine transporter 1 *Slc7a10* (*Asc-1*) is downregulated in our study by amphetamine in all strains. Loss of function of this gene in mice induces tremors and seizures (Xie *et al*, 2005). It is well known that acute, high doses of several classical stimulants including amphetamines cause seizures in mice, rats, and humans (Golden *et al*, 2001; Hanson *et al*, 1999; Zagnoni and Albano, 2002). As a cautionary note we like to point out that the analyses presented here are based on microarray data, and the changes in expression for specific genes were not independently confirmed with, for example, qRT-PCR. Nevertheless, because our gene signature approach considered only those genes that changed the most and because we have shown that the most significant changes detected by microarray analysis are routinely confirmed by qRT-PCR (Maret *et al*, 2007), we feel confident about the gene pathway we identified. For the same reason, the gene expression-EEG phenotype correlations should be interpreted with caution. In addition, as is the case with any correlation, the analyses do not prove causality.

CONCLUSION

We conclude that different stimulants leave different behavioral, sleep, EEG, and brain gene-expression signatures, indicating that complex molecular and neuronal pathways differentially and/or in combination are required to keep the brain awake. YKP10A is an atypical wake-promoting drug that may prove to be a useful medication in humans. Its mode of action differs greatly from that of both *d*-amphetamine and modafinil. This study also underscores that taking genetic background and the gene-expression signature into account is paramount to estimate drug efficacy in a therapeutic discovery process, especially for sleep-wake phenotypes in which genetic factors play an important role.

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DISCLOSURES/CONFLICT OF INTEREST

MT declares that over the past 3 years he has received compensation from UCB Pharma and Servier. AA and WD declare their full-time employment at Johnson and Johnson, Pharmaceutical Research and Development, Beerse, Belgium. SH, SP, and PF declare that, except for income received from their primary employers, no financial support or compensation has been received from any individual or corporate entity over the past 3 years of research or professional service, and they have no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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