The Roles of Dopamine Transport Inhibition and Dopamine Release Facilitation in Wake Enhancement and Rebound Hypersomnolence Induced by Dopaminergic Agents

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Study Objective: Rebound hypersomnolence (RHS: increased sleep following increased wake) is a limiting side-effect of many wake-promoting agents. In particular, RHS in the first few hours following wake appears to be associated with dopamine (DA)-releasing agents, e.g., amphetamine, but whether it can also be produced by DA transporter (DAT) inhibition alone is unknown. In these studies, DA-releasing and DAT-inhibiting agents and their interaction were systematically examined for their ability to increase wake and induce RHS.

Design: Chronically implanted rats were evaluated in a blinded, pseudo-randomized design.

Participants: 237 rats were used in these studies with 1 week between repeat tests.

Interventions: Animals were habituated overnight and dosed the next day, 5 h after lights on, with test agents.

Measurements and Results: Sleep/wake activity and RHS were evaluated using EEG/EMG recording up to 22 h post dosing. In vitro dopamine release was evaluated in rat synaptosomes. At doses that produced equal increases in wake, DA-releasing (amphetamine, methamphetamine, phentermine) and several DAT-inhibiting agents (cocaine, bupropion, and methylphenidate) produced RHS during the first few hours after the onset of sleep recovery. However, other DAT-inhibiting agents (mazindol, nomifensine, GBR-12909, and GBR-12935) did not produce RHS. Combination treatment with amphetamine and nomifensine produced waking activity greater than the sum of their individual activities alone while ameliorating the amphetamine-like RHS. In rat synaptosomes, nomifensine reduced the potency of amphetamine to induce DA release ~270-fold, potentially explaining its action in ameliorating amphetamine-induced RHS.

Conclusions: All DA-releasing agents tested, and some DAT-inhibiting agents, produced RHS at equal wake-promoting doses. Thus amphetamine-like DA release appears sufficient for inducing RHS, but additional properties (pharmacologic and/or pharmacokinetic) evidently underlie RHS of other DAT inhibitors. Enhancing wake while mitigating RHS can be achieved by combining DAT-inhibiting and DA-releasing agents.

Keywords: Sleep/wake, dopamine transporter, amphetamine, hypersomnolence, pharmacokinetic, rat

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per, “DA-releasing agent” will specifically refer to agents that induced DA release in the synaptosomal preparation. The in vivo effects of these compounds on specific measures of RHS, primarily within the first 7 h after dosing in rats (up to the time of lights off), were then studied at doses that produced comparable wake activity. These studies revealed that while DA-releasing agents produced RHS, some DAT inhibitors also produced RHS, while others (e.g., nomifensine) did not. Furthermore, the combination of amphetamine and nomifensine ameliorated the amphetamine-induced RHS and surprisingly produced more wake activity than expected from either agent alone. A mechanistic explanation for this interaction supported by the finding that nomifensine inhibits amphetamine-induced DA release in a rat synaptosomal assay is discussed.

MATERIALS AND METHODS

Test Agents

Test compounds included (with salts indicated in parentheses): (+)-amphetamine (d-amphetamine) (SO₄) (hereafter “amphetamine”), (+)-methamphetamine (HCl) (hereafter “methamphetamine”), nomifensine (CH₃O₂), mazindol, bupropion (HCl), cocaine (HCl), GBR-12909 (2HCl), and GBR-12935 (2HCl), methylphenidate (HCl), and phentermine (HCl) (Sigma-Aldrich, St. Louis, MO). For in vivo experiments, compounds were formulated for intraperitoneal administration using 0.5% methylcellulose (Methocel A15 Premium, Dow Chemical Co., Midland, MI) / 0.2% Tween-80 (Fisher Scientific, Fair Lawn, NJ) in sterile water and administered in a volume of 5 mL/kg.

In Vitro DA Release Experiments

DA release was evaluated using ³H-DA pre-labeled rat striatal synaptosomes prepared using a modification of published protocols. All test drug solutions were prepared on the day of the experiment. The protocol was designed to allow incubation of the P2 synaptosome fraction with ³H-DA to minimize radioactive contamination of equipment.

Buffer Solutions

The release buffer contained the following: 25 mM HEPES buffer, 120 mM NaCl, 5 mM KCl, and 1.2 mM MgSO₄. Uptake buffer was identical to release buffer with the addition of 2.5 mM CaCl₂. On the day of the assay, the following was added per 20 mL uptake buffer: 20 µL pargyline (1 µM), 40 mg D-glucose (11.1 mM), and 4 mg ascorbic acid (1.14 mM).

Crude Synaptosomal Preparations

Anesthetized rats (Nembutal, 45 mg/kg ip, Abbott Laboratories, Abbott Park, IL) were decapitated and their brains removed to isolate striatal tissue (~85 mg). The striata were homogenized in 25 mL of ice-cold 0.32 M sucrose buffer using 10 strokes of a Teflon pestle (Heidolph model RZR1, Federal Republic of Germany). The homogenate was centrifuged (Sorvall RC5B, DuPont, DE) at 1000 g (2854 rpm) for 10 min at 4°C and the supernatant decanted. The supernatant was brought to a volume of 30 mL by adding cold 0.32 M sucrose buffer and centrifuged at 20,000 g (13,000 rpm) for 30 min at 4°C to obtain a synaptosomal pellet. The pellet was re-suspended in 25 mL of cold 0.32 M sucrose buffer and again centrifuged at 20,000 g for 30 min. The final pellet was then re-suspended in 16.7 mL uptake buffer. A protein concentration of 1 mg/mL (~20,000 CPM) was chosen as being in the linear uptake range using a 10-min incubation period.

The total synaptosomal volume was divided into cold (synaptosomes only), mazindol control (synaptosomes + 10 nM mazindol + ³H-DA), and test (synaptosomes + ³H-DA) portions. The mazindol control portion, for determining non-specific binding, was made by adding mazindol to the synaptosomes prior to adding ³H-DA. Mazindol was initially dissolved in 350 µL DMSO and then 650 µL release buffer added for a final concentration of 1 mg/mL. Synaptosomes were made by adding 2.85 µL/mL for a final concentration of 10 µM mazindol and 0.1% DMSO. Mazindol control and test synaptosomes were loaded with labeled DA by incubating with 30 nM ³H-DA (~1.7 µL/mL) (Perkin Elmer, Boston, MA, Cat# NET673001MC, ~1 mCi/mL) for 10 min, after which they were returned to ice.

All synaptosomal portions were then washed twice by centrifuging in 1.5 mL portions in a microcentrifuge at 11,950 g (1000 rpm) for 10 min at 4°C and resuspending the pellets in release buffer (including mazindol in the case of the mazindol control portion). After the second wash, the corresponding cold, mazindol control, and test synaptosome tubes were pooled and then pipetted onto a 96-well plate (50 µL/well). Test drugs dissolved in release buffer or plain buffer were then added to appropriate wells (500 µL/well). Control wells consisted of test synaptosomes to which only release buffer was added. All wells contained a final concentration of 0.1% DMSO. After cold, control, and test synaptosomes were pipetted onto the plates, they were incubated for 30 min at 37°C.

Amphetamine-Induced DA Release Assay

Inhibition of amphetamine induced DA release, was evaluated by adding nomifensine, bupropion, cocaine, methylphenidate, or mazindol at 100 µM to the test synaptosomes. Amphetamine (in 500 µL release buffer) was then added at varying concentrations to appropriate wells containing synaptosomes (50 µL). The final concentration of test compounds was 10 µM. All other procedures were as described above.

Following incubation, plates were immediately placed on ice and harvested in a tissue harvester (Brandel model# CH-055; Gaithersburg, MD) onto a GF/C filter plate (cat# 1450-525, Perkin Elmer, Wellesley, MA) soaked in 0.4% PEI (polyethylenimine, Sigma-Aldrich). The initial vacuum harvest was followed by 2 cold saline flushes. The filter plates were dried at 60°C and then 50 µL scintillant (Betaplate, Perkin Elmer, Wellesley, MA) was added to each well. Radioactive counts were performed using a Microbeta Scintillation Counter (Wallach model 1450, Downers Grove, IL).

Data Analysis

Effects of nomifensine were evaluated in at least 3 separate experiments, each sample being run in quadruplicate on each plate. Control counts values were typically 6000–7000 counts. Cold counts (no ³H-DA) were typically 0.3%–0.5% of mean control values. Within each experiment, DA release was evaluated as follows. First, a baseline count for each plate was calculated by averaging the mazindol control samples. Second, a mean maximal count was calculated as the average of the control wells containing only ³H-DA. Third, the count for each in-
Animal Preparation

Waking activity was evaluated in rats using standard methodology. Rats were anesthetized (Nembutal, 45 mg/kg ip) and chronically implanted with cortical screw and intramuscular wire electrodes for recording cortical EEG and nuchal electromyographic (EMG) activity. After shaving the head, Lidocaine (2%, Phoenix Pharmaceuticals, Burlingame, CA) was applied subcutaneously, 50 mg/mL solution; Fort Dodge, Overland Park, KS) was administered. Following recovery from surgery, the skin was sutured with 5-0 catsgut, and wound clips were used to close the incision (4.0 mm posterior to bregma, ± 2.0 mm medio-lateral), and over the hippocampal region (4.0 mm posterior to bregma, ± 2.0 mm medio-lateral). Two additional anchoring screws were placed 3 mm anterior to the frontonasal sinuses, and at the posterior margin of the skull. Two EMG recording electrodes (Plastics One) were positioned intramuscularly into the nuchal muscles just caudal to the skull. The electrode leads were inserted into a plastic connector pedestal, and the pedestal, screws, and wires were affixed to the skull with dental acrylic. The skin was sutured around the plastic skullcap, and antibiotic cream (neomycin, Phoenix Pharmaceuticals, St. Joseph, MO) was applied to the wound edges. A sterilized temperature/activity transmitter (TA10TA-F40, Data Sciences International, N. St. Paul, MN) was then implanted into the peritoneal cavity through an abdominal skin incision. After suturing the peritoneum closed, wound clips were used to close the skin. Finally, a prophylactic antibiotic (amikacin sulfate, 0.2 cc subcutaneous, 50 mg/mL solution; Fort Dodge, Overland Park, KS) was administered. Following recovery from surgery, the animals were housed in pairs in standard rat cages. Recording sessions were begun no sooner than one week later.

Sleep/Wake Activity Recording

Animals were handled several times prior to final testing. The day prior to a recording session, each rat was placed in a separate Nalgene container (31 x 31 x 31 cm) with a wire-mesh top and connected by cables to the recording equipment. The animals were not disturbed thereafter until the end of recording, except for dosing. Food and water were available *ad libitum*, ambient temperature was ±22°C, and humidity was 45% ± 5%.

Typically, 16 rats were monitored simultaneously during a single session. EEG and telemetry recording were automatically initiated 3 h after lights on (Zeitgeber time 3 or ZT-3) and continued for 24 h (except in the case of GBR-12935, in which case recording lasted only 11 h). Treatment administration was pseudo-randomly distributed among the animals taking into account treatment and cage location. Drugs were administered at ZT-5. To insure proper and non-stressful ip injections, they were administered by 2 investigators: one held the animal with the hind limbs extended, while the second stroked the abdomen to relax the muscles prior to injecting the drug. Drug administration and handling awakened the animals, after which they gradually returned to their normal “quiet” activity level (~20% awake) over the next 20–30 min. The animals were placed back in their home cages the next morning.

Each recording container was placed in a sound attenuation cabinet (#ENV-018V, Med Associates, Inc. St. Albans, VT). Each cabinet had a ceiling light (14 lux at the floor of the cabinet), which was on from 07:00 to 19:00 and a ventilation fan mounted externally to minimize ambient noise. Convoluted acoustic foam (# 3002Bfl, Foamorder.com, San Francisco, CA) was added outside the cabinets to further reduce ambient sounds. Random background sound which by itself had no impact on sleep/wake activity (data not shown) was provided inside each cabinet to reduce the impact of any external disturbance.

Cortical EEG activity was recorded differentially between the left frontal and the right posterior EEG electrodes, with the right frontal electrode serving as ground. The EEG and EMG signals were conducted via cables to a commutator (Plastics One) and then to pre-amplifiers (model 1700, A-M Systems, Carlsborg, WA). EEG and EMG signals were amplified (10K and 1K respectively) and band pass filtered (0.3 to 500 Hz for EEG and 10 to 500 Hz for EMG). These signals were digitized at 128 samples per second using ICELUS sleep scoring software (Mark Opp, U. Michigan, Ann Arbor, MI) under National Instruments (Austin, TX) data acquisition software (Labview 5.1) and hardware (PCI-MIO-16E-4).

Sleep/Wake Activity Scoring and Analysis

EEG and EMG data were scored using standard protocols. EEG and EMG data were manually classified into one of 3 sleep/wake states: awake, REMS, or slow wave sleep (SWS) according to visual analysis of EEG frequency and amplitude characteristics and EMG activity. Waking activity was defined as consisting of relatively low-amplitude EEG activity with low power in the low-frequency bands from 0.3–6 Hz, accompanied by moderate to high level EMG activity. “Theta-waking,” in which EEG power was concentrated in the 6–9 Hz (θ) range and significant EMG activity was always present, was scored as awake. SWS was defined as generally high-amplitude EEG activity with greater power in the low-frequency bands from 0.3–6 Hz, accompanied by minimal EMG activity. REM sleep was characterized by moderate and constant amplitude EEG activity focused in the 0 (6–9 Hz) range with no (or only sporadic) EMG activity, and was always preceded by SWS.

Sleep/wake stage scoring was performed by an investigator blinded to treatment. Records were scored in 6-sec epochs as awake, SWS, or REM states using the ICELUS software. Animals having prolonged periods of artifact were eliminated. The principal outcome measure was percent time awake for 30-min periods. For each animal, cumulative wake time (CWT) following test agent...
administration was calculated. Cumulative wake surplus (CWS), defined as CWT minus the corresponding mean vehicle CWT value at a given time point, was also calculated. Two measures were used to compare treatment efficacy: (1) CWT at 4 h post dosing derived from the 4-h area under the curve (i.e., a 4 h AUC) and (2) maximal CWS (typically within 10 h of injection). In some animals, CWS continued to increase slowly to a maximum value several hours after the group mean maximal CWS. In such cases, the time at which CWS reached a plateau value near the time of maximal CWS for the group mean was chosen as maximal CWS.

RHS was assessed using 4 principal measures. (1) Sleep recovery slope for each animal reflected the slope of the wake recovery curve from maximal wake (~100%) down to approximately 30% wake and was calculated using half-hour time points. (2) Comparisons of drug versus vehicle group CWT means were performed for all half-hour time points using individual unpaired, 2-tailed \( t \) tests. Hypersomnolence was defined as occurring when the mean drug value was less than the mean vehicle value for a given time point. (3) Cumulative wake time for 3 h relative to the vehicle group following maximal CWS was calculated. After maximal CWS, the slope of the CWS curve becomes negative as sleep recovery begins and the percent wake time for the drug group falls below that of the vehicle group. If a maximal CWS point did not occur at an appropriate time, the time of closest return of the drug to the vehicle percent wake time curve was used. (4) CWS at 22 h post dosing (calculated as the average of the CWS values at 21.5 and 22 h); also, this value was calculated as a percentage of maximal CWS. These measurements are illustrated in Figure 4.

For purposes of analysis, additional animals from single-dose studies were added to vehicle, nomifensine, and amphetamine treatment groups in these experiments for totals of 13 vehicle, 14 nomifensine, and 11 amphetamine animals per group after confirming that the data were consistent for the respective treatment groups.

**Motor Activity Recording and Analysis**

Motor activity was recorded using the Dataquest A.R.T. v4.0 (Data Sciences International, N. St. Paul, MN) integrated hardware/ software system. Each recording container sat on a receiver plate which picked up the signal from the transmitter in the animal’s abdominal cavity. Average body and motor activity signals were saved by the computer every 2 min. Motor activity included any movement causing the transmitter to be displaced relative to the receiver, such as locomotor activity and rearing. Recording started the day prior to dosing and continued until the animal was removed the day after dosing. Motor intensity was calculated by dividing the average motor activity for the first 2 h after dosing by the corresponding mean time awake in minutes. Thus motor intensity = MI = \((MA / WA)\), where \(MA\) = total motor activity units over 2 h post dosing, and \(WA\) = average time awake in min for 2 h post dosing.¹⁹

**Evaluation of Plasma and Brain Drug Levels**

Twelve rats (Sprague-Dawley, ~300 g) were dosed with amphetamine (1 mg/kg ip), nomifensine (3 mg/kg ip), or both drugs (separate ip injections to the right and left sides), \( N = 4 \) per group. Plasma levels were evaluated at 0.25, 0.5, 1, 2, 4, and 6 h after dosing by tail-vein blood withdrawal. An additional 9 groups of 4 rats were administered amphetamine, nomifensine, or both drugs as before but sacrificed at 1, 3, and 6 h, at which time a blood sample was taken and the brain removed and homogenized as described previously.³⁴

**STATISTICAL ANALYSIS**

Treatment groups were compared using unpaired \( t \) tests or analysis of variance (ANOVA) with Dunnett or Bonferroni/ Dunn post hoc tests for multiple comparisons where appropriate (SigmaStat v3.5, SPSS, Inc., Chicago, IL). Repeated-measures ANOVA was used to compare wake curves across multiple time points. Significance was set at \( P \leq 0.05 \) for all tests. For evaluating motor activity and intensity across compounds, unpaired \( t \) tests versus vehicle were used since the various individual experiments comprised different numbers of compounds and doses. Additionally, Pearson correlation was applied for assessing the relationship between 2 h RHS and the magnitude of the change in motor activity produced by test compounds. Plasma and brain PK parameters were calculated using WinNonlin v4.1.A (Pharsight Corp., Mountain View, CA). In the case of brain half-life values, WinNonlin could not calculate SEMs for the mean half-life values because different animals were used for each time point, unlike the case for plasma half-life.

**RESULTS**

**Synaptosomal DA Release**

Amphetamine, methamphetamine, phentermine, bupropion, cocaine, methylphenidate, mazindol, and nomifensine were characterized for DA release activity and ability to inhibit amphetamine-induced DA release. Amphetamine was included as a positive control in every experiment. The mean amphetamine \( EC_{50} \) value for DA release across all experiments was 0.095 \( \mu M \) (0.071–0.126, 95% confidence limits) and did not differ across experiments \((P = 0.67, ANOVA)\). Methamphetamine, amphetamine, and phentermine, had \( EC_{50} \) values less than 1.0 \( \mu M \), GBR-12909, and GBR-12935 had \( EC_{50} \) values between 1 and 10 \( \mu M \); and bupropion, cocaine, methylphenidate, mazindol, and nomifensine had \( EC_{50} \) values > than 100 \( \mu M \) (Table 1).

**Effects of DA-Releasing versus DAT-Inhibiting Agents on Sleep/Wake Measures**

As seen in Table 2, all compounds tested significantly enhanced waking at various doses. Examples of activity time courses for several doses of phentermine and nomifensine are presented in Figures 1 and 2, respectively. Phentermine at 3 and 10 mg/kg ip increased waking for 2.5 and 4.5 h, respectively \((173 \pm 11 \text{ and } 236 \pm 2.2 \text{ min awake respectively in the first } 4 \text{ h after dosing versus } 85 \pm 6 \text{ min for the vehicle group, Figure 1})\). Phentermine, which enhanced wake at 3 and 10 mg/kg ip, showed sleep recovery onsets of approximately 2 and 3.5 h, respectively, and also produced subsequent RHS (wake activity below vehicle at 4.5–5 h and 5.5 h, respectively). In contrast, nomifensine at similar wake-enhancing doses \((1–10 \text{ mg/kg ip})\) did not produce RHS. Specifically, nomifensine at 10 mg/kg ip produced over 5.5 h of increased waking with no sleep rebound up to 11 h after dosing (Figure 2).

In order to assess whether RHS was specifically dependent on DA release as opposed to DAT inhibition, equal wake promoting
Table 1—Summary of EC50 Values (in µM) for Dopaminergic Compounds to Induce DA Release in Synaptosome Assay

| Compound      | N   | EC50 (µM) | 95% Confidence Intervals |
|---------------|-----|-----------|--------------------------|
| Amphetamine   | 19  | 0.095     | 0.071–0.126              |
| Methamphetamine | 3  | 0.062     | 0.047–0.082              |
| Phenetermine   | 3   | 0.310     | 0.128–0.075              |
| GBR-12909     | 4   | 4.23      | 2.465–6.409              |
| GBR-12935     | 5   | 3.047     | 1.765–5.260              |

N represents number of repetitions in each EC50 determination. EC50 values determined by sigmoidal curve fit. EC50 values for bupropion (N = 4), cocaine (N = 3), mazindol (N = 5), methylphenidate (N = 3), and nomifensine (N = 7) were all greater than 100 µM.

doses for each compound were determined by evaluating the 4 h CWT and maximal CWS values. Doses that produced comparable 4-h CWT (P = 0.160; ANOVA) and maximal CWS (P = 0.334; ANOVA) for each compound are listed in Table 3. Examination of the mean wake activity curves for these compounds at the chosen doses (Table 2) showed that in all cases, sleep recovery began at nearly the same time, 1.5 to 2 h post dosing (Figure 3). However, when the mean wake activity curves for all compounds tested were compared with the mean vehicle curve (average for all vehicle animals), there was a significant treatment effect (P = 0.008; repeated-measures ANOVA) in sleep recovery during the interval from 2 to 5 h after reaching maximal CWS.

Differences in sleep recovery produced by different compounds were further investigated by comparatively evaluating four relatively independent measures of sleep recovery (see Materials and Methods for details). An example of evaluation of these parameters for bupropion is illustrated in Figure 4. Significant half-hour periods following dosing for each compound up to 12 h post dosing are shown in Table 4 (see also Figure 3, solid points). The DA-releasing agents (amphetamine, methamphetamine, and phenetermine) all showed ≥ 2 half-hour periods that exhibited RHS in the first 7 h post dosing (up to the time of lights off). Amphetamine also showed a period of hyper-somnolence at 9–10 h. Interestingly, the DAT-inhibiting compounds methylphenidate, cocaine, and bupropion also showed some periods of RHS up to 7 h post dosing, while nomifensine, mazindol, and GBR-12909 did not (Table 4, Figure 3). GBR-12935 showed a single period of hypersomnolence at 7 h due to a transient increase in the within-experiment vehicle group.

The mean values for the other hypersomnolence measures are listed in Table 5. ANOVA performed over all the treatments revealed significant treatment effects for sleep recovery slope (P = 0.002) and 3 h cumulative hypersomnolence (P < 0.001). A Bonferroni/Dunn post hoc test for sleep recovery slope revealed that the value for the mazindol group was less than for the amphetamine, methamphetamine, methylphenidate, and bupropion groups, and the nomifensine value was less than that for methylphenidate (P < 0.05). The lower slope values for mazindol and

Table 2—Doses Evaluated for Each Compound with Minimum Effective Dose for Each Wake State

| Compound      | Doses tested | Minimum Effective Dose |
|---------------|--------------|------------------------|
|               | mg/kg (N)    | Wake       | SWS       | REMS      |
| Amphetamine   | Veh (4); 0.1 (4); 0.3 (4); 1 (16) | 0.3 | 0.3 | 0.3 |
| Bupropion     | Veh (7); 1 (4); 3 (3); 10 (4); 30 (8); 60 (3) | 30 | 30 | 30 |
| Cocaine       | Veh (17); 3 (8); 10 (8); 15 (7) | 3 | 3 | 3 |
| GBR-12909     | Veh (7); 1 (4); 3 (8); 6 (10); 10 (8); 30 (3) | 1 | 1 | 1 |
| GBR-12935     | Veh (5); 0.3 (4); 1 (3); 3 (9); 10 (10) | 10 | 10 | 10 |
| Mazindol      | Veh (7); 0.3 (2); 1 (8); 3 (7) | 0.3 | 1 | 0.3 |
| Methamphetamine | Veh (9); 1 (7) | 1 | 1 | 1 |
| Methylphenidate | Veh (9); 3 (4); 6 (8) | 3 | 3 | 3 |
| Nomifensine   | Veh (12); 0.1 (4); 0.3 (4); 1 (14); 3 (10); 10 (10) | 1 | 1 | 0.3 |
| Phenetermine  | Veh (8); 3 (9); 10 (9) | 3 | 3 | 3 |

The values in parentheses indicate the group number (N) of animals tested for vehicle (Veh) and each dose tested. The lowest dose (Minimum Effective Dose) at which a significant effect was determined for wake, SWS, and REMS by Dunnett test versus vehicle following significant main effect in ANOVA.
nomifensine indicate relatively reduced rates of recovery of sleep lost during drug-induced wake. In the case of 3 h cumulative hypsomnolence, effects produced by mazindol were significantly smaller than those produced by amphetamine, methamphetamine, phenetermine, methylphenidate, and bupropion, while the effect produced by nomifensine was significantly smaller than those produced by amphetamine and bupropion. Again, lower values for mazindol and nomifensine indicate relatively reduced hypsomnolence. The 22 h CWS values (excluding GBR-12935, for which data past 8 h were not available) were not significantly different across treatments (P = 0.073; ANOVA).

ANOVA of the data represented in Table 5 was also performed using DA-release vs. DAT-inhibition designation as a factor (Table 3). However, since GBR-12909 and GBR-12905 acted as weak releasing agents (albeit more than 30-fold less potent than amphetamine), statistical analysis was carried out with them treated as a separate category. Maximum CWS and 4 h CWt were not significant across treatment groups for this factor (P > 0.6; ANOVA). This analysis did not show an effect for slope (P = 0.15), but 3 h cumulative hypsomnolence and 22 h CWS measures both showed significant effects (P = 0.004 and P = 0.031, respectively). In the last 2 cases, the DA-releasing compounds showed lower values consistent with greater hypsomnolence than the DAT-inhibitor group (P = 0.0005 and P = 0.009, respectively, Bonferroni/Dunn). The same pattern of statistical significance was obtained with the GBR compounds either excluded or treated as DAT inhibitors.

Since varied levels of motor activity produced by different agents could potentially influence RHS, effects of all compounds on motor activity for 4 h after dosing were analyzed at doses that produced equal 4 h wake enhancing effects (Table 3).

### Table 3—Equivalent Wake Enhancing Doses of Dopamine Releasing and Dopamine Transport Inhibiting Agents

| Treatment | Dose mg/kg | 4 h CWt min | MaxCWS min |
|-----------|------------|-------------|-------------|
| Amphetamine | 1 | 171.4 ± 5.2 | 99.5 ± 4.0 |
| Methamphetamine | 1 | 164.1 ± 3.0 | 97.9 ± 2.2 |
| Phentemrine | 3 | 172.5 ± 11.1 | 93.0 ± 9.6 |
| Bupropion | 30 | 175.0 ± 11.4 | 107.1 ± 9.0 |
| Cocaine | 15 | 151.7 ± 9.9 | 82.2 ± 7.6 |
| GBR-12909 | 6 | 170.9 ± 9.4 | 97.0 ± 8.3 |
| GBR-12935 | 10 | 172.8 ± 11.0 | 104.5 ± 14.5 |
| Mazindol | 1 | 194.0 ± 5.3 | 117.3 ± 4.7 |
| Methylphenidate | 6 | 160.2 ± 5.6 | 94.7 ± 4.4 |
| Nomifensine | 3 | 182.5 ± 10.0 | 106.5 ± 8.4 |

In addition, motor intensity was analyzed for 2 h after dosing\(^{(19)}\) (Table 6). Except for nomifensine, mazindol, and GBR-12935, the compounds increased motor activity (P < 0.05, unpaired \(t\) test versus vehicle). There was, however, no correlation (\(R^2 = 0.200, P = 0.579\); Pearson correlation) between 3 h RHS values (Table 5) and the magnitude of the change in motor activity produced by these compounds (Table 6). Motor intensity was increased by amphetamine and decreased by mazindol. The remaining drugs, regardless of their effect on RHS, had no effect on motor intensity (Table 6).

### Combined Effect of Nomifensine and Amphetamine on Sleep/Wake Measures

While RHS did not appear to be exclusively related to DAT inhibition versus DA release activity alone, the robust difference in RHS between amphetamine and nomifensine prompted us to examine the physiological interaction of these two compounds administered together at doses that produced equal wake activity (3 mg/kg ip nomifensine and 1 mg/kg ip amphetamine). Two experiments were conducted to investigate whether the order of administration would affect the outcome. Thus nomifensine was administered 15 min prior to amphetamine in 10 rats and 15 min after amphetamine in another 10 rats. Treatment order had no effect on outcome (P = 0.68; repeated measures ANOVA on time points, −2 to 21.5 h post dosing); therefore, the 20 animals administered both drugs were combined into one treatment group. The results for the individual treatments, shown in Figure 5A, were consistent with the data described previously. Specifically, nomifensine and amphetamine had similar effects on wake measures such as CWt (4 h CWt: 165.4 ± 6.8 vs 186.3 ± 8.1 min), maximal CWS (91.1 ± 5.4 vs 102.3 ± 7.0 min), and SWS onset latency (151.6 ± 7.3 vs 166.8 ± 8.8 min) (P > 0.05, unpaired \(t\) test), although REMS onset latency was later for amphetamine (205.8 ± 9.2 vs 280.1 ± 21.5 min; P = 0.002; unpaired \(r\) test). While the onset of sleep recovery
particularly evident from the CWS curves (Figure 5B). Note that (P > 0.05, unpaired) and therefore treatment group values were not different from each other (P = 0.02, unpaired t test) and the cumulative wake time relative to vehicle following maximal CWS was lower (−20.9 ± 4.1 min, P = 0.02, unpaired t test). In contrast to the individual treatments, the combined treatment group showed enhanced wake for 6.5 h after dosing with no rebound hypersomnolence for 2 h after returning to vehicle values (Figure 5A). However, hypersomnolence was observed later between 9 and 11.5 h post dosing (−39.3 ± 5.7 min, P < 0.05 vs. vehicle, Dunnett’s). The group treated with amphetamine alone showed a similar period of hypersomnolence during this period (−24.3 ± 5.3 min, P < 0.05 vs. vehicle) in addition to the early RHS at 3–5 h; the amphetamine and combined treatment group values were not different from each other (P > 0.05, unpaired t test).

Differences between the 3 drug treatment groups are particularly evident from the CWS curves (Figure 5B). Note that amphetamine-induced wake reversed rapidly starting at 3 h, while wake induced by nomifensine alone showed a plateau between 4 and 6 h before reversing. Based on individual values for each animal, the mean maximal CWS of the combined amphetamine + nomifensine group (224.2 ± 7.7 min) was significantly greater than the sum of the maximal CWS means (193.4 ± 4.7, P < 0.001; t test) produced by amphetamine (91.1 ± 5.4) and nomifensine (102.3 ± 7.0) alone. Furthermore at 22 h post dosing, amphetamine retained only 7% of its maximal CWS, whereas nomifensine retained 64%, and the combined amphetamine + nomifensine treatment retained 50%.

Evaluation of SWS and REMS latencies produced results analogous to those for wake activity. For both outcome measures, all treatment combinations were significantly different from each other (P < 0.001; Bonferroni/Dunn) except for amphetamine alone versus nomifensine alone. Furthermore, the SWS onset latency of the combined treatment (379.5 ± 18.6 min) was greater than the sum (318.4 ± 6.0 min, P = 0.002; t test) of the individual amphetamine (151.6 ± 7.3) and nomifensine (166.8 ± 8.8) latencies, indicating more prolonged waking with the combined treatment.

Figure 3—Percent time awake produced by DA-releasing and DAT-inhibiting agents. Each point represents group mean ± SEM. Separate vehicle controls were included during testing of each compound; solid points were significantly different from the within-experiment vehicle (not shown). The gray curve (“Average vehicle”) represents a composite average vehicle curve of all vehicle-treated animals in these experiments since individual vehicle groups were statistically comparable (P = 0.22; repeated-measures ANOVA). Hypersomnolence (excess sleepiness relative to vehicle) was produced by amphetamine, methamphetamine, phentermine, cocaine, methylphenidate, and bupropion immediately after recovery, but not by nomifensine, mazindol, GBR-12909, and GBR-12935. AMP = amphetamine; MAMP = methamphetamine, MPH = methylphenidate, PHN = phentermine. Dose (in mg/kg ip) for each compound is given after compound name in legend; the number of rats per group is given in Table 2. Black bar indicates lights off.
The potentiation of wake activity produced by the combination of amphetamine and nomifensine might have partly resulted from reduced sleep pressure, as the rats were approaching the time of lights off during which they would typically be awake. Therefore, the effects produced by twice the initial doses of amphetamine and nomifensine (e.g., 2 and 6 mg/kg, respectively) given separately were evaluated. Amphetamine and nomifensine (3 mg/kg ip) was still greater than the values for the double doses of amphetamine and nomifensine alone (comparison of all 3 treatment groups: P < 0.0001, ANOVA; P < 0.05 for amphetamine and nomifensine versus combination treatment, P > 0.05 for amphetamine versus nomifensine alone; Bonferroni/Dunn).

### Combined Effects of Nomifensine and Amphetamine on Motor Activity and Intensity

The combination of amphetamine (1 mg/kg ip) and nomifensine (3 mg/kg ip) increased 4 h CWT motor activity values to a level approximately equal to the sum of their individual effects. It may be noted however that the combination treatment increased motor activity relative to the vehicle group up to 7 h post dosing (up to the time of lights off; data not shown), while the individual amphetamine and nomifensine treatments increased it only for 2.5 h. Furthermore, motor intensity was increased by the combination treatment (Table 6).

### Effect of Nomifensine and Amphetamine on Synaptosomal Dopamine Release

One explanation for the increased duration of amphetamine-induced wake activity produced by nomifensine is that by blocking DAT, the rate at which amphetamine is transported into the terminal is reduced, thereby blunting and perhaps prolonging amphetamine’s action in raising extracellular DA. In order to investigate this proposition, the ability of nomifensine to inhibit amphetamine-induced DA release from synaptosomes was evaluated. Nomifensine alone had no effect on DA release (EC50 > 100 µM; Figure 7A). The mean EC50 value for amphetamine-induced DA release was 0.065 µM (0.034 to 0.126, 95% confidence limit).

| Table 4—Time-Course of Wake Activity of Dopaminergic Agents Compared to Within-Experiment Vehicle Group |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------|
| **Compound \ Time** | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | 3.5 | 4.0 | 4.5 | 5.0 | 5.5 | 6.0 | 6.5 | 7.0 |
|----------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Amphetamine          | w   | w   | w   | w   | w   | h   | h   | h   | h   | h   | h   | h   | h   | h   | h   |
| Methamphetamine     | w   | w   | w   | w   | w   | w   | h   | h   | h   | h   | h   | h   | h   | h   | h   |
| Phentermine          | w   | w   | w   | w   | w   | w   | h   | h   | h   | h   | h   | h   | h   | h   | h   |
| Cocaine              | w   | w   | w   | w   | w   | w   | h   | h   | h   | h   | h   | h   | h   | h   | h   |
| GBR12909             | w   | w   | w   | w   | w   | w   | w   | h   | h   | h   | h   | h   | h   | h   | h   |
| GBR12935             | w   | w   | w   | w   | w   | w   | w   | w   | h   | h   | h   | h   | h   | h   | h   |
| Mazindol             | w   | w   | w   | w   | w   | w   | w   | w   | w   | w   | w   | w   | w   | w   | w   |
| Methylphenidate      | w   | w   | w   | w   | w   | w   | w   | w   | w   | w   | w   | w   | w   | w   | w   |
| Nomifensine          | w   | w   | w   | w   | w   | w   | w   | w   | w   | w   | w   | w   | w   | w   | w   |

The "0" time point is the time of drug administration. See Table 3 for doses of each drug. "w" donates significant increases in wake, "h" donates significant hypersomnolence. w, h: P < 0.05, unpaired t test versus within-study vehicle.
Table 5—Measures of Hypersomnolence for DA-Releasing and DAT-Inhibiting Agents

| Treatment                  | Slope min/h | 3h RHS min | 22 h CWS min | Maintained CWS % |
|----------------------------|-------------|------------|--------------|------------------|
| Amphetamine (1)            | -26.1 ± 2.5 | 27.5 ± 4.1 | 21.9 ± 11.6  | 22.6 ± 11.6      |
| Methamphetamine (1)        | -27.7 ± 4.4 | 25.8 ± 7.2 | 47.3 ± 21.1  | 48.8 ± 21.6      |
| Phentermine (3)            | -22.6 ± 1.5 | 30.6 ± 4.7 | 41.3 ± 15.7  | 50.0 ± 20.3      |
| Bupropion (30)             | -25.8 ± 4.1 | 22.9 ± 7.4 | 74.3 ± 16.1  | 71.3 ± 15.3      |
| Cocaine (15)               | -23.8 ± 5.0 | 8.0 ± 6.5  | 45.9 ± 35.7  | 45.5 ± 26.2      |
| GBR-12909 (6)              | -21.7 ± 2.2 | 5.7 ± 6.1  | 53.7 ± 24.7  | 54.8 ± 26.3      |
| GBR-12935 (10)             | -23.3 ± 1.7 | 1.5 ± 14.3 | -             | -                |
| Mazindol (1)               | -10.2 ± 1.1 | 2.3 ± 5.2  | 103.7 ± 24.4 | 91.9 ± 22.4      |
| Methylphenidate (6)        | -32.0 ± 5.4 | 20.9 ± 6.4 | 38.7 ± 25.0  | 45.5 ± 50.6      |
| Nomifensine (3)            | -13.7 ± 1.5 | -13.3 ± 9.2| 94.8 ± 19.2  | 85.2 ± 19.3      |

Compounds were tested at equal wake-promoting doses (doses are listed in parentheses; see Table 2 for the number of animals tested per each dose). Data represent group mean (± SEM) for rate of sleep recovery after onset of sleep recovery (Slope, in min/h); rebound hypersomnolence (cumulative wake deficit) relative to vehicle for 3 h period following maximal CWS (3h RHS, in min); CWS at 22 h post dosing (22 h CWS, in min); and percent of maximal CWS maintained at 22 h post dosing (Maintained CWS, in %). The 22 h CWS and % Maintained SWS data were not available for GBR-12935 as recording was only carried out for 11 h for this drug. See Materials and Methods for additional details of measurements.

its) (Figure 7B). At 10 µM, nomifensine increased the EC_{50} value for amphetamine-induced DA release by over 270-fold (Figure 7B). Likewise, bupropion, cocaine, methylphenidate, and mazindol increased the EC_{50} value for amphetamine-induced DA release to 2.5 (1.44–4.34), 3.4 (1.36–8.70), 10.4 (2.44–44.0), and 47.1 (23.0–96.5) µM respectively (data not shown).

Pharmacokinetics of Nomifensine and Amphetamine Alone and in Combination

Given the prolonged wake duration following combined amphetamine and nomifensine treatment, the concern arose that the plasma and/or brain levels of one or both compounds could be significantly altered. Therefore, plasma and brain concentrations of amphetamine and/or nomifensine were evaluated up to 6 h after administration of amphetamine (1 mg/kg), nomifensine (3 mg/kg), or combined treatment. By themselves, the drugs each had a plasma half-life of 0.9 ± 0.1 h and a CMax time of 0.3 h (Table 7). When administered together, the plasma half-life of amphetamine increased by 78% to 1.6 ± 0.1 h (P = 0.02, unpaired t test), and that of nomifensine increased 22% to 1.1 ± 0.1 h (P > 0.05, unpaired t test). The brain levels of nomifensine were unaltered by the presence of amphetamine (P > 0.2, unpaired t test at 1, 3, and 6 h after dosing). In contrast, the brain concentration of amphetamine in the presence of nomifensine was significantly increased at 1, 3, and 6 h (P < 0.01) and the brain half-life doubled from 1.1 to 2.3 h. The brain/plasma ratio for amphetamine at 1 h after dosing by itself or with nomifensine was not different (7.2 ± 0.9 vs. 8.3 ± 0.5, P = 0.36, unpaired t test). Likewise, the brain/plasma ratio for nomifensine at 1 h after dosing by itself or with amphetamine was not different (10.2 ± 0.5 vs. 11.2 ± 0.9, P = 0.33).

DISCUSSION

The results of the present experiments provided several confirmatory and novel findings relevant to the wake-promoting properties of dopaminergic agents. First, we confirmed the relative potencies of several dopaminergic compounds in a systematic comparison of their effects on spontaneous DA release in rat synaptosomes and found that all non–DA-releas-
ing compounds inhibited amphetamine-induced DA release. Second, we confirmed robust wake-promoting properties of several DA-releasing and DAT-inhibiting agents and report for the first time that phentermine and GBR-12935 exhibit similar wake-promoting activity in rats. Third, head-to-head comparisons of RHS produced by equal wake-promoting doses of these agents indicate that DA-release by itself (as determined by the synaptosomal preparation), but not DAT-inhibition alone, appears to be sufficient to produce RHS. Thus, this study provides evidence against the hypothesis that RHS is associated solely with amphetamine-like DA-release. Fourth, we demonstrated that motor stimulatory effects of DA agents did not correlate with RHS. Fifth, nomifensine was shown to potentiate the wake-promoting effects of amphetamine and eliminated amphetamine-induced RHS. A mechanistic basis for this interaction is supported by synaptosomal DA release experiments showing inhibition of amphetamine-induced DA release by nomifensine.

**Relative Potency for Synaptosomal DA Release**

Although $K_i$ values for DAT inhibition and DA release are available for the compounds in this study, a systematic comparison in synaptosomes seemed prudent to confirm relative potencies for DA release, and inhibition of amphetamine-induced DA release by DAT inhibitors. The relative potencies for DA release obtained in the present study were consistent with published values. In the case of GBR-12909, it is noteworthy that the present results are consistent with those of Easton et al., using ex vivo slices in showing release potency intermediate between amphetamine and methylphenidate, whereas Rothmann and Baumann reported essentially no release by GBR-12909 ($K_i > 10,000$) probably due to the short incubation time (5 min, versus 30 min in the present study).

**Wake Enhancement and Sleep Recovery Produced by DA-releasing and DAT Inhibiting Agents**

As expected, the DA-releasing agents amphetamine and methamphetamine enhanced wake, with a similar result was obtained for phentermine which, while not unexpected based on its DA-releasing characteristics as noted above, does not appear to have been previously reported in animal studies. Likewise, dose-dependent wake enhancement of several DAT inhibitors was confirmed, and we further report similar effects produced by GBR-12935. Comparison of the effects of all agents tested using the DA-release/ DAT-inhibition designation as a factor did not support the initial hypothesis that RHS might be solely associated with...
amphetamine-like DA release. Thus RHS was produced by the DA-releasing agents (amphetamine, methamphetamine, and phentermine) as well as by some DAT-inhibiting agents (methylphenidate, cocaine, and bupropion) at equal wake-promoting doses. However, comparison across all the compounds independent of drug category revealed significant treatment effects. Specifically, recovery slope and cumulative hypersomnolence were significant across all groups, and both nomifensine and mazindol were different from amphetamine on the 3 h cumulative hypersomnolence measure. There were also qualitative differences within the DAT-inhibiting group. Some DAT-inhibiting agents (e.g., cocaine, methylphenidate, and bupropion) produced more RHS and retained less cumulative wake than others, in particular mazindol and nomifensine, after the onset of sleep recovery.

Several factors besides DA release or DAT inhibition per se might be involved in RHS. For example, different modes by which DAT-inhibiting agents interact with DAT could contribute to their different pharmacological profiles. Also, amphetamine, methamphetamine, and phentermine promote release of norepinephrine and serotonin as well as dopamine, and all the compounds tested have differential affinities for DAT, norepinephrine transporter (NET), serotonin transporter (SERT), and other targets. While cocaine blocks DAT, NET, and SERT relatively equally, bupropion, amphetamine, methamphetamine, and phentermine are relatively weak at SERT indicating this transporter is unlikely to play a major role in RHS. Also, neither inhibition of NET alone nor direct adrenergic agonist activity appear to be potent factors in wake promoting activity. Furthermore, DSP-4 lesion of the noradrenergic system did not appear to block methamphetamine-induced SWS rebound, suggesting NE is not critical for RHS. Specific studies would be required to confirm these conjectures.

It is notable that cocaine did not induce dopamine release in the synaptosomal preparation, yet it clearly does so both in vivo and in the electrically stimulated slice preparation. We did not observe alterations in cocaine-induced DA release when synaptosomes were depolarized by elevating potassium (5–30 mM) or adding 4-aminopyridine (0.3, 1 μM) (unpublished observations). This and other evidence suggest that RHS following cocaine administration might be produced by DA release that is mechanistically different from that produced by amphetamine.

Excessive DA release by whatever means can result in after-effects such as vesicle depletion, D2 receptor activation, and/or D1 receptor activation and subsequent desensitization, which can eventually lead to low basal levels of DA release and depressed postsynaptic DA signaling. If RHS is indeed a result of supranormal dopamine release and subsequent desensitization, then a more gradual increase in DA would be expected to produce less of a disturbance of synaptic homeostatic mechanisms, and therefore a smaller or negligible RHS. The rate and magnitude of change in synaptic DA concentration has also been related to the abuse potential of dopaminergic psychostimulants. It is thus notable that drugs that produced larger RHS in the present study (e.g., amphetamine, cocaine) appear also to have higher abuse potential than those that produced smaller RHS (mazindol and nomifensine). Additional experiments to establish whether RHS could serve as a surrogate marker of, or even be mechanistically related to, drug abuse liability appear warranted.

### Motor Activity and Intensity of DA-releasing and DAT-inhibiting Agents

Different degrees of motor activation were produced by the compounds tested in the present study. The findings are consistent with other studies of DA agents in mice showing increased motor activity produced by DA-releasing and DAT-inhibiting agents. The results also demonstrate that RHS was not correlated with changes in motor activity or intensity. Specifically, there was no correlation between 3 h RHS and the magnitude of the change in motor activity produced by these compounds. Motor intensity was increased by amphetamine and decreased by mazindol, while the remaining drugs had no effect on motor intensity regardless of their effect on RHS. Importantly, these findings indicate that RHS was not simply a result of excessive motor activation preventing animals from going to sleep or leading to exhaustion and rapid sleep recovery.

### Combined Effect of Nomifensine and Amphetamine on Sleep/Wake Measures

It is generally accepted that amphetamine induces DA release from terminals by first being taken up via DAT, and subsequently entering synaptic vesicles where it promotes release of DA into the cytosolic milieu. The increased cytosolic DA concentration ultimately drives the DAT in a reverse direction, resulting in increased synaptic DA levels. It is thus presumed...
that blockade of DAT by nomifensine (for example) should prevent amphetamine from entering the terminals and thereby decrease the uptake of amphetamine and attenuate its effects on wake and hypersomnolence. Therefore, the absence of RHS produced by the combination of amphetamine and nomifensine was not unexpected, while the enhancement of wake promotion was surprising. This enhancement is best illustrated by the mean maximal CWS which for the combined amphetamine and nomifensine group was greater than the sum of the maximal CWS means produced by each compound alone. Furthermore, the group treated with amphetamine and nomifensine retained approximately 50% of its maximal CWS, while the group treated with amphetamine alone retained only 7% of maximal CWS at 22 h post dosing. The treatments were further distinguished by the rate of initial reversal of sleep recovery, which for amphetamine alone was abrupt, while for nomifensine alone or in combination with amphetamine was gradual, with correspondingly lower magnitude slope values.

Since the duration of wake activity produced by the combined treatment was much longer than that of amphetamine and nomifensine alone, differences in sleep pressure must be considered. Combined amphetamine and nomifensine treatment extended wake from 3 h post dosing to 7 h post dosing. During this time period, sleep pressure is normally decreasing as the rats approach their physiological active phase starting at 7 h post dosing (lights off). This could reduce the rate of sleep recovery in the combination treatment group. However, prolonged wake activity also should increase sleep debt, which would be expected to have an opposite effect. Although the net effect of the contributions of these 2 opposing processes is hard to predict, there was no immediate RHS in the combination group between 6.5 and 8.5 h analogous to that seen in the amphetamine alone group at 3–5 h. At 22 h after dosing, the combination treatment produced substantially greater surplus wake activity (112 min) than the individual effects combined (72 min). Furthermore, comparison of the separate effects of 2 mg/kg ip amphetamine or 6 mg/kg ip nomifensine versus combined treatment with 1 mg/kg ip amphetamine plus 3 mg/kg ip nomifensine also indicates that the combination of the 2 drugs was more efficacious in inducing wake than each drug alone even at 2-fold higher doses.

There were later periods of hypersomnolence in the amphetamine alone and combined amphetamine and nomifensine groups, from 9 to about 11 h after dosing. The similarity in the time-course of this period of late hypersomnolence between those 2 groups and the absence of late hypersomnolence in the nomifensine alone group suggest that this effect was chiefly produced by amphetamine. The physiological significance and mechanistic explanation of this late hypersomnolence produced by amphetamine remain unclear at present. Clearly, it did not appear to depend on the duration of wake activity or the presence of earlier RHS.

Other factors that might explain or contribute to the enhanced wake activity of the combination therapy include a metabolic interaction, pharmacokinetic effects such as rapid clearance, or an effect on cerebral blood flow in which plasma and brain levels of amphetamine are altered by co-administration of nomifensine. We have no evidence that differences in clearance or half-life at the time of onset of sleep recovery contrib-

ute to RHS, but this requires further investigation to rule out. Pharmacokinetic data indicate that increased brain concentration of amphetamine must be considered along with the competitive interaction between nomifensine and amphetamine at the DAT. Amphetamine did not significantly alter the plasma or brain concentrations of nomifensine. In contrast, in the presence of nomifensine, the plasma and brain concentrations of amphetamine increased, and the respective half-lives increased 1.8 and 2.1 times, while the brain/plasma ratio of amphetamine did not change. The increase in amphetamine concentrations could arise from reduced elimination or degradation in the liver. Additionally, some metabolism of amphetamine occurs in the brain, but the relative contributions of cytosolic versus extrasynaptic degradation is not clear. Thus the increased brain concentration of amphetamine in combination with (and perhaps even because of) its reduced synaptic uptake could result in a low but sustained level of synaptic DA sufficient to prolong wake activity, but insufficient for inducing after-effects such as RHS and/or depressed postsynaptic DA signaling. This scenario is consistent with demonstrations that, via DAT inhibition, nomifensine and cocaine can antagonize amphetamine-induced DA release in vitro, and that GRB-12909 can antagonize methamphetamine-induced DA-release in the nucleus accumbens in vivo. The present study similarly demonstrated that nomifensine blocked amphetamine-induced DA release in the rat synaptosome assay, decreasing the potency of amphetamine 270-fold. The nearly 2-fold increase in brain levels of amphetamine at 1 h in the combination treatment could also explain the increase in motor activity and intensity in this group. Such an increase in brain amphetamine concentration, however, would still be expected to produce evidence of RHS around 7–9 h after dosing, which was not observed. Additional combination studies at lower doses and earlier dosing times are needed to determine whether the absence of detectable RHS is due to reduced DA release and/or to increased wake pressure near the lights off time.

In conclusion, the present data demonstrate that synaptic somal DA-releasing activity appears to be sufficient for producing RHS after increased wake. Differential effects of DAT inhibitors on RHS suggests that other pharmacologic and/or pharmacokinetic properties, including non-amphetamine-like DA release and/or effects on other monoamine transporters, are also capable of affecting RHS. Furthermore, reduction of the rate of amphetamine-induced DA release by nomifensine resulted in increased wake while mitigating RHS. This result provides a rationale for modulation of DA pharmacokinetics by combining DA-releasing and DAT-inhibiting agents as an approach to treating disorders associated with excessive sleepiness, as well as other disorders such as attention deficit hyperactivity disorder, currently treated with dopaminergic stimulants.

**ABBREVIATIONS**

Amphetamine, d-amphetamine; ANOVA, analysis of variance; ZT, Zeitgeber time; CWS, cumulative wake surplus; CWT, cumulative wake time; DA, dopamine; DAT, dopamine transporter; RHS, rebound hypersomnolence; NET, norepinephrine transporter; REMS, rapid eye movement sleep; SERT, serotonin reuptake transporter; SWS, slow wave sleep.
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