Increased Dopaminergic Transmission Mediates the Wake-Promoting Effects of CNS Stimulants

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Amphetamine-like stimulants are commonly used to treat sleepiness in narcolepsy. These compounds have little effect on rapid eye movement (REM) sleep-related symptoms such as cataplexy, and antidepressants (monoamine uptake inhibitors) are usually required to treat these symptoms. Although amphetamine-like stimulants and antidepressants enhance monoaminergic transmission, these compounds are non-selective for each monoamine, and the exact mechanisms mediating how these compounds induce wakefulness and modulate REM sleep are not known. In order to evaluate the relative importance of dopaminergic and noradrenergic transmission in the mediation of these effects, five dopamine (DA) uptake inhibitors (mazindol, GBR-12909, bupropion, nomifensine and amineptine), two norepinephrine (NE) uptake inhibitors (nisoxetine and desipramine), d-amphetamine, and modafinil, a non-amphetamine stimulant, were tested in control and narcoleptic canines. All stimulants and dopaminergic uptake inhibitors were found to dose-dependently increase wakefulness in control and narcoleptic animals. The in vivo potencies of DA uptake inhibitors and modafinil on wake significantly correlated with their in vitro affinities to the DA and not the NE transporter. DA uptake inhibitors also moderately reduced REM sleep, but this effect was most likely secondary to slow wave sleep (SWS) suppression, since selective DA uptake inhibitors reduced both REM sleep and SWS proportionally. In contrast, selective NE uptake inhibitors had little effect on wakefulness, but potently reduced REM sleep. These results suggest that presynaptic activation of DA transmission is critical for the pharmacological control of wakefulness, while that of the NE system is critical for REM sleep regulation. Our results also suggest that presynaptic activation of DA transmission is a key pharmacological property mediating the wake-promoting effects of currently available CNS stimulants.

CURRENT CLAIM: Increased dopaminergic transmission mediates the wake-promoting effects of CNS stimulants.

Narcolepsy is a sleep disorder characterized by excessive daytime sleepiness (EDS) and dissociated manifestations of REM sleep, namely cataplexy (sudden onset of muscle atonia induced by emotional excitation), hypnagogic hallucinations, and sleep paralysis (Aldrich, 1992; Guilleminault, 1994; Mitler et al., 1990; Nishino and Mignot, 1997). Central nervous system (CNS) stimulants, amphetamines and amphetamine-like compounds (methylphenidate and pemoline), are the most commonly used pharmacological treatments for EDS in narcolepsy (Mitler et al., 1990, 1994; Parkes et al., 1975; Thorpy and Goswami, 1990). Amphetamine-like stimulants at doses effective to treat EDS, however, have little beneficial effects on REM sleep-related symptoms, and antidepressants or monoamine oxidase inhibitors are thus also required to treat these symptoms (Mitler et al., 1990, 1994; 1990; Parkes et al., 1975; Thorpy and Goswami, 1990). The success of these pharmacological approaches, however, is limited by the occurrence of multiple side effects and the development of drug tolerance (see Mitler et al., 1990, 1994; Parkes et al., 1975; Thorpy and Goswami, 1990).

Pharmacological, neurochemical, and neurophysiological studies using a canine model of narcolepsy (see Baker and Dement, 1985; Mignot et al., 1992; Mignot and Mignot, 1997; Nishino et al., 1994 for review) have shown that high cholinergic activity and/or low monoaminergic activity in the brainstem and the basal forebrain are critically involved in mediating EDS and abnormal REM sleep in narcolepsy (Nishino and Mignot, 1997; Nishino et al., 1994, 1995; Reid et al., 1994a, 1994b, 1996). In contrast to the therapeutic applications of monoaminergic compounds in narcolepsy, attempts to use cholinergic antagonists for the treatment of human narcolepsy have generally failed, partially due to the peripheral side effects induced by the compounds (see Gillin et al., 1976; Nishino and Mignot, 1997).

Amphetamine-like stimulants are the most potent and efficacious wake-promoting compounds currently available, but little is known regarding their mode of action on sleep and wakefulness. These agents have multiple pharmacological properties, such as increasing monoamine release, blocking monoamine reuptake and inhibiting monoamine oxidase (see Parkes, 1985b for review). These properties contribute to the global enhancement of central monoaminergic transmission and are not selective for any single monoamine (DA, NE, or serotonin [5-HT]) (see Parkes, 1985b for review). The specific pharmacological property by which these compounds enhance wakefulness is still being debated (see Nishino and Mignot, 1997 for review) and either or both increased NE or DA transmission have been suggested to be involved (see Baldessarini, 1972; Parkes, 1985b). For many years, pharmacologists have studied the effects of these compounds...
on locomotor activity or on barbiturate-induced locomotor depression in rodents and used these effects as an index of their “alerting” effects (see Baldessarini, 1972; Ogren et al., 1983; Taylor and Robbins, 1984). Amphetamine-like stimulants, DA uptake inhibitors, and DA agonists at high doses, have similar CNS stimulant effects, suggesting dopaminergic mediation of wake promotion (see Koob and Swerdlow, 1988 for review). Other investigators have also suggested adrenergic mediation of CNS stimulants for locomotor activation (Ogren et al., 1983; Taylor and Robbins, 1984), but this effect may not directly represent the wake-promoting effects of these compounds. Furthermore, much higher doses of CNS stimulants are generally required to increase locomotor activity versus those to induce wakefulness in vivo (see also Shelton et al., 1995). In addition, some wake-inducing compounds such as modafinil promote wakefulness, as evidenced by polygraphic recordings, without significantly increasing locomotor activity (Edgar et al., 1994; Shelton et al., 1995). Additional studies using selective DA and NE compounds and EEG recordings are thus needed to address these controversies.

Previous studies have shown that NE uptake inhibitors reduce canine cataplexy much more potently than do 5-HT or DA uptake inhibitors (Mignot et al., 1993; Nishino et al., 1993). These results suggest that the NE system is the most important monoaminergic system for the control of cataplexy (Mignot et al., 1993; Nishino et al., 1993), and that antidepressant therapy alleviates cataplexy via adrenergic uptake inhibition. Selective DA uptake inhibitors had no effect on canine cataplexy, but subjective observation indicated an increase in wakefulness in narcoleptic animals (Mignot et al., 1993; Nishino and Mignot, 1997). We therefore hypothesized that increased dopaminergic transmission is a key pharmacological property mediating the wake-inducing effects of amphetamine-like stimulants, while adrenergic effects are more important for the regulation of REM sleep and REM sleep-related symptoms.

In the current study, the effects of DA uptake and NE uptake inhibition on wake and REM sleep were examined in genetically narcoleptic and control Doberman pinschers. A greater understanding of the basic mechanisms of these two monoaminergic systems and their pharmacological control of the sleep-wake process would lead to better treatments not only for EDS but also for various neurological and neuropsychiatric disorders associated with sleep problems.

**METHODS**

**Drugs**

Five DA uptake inhibitors [amineptine (1.00, 4.00 mg/kg, n = 5), bupropion (1.00, 4.00 mg/kg, n = 4), GBR-12909 (0.25, 1.00 mg/kg, n = 5 for narcoleptic and n = 4 for control animals), nomifensine (0.25, 1.00 mg/kg, n = 4), and mazindol (16.00, 64.00 µg/kg, n = 4)], 2 NE uptake inhibitors [nisoxetine (0.125, 0.50 mg/kg, n = 5 for narcoleptic and n = 4 for control animals) and desipramine (0.10, 0.40 mg/kg, n = 4)], and 2 wake-promoting compounds [d-amphetamine (0.1, 0.2 mg/kg, n = 4) and modafinil (1.25, 5 mg/kg, n = 4)] were included for the sleep recording study. Although the effects of d-amphetamine and modafinil on sleep in narcoleptic dogs were previously published by our group (Shelton et al., 1995), the sleep recording data for d-amphetamine (same dose range) and modafinil (lower dose range) included in this study were newly collected using the same implanted animals used for the polygraphic recordings of DA and NE uptake inhibitors.

Amineptine was obtained from Servier, Paris, France. Bupropion, GBR-12909, nomifensine, mazindol, nisoxetine, and d-amphetamine were purchased from Research Biochemical International, Natick, MA. Desipramine was purchased from Sigma, St Louis, MO. Modafinil was obtained from Laboratorie L. Lafon, Maisons Alfort, France. Each drug was tested using 2 different doses (shown in parentheses above) and a baseline session was carried out for each compound using either saline (for d-amphetamine and bupropion) or DMSO (for all other compounds), depending on the appropriate vehicle used for the corresponding drug. All drug solutions were prepared on each experimental day. All compounds were administered intravenously through the cephalic vein, and the order of the injections (vehicle, low dose, high dose) was randomized for each session. At least 2 days were allowed between each drug injection and the following recording session to allow for drug washout. Since the order of the injections was randomized, any residual drug effects were considered to be negated for the analysis of the drug effect of each dose.

**In vitro DAT and NET binding**

In order to characterize the selectivity and potency of each compound for the DA and NE uptake sites (DAT and NET), in vitro binding affinities for the DAT and NET were assessed using canine brain tissue. Frozen control canine brain samples (purchased from Pel-Freeze, Rogers, AR) were dissected, and the cortex (for NET binding) or caudate (for DAT), was homogenized in 15 ml of ice-cold buffer (sodium phosphate buffer 25 mM [final sodium concentration 48 mM], pH 7.7 for DAT binding, 50 mM Tris-HCl, 300 mM NaCl, 5 mM KCl buffer, pH 7.4 for NET binding) using a Polytron tissue grinder. Homogenates were centrifuged at 46,000 g x 15 min at 4°C. Supernatants were discarded, and the pellets were washed twice by re-suspension and re-centrifugation. The final pellet was re-suspended (3 mg/ml) in ice-cold buffer.

Scatchard analyses for DAT and NET were first carried out using [3H]-WIN 35,428 (specific activity 84 Ci/mmol; NEN Product, Boston, MA) and [3H]-nisoxetine (specific activity 85 Ci/mmol; Amersham, Arlington Heights, IL), and single binding sites and dissociation constants (Kd) of 5.4 nM for DAT and 4.0 nM for NET were obtained. Displacement experiments were therefore carried out using [3H]-WIN 35,428 (2 nM) for DAT binding and [3H]-nisoxetine (1 nM) for NET binding. Specific binding for the DAT or NET is defined as the radioactivity that can be displaced by cocaine (30 µM) or mazindol (1 µM), respectively. Total binding and non-specific binding were measured in duplicate. Displacement experiments were performed to obtain inhibition constants (Ki) for various displacing agents (DA and NE uptake inhibitors and CNS stimulants). Binding is initiated by adding 450 µl of tissue suspension to 25 µl of labelled ligand and 25 µl of buffer
In vitro

Table 1

In vitro Binding Affinities to DAT and NET of Various DA and NE Uptake Inhibitors, d-amphetamine and Modafinil

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$K_i$-(DAT)</th>
<th>$K_i$-(NET)</th>
<th>Designated Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>bupropion</td>
<td>3.10E-07</td>
<td>1.04E-04</td>
<td>DA uptake inhibitor</td>
</tr>
<tr>
<td>GBR 12909</td>
<td>1.59E-08</td>
<td>2.29E-06</td>
<td>DA uptake inhibitor</td>
</tr>
<tr>
<td>amineptine</td>
<td>3.30E-06</td>
<td>3.56E-06</td>
<td>low potency DA uptake inhibitor</td>
</tr>
<tr>
<td>mazindol</td>
<td>8.60E-09</td>
<td>7.60E-09</td>
<td>DA+NE uptake inhibitor</td>
</tr>
<tr>
<td>nomifensine</td>
<td>4.40E-08</td>
<td>3.10E-08</td>
<td>DA+NE uptake inhibitor</td>
</tr>
<tr>
<td>desipramine</td>
<td>3.23E-06</td>
<td>1.14E-09</td>
<td>NE uptake inhibitor</td>
</tr>
<tr>
<td>nisoxetine</td>
<td>3.27E-07</td>
<td>8.02E-10</td>
<td>NE uptake inhibitor</td>
</tr>
<tr>
<td>d-amphetamine</td>
<td>3.30E-06</td>
<td>2.56E-08</td>
<td>amphetamine stimulant</td>
</tr>
<tr>
<td>modafinil</td>
<td>3.80E-06</td>
<td>&gt;E-4</td>
<td>non-amphetamine wake-promoting compound</td>
</tr>
</tbody>
</table>

Shown inhibition constants ($K_i$s) for DAT and NET were obtained by displacement experiments using [3H]-WIN 35,428 (2nM) and canine caudate membrane, and [3H]-nisoxetine (1nM) and canine cortex membrane, respectively. Specific binding for DAT and NET bindings is defined as the radioactivity that can be displaced by cocaine (30 µM) and mazindol (1 µM), respectively. Data were analyzed assuming single binding site models by analysis of multiple files (3 to 6) simultaneously.

Six-hour daytime polygraph recordings

The dogs were habituated to the experimental room, wearing recording cables and a harness, for at least 2 days before recording. Injections were given between 9:00 and 10:00 a.m. Six-hour polygraph recordings on an 8-channel Grass polygraph began immediately following the injection. The dog was left alone in the recording room (3m x 3m) with the lights on while being continuously monitored from an adjacent room by a video camera.

Polygraph recordings were visually scored in 30-second epochs as described by Nishino et al. (Nishino et al., 1995). These criteria are based upon the frequency and amplitude patterns of 2 cortical tracings (fronto-frontal and fronto-parietal EEG), EMG, and EOG together with notes on behavior (behavior is continuously recorded either by direct observation or by use of a video camera). Briefly, wake includes all episodes with a low voltage mixed-frequency tracing in which the EMG was not inhibited. During this stage, canines lay down, sit up, stand or walk, and eyes are open. The drowsy state is scored when the animal lies quietly with eyes partially open and cortical EEG shows trains of theta waves (5-7 Hz) without the development of sleep spindles. In this vigilance state, synchronous waves at 4-7 Hz, 50-100 µV appear on a background of low voltage fast activity, and EMG amplitude shows a moderate decrease from wakefulness. In light sleep, canines are relaxed with prone posture, EEG patterns are more synchronous and are of higher amplitude than in the previous stage, and sleep spindles (10-14 Hz) and/or K complexes (in the fronto-parietal tracing) must be present. Deep sleep is scored when high amplitude slow waves (< 4 Hz) constitute more than 20% of a 30-sec epoch.
scored when a low voltage, mixed-frequency EEG tracing is observed together with rapid eye movements (REMs) and a significant drop in EMG activity. Animals are lying down and muscle twitches may occur, but are not always observed. In order to designate an epoch as REM sleep, the previous two epochs must have been scored as sleep (light sleep, deep sleep or REM sleep). REM sleep is considered to have ended when the EMG increases or sleep spindles and/or K complexes start to appear in the EEG tracings. Cataplexy is scored when an abrupt drop in EMG during wakefulness is observed. The dogs will often collapse if standing, with the back legs being especially likely to buckle. The EEG pattern is of low voltage, mixed frequency, with occasional REMs and muscle twitches. In contrast to REM sleep, cataplexy is scored if the previous two epochs were wake, drowsy, or cataplexy. A cataplectic attack is considered to have ended, when the EMG returns to its previous amplitude or if spindles (light sleep) begin to appear.

Statistical analyses

Friedman’s non-parametric repeated measures ANOVAs were performed for each sleep stage for the initial 4 hours, with the compound dose as the replicate, to determine the drug’s effects on sleep stages. For data presentation of the effects of compounds on sleep stages, the percent change from the baseline session for the mean value of 4-5 recording sessions are shown (see results sections, Figure 2-4), thus this parameter does not have any standard error values. When drug effects on wake were statistically significant ($p < 0.05$), the ED+40% (mg/kg i.v.), the dose that increased wakefulness to 40% above baseline were calculated from the dose-response slope by linear regression. The ED-60% (mg/kg i.v.), the dose which reduced REM/SWS ratio by 60% below baseline were also calculated. In vitro effects on sleep parameters ($\log ED+40\%$ or $ED-60\%$ [mol/kg i.v.]) were correlated with in vitro binding affinity ($\log Ki$ [M]) using Pearson’s correlation. All statistical computations were made using SYSTAT (Systat Inc., Evanston, IL) on a personal computer.

RESULTS

In vitro binding affinities of compounds for DAT and NET

All DA and NE uptake inhibitors displaced 100% of the specific binding with different affinities for the DAT ($Ki = 8.60$ $10^{-9}$ M to 3.30 $10^{-6}$ M) and the NET ($Ki = 8.02$ 10-10 M to 1.04 $10^{-4}$ M). The respective inhibition constants ($Ki$‘s) for each compound are listed in Table 1. The results indicate that desipramine and nisoxetine are potent and selective NE uptake inhibitors, and that GBR-12909 and bupropion are potent and selective DA uptake inhibitors. Mazindol and nomifensine were revealed to be potent for both the DA and NE uptake sites and are thus designated as “DA+NE uptake inhibitors”. Aminentpine showed low affinity for both DAT and NET and was designated as a “low affinity DA uptake inhibitor”. As we previously reported using guinea pig brain tissue (Mignot et al., 1994), modafinil also showed a low but selective affinity to the DAT in canine brain membrane ($Ki = 3.8$ $10^{-6}$ M) ($Ki$ for the NET is greater than $10^{-4}$ M). D-amphetamine exhibits relatively low affinity for the DAT and high affinity for the NET.

In vivo effects of compounds on sleep recordings

Typical hypnograms of 3 doses for DA, NE and DA+NE uptake inhibitors and modafinil are shown in Figure 1a.b. The hypnograms, which depict the sleep-wake architecture as determined by visual scoring, qualitatively show the dose response, time course of action, and the differences among DA, NE, and DA+NE uptake inhibitors and modafinil in their effects on the various arousal states. Under our experimental recording conditions, narcoleptic dogs are found to be asleep (light + deep + REM sleep) 44.3% of the time, and undergo REM sleep and cataplexy 7.2% and 1.0% of the time, respectively, during an average six-hour baseline recording ($n = 39$). The drug effects were generally most prominent within the first 4 hours of recording (Figure 1a.b). Thus, effects on sleep parameters for the initial 4 hours were used for further quantitative analysis presented in this manuscript. Figure 1b-i shows that bupropion, a selective DA uptake inhibitor, promoted wakefulness, almost completely suppressing sleep for the first 2 hours of the high-dose session. However, when sleep did occur in the third hour, REM sleep also appeared. Desipramine, a selective NE uptake inhibitor, had no effect on wakefulness, but greatly suppressed REM sleep (Figure 1b-ii). At the low dose, REM sleep did not appear until 3 hrs after injection, and even then, there were only a few short episodes of REM sleep in the entire 6-hour recording. At the higher dose, desipramine completely suppressed REM sleep until the middle of the fifth hour, and again, the episodes were few and brief. Nomifensine, a DA+NE uptake inhibitor, promoted wakefulness and also suppressed REM sleep. At the higher dose, there was almost complete suppression of sleep for 3 hours after injection, as well as complete suppression of REM sleep for the entire duration of the recording (Figure 1b-iii).

Both d-amphetamine (data not presented) and modafinil (Figure 1b-iv) also potently increased wake and moderately reduced REM sleep, as previously reported (Shelton et al., 1995).

Figure 1a. Effect of GBR-12909 on the sleep architecture of a narcoleptic dog, as displayed through hypnograms and EEG power spectra of a series of 6-hour daytime polygraph recordings. Under our experimental recording conditions, narcoleptic dogs are found to be asleep (light + deep + REM sleep) 44.3% of the time, and undergo REM sleep and cataplexy 7.2% and 1.0% of the time, respectively, during an average six-hour baseline recording ($n = 39$). The hypnograms (lower figures) show the sleep-wake architecture, in which the various arousal states were determined by visual scoring of 30-second epochs of polygraph data. The power spectra (upper figures) show the Fast Fourier Transformation analysis of the EEG trace. The power density at various frequencies (0 Hz to 16 Hz) is color coded, with the red end of the scale indicating highest density. During SWS (i.e., light + deep sleep), delta power [0-3Hz] increases, especially in deep sleep. In REM sleep and cataplexy, theta power [6-7 Hz] increases. A selective dopaminergic uptake inhibitor, GBR-12909, dose-dependently decreases the time spent in SWS and the delta power, especially in the first few hours after drug administration.
Figure 1b. Comparison of the effects of bupropion (DA uptake inhibitor), desipramine (NE uptake inhibitor), nomifensine (DA/NE uptake inhibitor), and modafinil (a new non-amphetamine stimulant) on the sleep architecture of a narcoleptic dog. These hypnograms show the dose response, time course of action, and the differences among DA, NE, and DA+NE uptake inhibitors in their effects on the various arousal states. (1b-i) Bupropion, a DA uptake inhibitor, promoted wakefulness, but did not reduce REM sleep completely. (1b-ii) Desipramine, a NE uptake inhibitor, did not affect wakefulness but suppressed REM sleep. (1b-iii) Nomifensine, a DA+NE uptake inhibitor, both increased wakefulness and suppressed REM sleep. (1b-iv) Modafinil also potently increased wake and moderately reduced REM sleep. For all drugs, the effects were dose-dependent and were generally most prominent in the first 4 hours.
Effects on wakefulness

Dose response effects of each compound on wake are presented as percent change in time spent in wake from the baseline sessions (Figure 2a). All DA and DA+NE uptake inhibitors and 2 CNS stimulants, d-amphetamine and modafinil, significantly increased wake in a dose-dependent fashion in narcoleptic dogs ($p < 0.05$). One selective DA uptake inhibitor, GBR-12909, was also tested in 2 control animals (2 sessions for each animal), and it was found to significantly increase wake, specifically for the first 4 hours of the recording session (+36.6%, 0.25 mg/kg and +59.8%, 1 mg/kg i.v., $p < 0.05$). In contrast, desipramine and nisoxetine, potent selective NE uptake inhibitors, had no significant effect on wake under the dose range tested in the narcoleptic animals ($p = 0.19$ and $p = .083$, respectively), although nisoxetine at the high dose (1mg/kg i.v.) moderately, but not significantly, increased wake (+15.2%) (Figure 2a). Similarly, 1mg/kg i.v. of nisoxetine moderately, but not significantly, increased
wakefulness in control animals (-1.1%, 0.25 mg/kg and +18.9%, 1 mg/kg i.v. for initial 4 hours, *p = 0.17). The moderate wake-promoting effects of nisoxetine may possibly be due to its moderately high affinity for the DA T (Table 1). From the dose-response curve, a drug dose which increased time spent in wake 40% above baseline (vehicle session) was calculated, and the order of potency of the compounds on arousal determined by EEG was then established to be: mazindol > (d-amphetamine) > nomifensine > GBR-12909 > amineptine > (modafinil) > bupropion (Figure 2a).

Correlation between the in vivo effects of the compounds on EEG arousal and their in vitro DAT and NET binding affinities

Although all DA uptake inhibitors and CNS stimulants significantly increased wake, their potency varied widely, as measured using ED+40% (mg/kg i.v.) (Figure 2). Similarly, the in vitro DAT and NET binding affinities varied among the compounds (Table 1). The correlation between in vivo effects on EEG arousal and in vitro DAT and NET binding affinities is plotted in Figure 3. All compounds except d-amphetamine fell along the regression line (*y = -9.7 + 0.92x*), and a statistically significant correlation between in vivo wake-promoting effects and in vitro DAT binding (*r = 0.78, n = 7, p < 0.05*) was observed for 5 DA uptake inhibitors and modafinil (Figure 2b), while the correlation between in vivo wake-promoting effects and NET binding affinities was not statistically significant for these compounds (*r = 0.61, n = 7, p = 0.15*) (Figure 2c). These results suggest that DA uptake inhibition is important for the EEG arousal effects of DA uptake inhibitors and modafinil. D-amphetamine, however, with a relatively low DAT binding affinity, potently promotes alertness (Figure 2b). It is also reported that d-amphetamine enhances monoamine release, or

Figure 2. (a) Dose-dependent effects on wakefulness of various DA and NE uptake inhibitors, and reference CNS stimulants (d-amphetamine and modafinil). (b, c) Correlation between the effects of various DA and NE uptake inhibitors and CNS stimulants on EEG arousal and their in vitro DAT and NET binding affinities. (a) All DA uptake inhibitors and CNS stimulants significantly increased wake. The percent change from baseline in the amount of time spent in wake for the mean value of 4-5 recording sessions is plotted for the low and high doses of each compound. Data presented here represent the first 4 hours of six-hour daytime (approximately 9:00-15:00) polygraph recordings (*p < 0.05, n = 4-5, by Friedman’s nonparametric ANOVA). (b) The correlation between in vivo effects on EEG arousal (log[ED+40% (mol/kg i.v.)]) and in vitro DAT binding affinities (log[Ki (M)]) demonstrates that all compounds except d-amphetamine fell near the regression line (*y = -9.7 + 0.92x*). A statistically significant correlation of in vivo alerting effects and in vitro DAT binding (*r = 0.78, n = 7, p < 0.05*) was observed for 5 DA uptake inhibitors and modafinil. D-amphetamine potently promotes alertness despite its relatively low DAT binding affinity. (c) The correlation between the in vivo alerting effects and the NET binding affinities was not statistically significant for these compounds (*r = 0.61, n = 7, p = 0.15*). That desipramine had no effect on wakefulness and nisoxetine only moderately increased it is consistent with the hypothesis that NE is less important in the regulation of wakefulness. In order to perform regression analysis, ED[ED+40%] of nisoxetine was also calculated and included.
DOPAMINERGIC TRANSMISSION AND ELECTROCOR TICAL AROUSAL

57

DOP AMINERGIC TRANSMISSION AND ELECTROCOR TICAL AROUSAL

inhibits monoamine oxidation (see Baldessarini, 1972; Parkes, 1985a). These properties may therefore also be involved in the EEG arousal effects of d-amphetamine. Thus the data for the effects of d-amphetamine on sleep parameters were not included for some analyses (See Figure 3-4).

Effect on REM sleep

All uptake inhibitors and modafinil dose-dependently reduced REM sleep (Figure 3a). This effect was statistically significant ($p < 0.05$) for NE uptake inhibitors (desipramine and nisoxetine), DA+NE uptake inhibitors (mazindol and nomifensine), and 1 DA uptake inhibitor (bupropion), while the effect was not statistically significant for the other compounds (GBR-12909, amineptine, and modafinil).

Nisoxetine also significantly reduced REM sleep in control dogs (-16.0%, 0.25 mg/kg and -71.4%, 1 mg/kg i.v. for the initial 4 hours, $p < 0.05$), but GBR-12909 had no significant effect on REM sleep in control animals (+31.6%, 0.25 mg/kg and +13.2%, 1 mg/kg i.v. for the initial 4 hours, $p = 0.17$), a finding that is consistent with the results obtained from narcoleptic animals. Because entry into REM sleep occurs generally after a bout of SWS, it is possible that the reduction in REM sleep by some of the compounds could actually be secondary to or an indirect effect of the suppression of SWS (or equivalently, due to an increase in wake). Thus, we also considered the REM/SWS ratio, or more specifically, the percent change from baseline of this ratio, as an indicator of the relative effects on REM sleep and SWS. As the percent change

Figure 3. (a) Dose-dependent effects on REM sleep of various DA and NE uptake inhibitors and modafinil. (b) Effects on wake, SWS, REM sleep, and REM/SWS ratio at selected doses, of various DA and NE uptake inhibitors. (a) The percent change from baseline in the amount of time spent in REM sleep for the mean value of 4-5 recording sessions is plotted for the low and high doses of each compound. Data presented here represent the first 4 hours of six-hour daytime (approximately 9:00-15:00) polygraphic recordings ($^*p < 0.05$, n = 4-5, by Friedman’s nonparametric ANOVA). (b) The percent change from baseline in the time spent in wake, SWS, and REM sleep, and the percent change from baseline of the REM/SWS ratio for the mean value of 4-5 recording sessions are shown. The doses were selected to represent the most similar alerting effects for the DA and DA+NE uptake inhibitors and modafinil, and the highest alerting effect for the NE uptake inhibitors. The data consist of the mean of the first 4 hours of 4 recording sessions.

b) Effect on Wake, SWS, REM and REM / SW Ratio at Selected Dose
in the REM/SWS ratio deviates from zero, the change in REM sleep relative to the change in SWS becomes less proportional (Figure 3b). Therefore, a large, negative value would imply that REM sleep was reduced much more than SWS, and thus a primary suppression of REM sleep is likely to have occurred.

In order to normalize the effects on wake among the compounds, the drug dose which produced a similar, mid-range wake-promoting effect for each compound was chosen, and the effects on REM sleep and on SWS were examined (Figure 3b). NE uptake inhibitors greatly suppressed REM sleep, but only had a small effect on SWS. REM sleep therefore seems to have been directly and selectively targeted. The DA uptake inhibitors reduced REM sleep and SWS proportionally, suggesting that the reduction of REM sleep was an indirect result of the suppression of SWS. The DA+NE uptake inhibitors had an effect on REM sleep intermediate to the effects of the selective DA and selective NE uptake inhibitors: they reduced SWS levels similar to the DA uptake inhibitors. Thus, some of the effect on REM sleep could be attributed to the reduction in SWS. However, they suppressed REM sleep to a greater extent than did the selective DA uptake inhibitors, indicating that there was also a primary suppression of REM sleep.

Dose response profiles of individual compounds on the REM/SWS ratio are also plotted in Figure 4a. This presentation more clearly distinguishes DA uptake blockers (GBR-12909, amineptine, bupropion) from compounds with potent NE or NE/DA uptake effects.

Correlation between the in vivo effects of the compounds on REM sleep (REM/SWS ratio) and their in vitro DAT and NET binding affinities

Correlations between the in vivo potency on REM sleep and in vitro DAT and NET affinities of the compounds are also presented in Figure 4bc, and the result further demonstrates that the affinity of each compound to the NET, but not to the DAT significantly correlates with the potencies of the compounds’ effects on the REM/SWS ratio (ED-60% [mol/kg i.v.]) \((r = 77.5, n = 7, p < 0.05, \text{Pearson’s correlations})\).
suggested that presynaptic modulation of NE transmission is important for the pharmacological regulation of REM sleep.

**DISCUSSION**

This study demonstrates that DA and NE uptake inhibitors have preferential effects on wake and REM sleep, respectively. All DA uptake inhibitors tested promoted wake, as assessed by polygraphic recordings. For example, GBR-12909, a highly selective DA uptake inhibitor (see Nissbrandt et al., 1991), significantly increased alertness in control and narcoleptic dogs. The wake-promoting effects of GBR-12909 were also recently reported in rats (Seidel et al., 1996). The wake-promoting effects of most DA uptake inhibitors were as potent as those of d-amphetamine or modafinil, two reference CNS stimulants currently used for the treatment of EDS in human narcolepsy (Bastuji and Jouvet, 1988; Besset et al., 1993; Boivin et al., 1993; Mittler et al., 1990, 1994; Parkes et al., 1975; Thorpy and Goswami, 1990). We further found a significant correlation between the in vivo wake-promoting potency of DA uptake inhibitors (and modafinil) and their in vitro binding affinity to the DAT, but not to the NET. DA uptake inhibition is thus likely to be the most important pharmacological property mediating EEG arousal in normal and narcoleptic animals. Considering the fact that other amphetamine-like compounds, such as methylphenidate and pemoline not only bind to the DAT (Ki = 2.85 × 10−8 nM, and Ki = 9.32 × 10−7 nM, respectively, obtained from [3H]-WIN 35,428 binding using canine caudate) but also release DA in vivo (e.g., like amphetamine) presynaptic activation of DA transmission may be involved in the mode of action of all CNS stimulants currently used in clinical practice.

Most DA uptake inhibitors also reduced REM sleep moderately (Figure 4), but this effect was likely to be an indirect result of the suppression of SWS. In order to minimize this influence, the ratio for REM/SWS was calculated and it was found that DA uptake inhibitors, such as GBR-12909, bupropion or amphetamine have little or no effect on REM sleep at the dose that significantly increased wake (Figure 4b). In contrast, both NE uptake inhibitors, nisoxetine and desipramine, reduced REM sleep to a much greater extent than SWS (Figure 4b), indicating that the suppression of REM sleep occurred independently of the effect on SWS. Furthermore, a significant correlation between the in vivo potencies of the REM/SWS ratio and in vitro affinity of the compounds to the NET, but not to the DAT (Figure 4), suggests that presynaptic modulation of NE transmission is important for the pharmacological control of REM sleep; this may explain why most monoamine uptake inhibitors and monoamine-oxidase inhibitors strongly reduce REM sleep in humans and experimental animals (Akindele et al., 1970; Scherschlicht et al., 1982; Schneeberger and Haefely, 1979). A large body of experimental evidence suggests that brainstem monoaminergic (NE and 5-HT) and cholinergic systems are important for the control of REM sleep. The firing rate of NE neurons in the locus coeruleus (LC) is known to be dramatically depressed during REM sleep in rats (Aston-Jones and Bloom, 1981), a phenomenon also observed during cataplectic episodes in narcoleptic canines (Wu et al., 1996). Conversely, the activity of a subset of cholinergic neurons (PS-on) in the dorsal pons increases during REM sleep in cats and rats (El Mansari et al., 1989; Kayama et al., 1992; Steriade et al., 1990), thus suggesting reciprocal interaction for monoaminergic and cholinergic systems during REM sleep (see Hobson, 1990; Hobson et al., 1975). In this model, pontine PS-on cholinergic neurons are secondarily activated during REM sleep by a reduction in brainstem monoaminergic activities. In agreement with this hypothesis, it has recently been demonstrated that the application of NE in the laterodorsal tegmentum, which receives NE afferents from the LC, inhibits cholinergic neurons, both in vivo (Koyama and Kayama, 1993) and in slice preparations (Williams and Reiner, 1993). Systemic administration of NE uptake inhibitors are known to inhibit NE neurons in the LC, but also enhance NE transmission at the terminal level (Nissbrandt et al., 1991). NE uptake inhibitors may therefore directly inhibit pontine cholinergic PS-on (and cataplexy-active) neurons, an effect that may reduce REM sleep and cataplexy. It is surprising however, that NE uptake inhibitors did not increase wake significantly in narcoleptic and control animals, since NE systems are also believed to be involved in the control of wakefulness (see Jones, 1994 for review). Our results rather suggest that presynaptic modulation of DA but not NE transmission is critically involved in the control of wakefulness. These data are consistent with earlier studies that suggested the involvement of the dopaminergic and/or dopaminergic output neurons for the regulation of SWS. For example, removal of the caudate produces a month-long reduction of SWS in cats (Villablanca et al., 1976), and the DA metabolism in the striatum significantly decreases during SWS (Kovacevic and Radulovacki, 1976).

DA neurons in the ventral tegmental area (VTA) and substantia nigra (SN) in the midbrain, however, do not change their activity during the sleep cycle (Miller et al., 1983; Steinfels et al., 1983). In rats, mesolimbocortical projections of DA neurons are limited to the prefrontal cortex, in contrast to NE’s wide projections to the entire cortex (Jones, 1994). This has led most investigators to believe that DA systems were not involved in natural wake/sleep processes. However, we have recently reported that dopaminergic D2 or D3-type compounds injected into the VTA and the SN significantly modifies cataplexy and/or alertness in canine narcolepsy (Reid et al., 1996; Honda et al., 1998). These findings suggest that a reduction of activity in midbrain DA neurons enhances the occurrence of cataplexy and/or induces sleepiness. Interestingly, Trulson (1985) reported that unit activity of the midbrain DA neurons does not show significant changes during the sleep cycle, consistent with the report by Miller and Steinfels (Miller et al., 1983; Steinfels et al., 1983), but that terminal release of DA in the post-synaptic target regions of the striatum in the same animals decreased significantly during SWS. Anatomical studies in primates suggest that mesocortical DA projections more widely terminate in the temporal and/or visual cortex (Brown and Goldman, 1977). Furthermore, recent clinical sleep studies on patients with Huntington’s...
disease, where degeneration of the striato-nigral gammaaminobutyric acid (GABA) pathway results in the disinhibition of the nigro-striatal DA neurons, have shown significant reductions in SWS (Wiegand et al., 1991). We therefore believe that the putative role of DA systems for the control of wakefulness warrants further investigation.

The finding that systemic administration or local VTA perfusion of D3 agonists aggravates cataplexy (Reid et al., 1996) does not correspond well with the absence of any significant effects of DA uptake blockers on cataplexy (Mignot et al., 1993) and REM sleep (Figure 4). However, it fits well with the observation that D2/D3 agonists or antagonists but not DA uptake blockers have potent effects on REM sleep in rodents (Kafi and Gaillard, 1976; Mereu et al., 1978; Seidel, personal communication). A possible explanation might be that the dopaminergic projections that are involved in the regulation of cataplexy (and REM sleep) are much less sensitive to DA uptake inhibition. In agreement with this hypothesis, discrepancies between distribution of tyrosine hydroxylase (TH)-containing neurons and DAT immunoreactivity in the brain have recently been reported (i.e., TH-staining cells are enriched in the medial VTA, but this structure lacks DAT immunoreactivity) (Ciiali et al., 1995). In contrast, distribution of NET immunoreactivity and TH-containing neurons are reported to be generally well-matched (Charnay et al., 1995). Furthermore, electrophysiological and pharmacological studies have also shown that DA re-uptake is of physiological importance for the elimination of DA from the synaptic cleft in the cortical hemispheres, limbic forebrain and striatum, but not in the midbrain DA neurons (Nissbrandt et al., 1991). It is thus possible that DA uptake inhibitors mostly act on DA terminals in the cortical hemispheres, limbic forebrain, and striatum to induce alertness, but have little effect on pathways involved in the regulation of cataplexy.

In conclusion, our current results demonstrate that increased DA transmission using uptake inhibitors or release enhancers preferentially modulates EEG arousal in normal and pathological conditions. In contrast, presynaptic modulation of NE systems has preferential effects on REM sleep and REM sleep-related phenomena. The two axial symptoms of narcolepsy, EDS and cataplexy, are thus pharmacologically regulated differently, and dysfunctions of both the dopaminergic and the noradrenergic systems may be involved in the pathophysiology of narcolepsy. This interpretation further explains why two different types of drugs, namely amphetamine-like stimulants and tricyclic antidepressants must be used for the treatment of EDS and REM sleep-related symptoms, respectively, in most human narcoleptic subjects.

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