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Effect of the Wake-Promoting Agent Modafinil on Sleep-Promoting Neurons from the Ventrolateral Preoptic Nucleus: an In Vitro Pharmacologic Study

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Study Objectives: The pharmacologic profile of modafinil, an increasingly popular wake-promoting drug for narcolepsy treatment, differs from those of classic psychostimulants such as amphetamine. However, its brain targets are still a matter of debate. We hypothesized that modafinil could increase waking by inhibiting the sleep-promoting neurons from the ventrolateral preoptic nucleus (VLPO). Such action could be direct or indirect via the potentiation of inhibition mediated by waking neurotransmitters. We thus studied the effect of modafinil on the membrane potential and firing rate of VLPO neurons recorded in rat-brain slices. We further determined whether pretreatment with modafinil modifies the effect of noradrenaline, carbachol, serotonin, histamine, dopamine, or clonidine.

Measurements and Results: Pretreatment with modafinil specifically increased the inhibition of VLPO neurons induced by noradrenaline but had no effect when applied alone or in combination with other substances. Pretreatment with nisoxetine, a selective noradrenaline reuptake blocker, similarly increased the noradrenaline-induced inhibition of VLPO cells. Further, the potentiation by modafinil was minimized when modafinil and nisoxetine were applied together.

Conclusions: These results suggest that modafinil blocks the reuptake of noradrenaline by the noradrenergic terminals on sleep-promoting neurons from the VLPO. Such a mechanism could be at least partially responsible for the wake-promoting effect of modafinil.

Key Words: rat, nisoxetine, potentiation, VLPO, noradrenaline reuptake transporter

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INTRODUCTION

MODAFINIL IS CURRENTLY USED FOR THE TREATMENT OF EXCESSIVE SLEEPINESS, SUCH AS NARCOLEPSY. It increases wakefulness in many species,[1-5] including humans,[6] without the central and peripheral side effects associated with dopaminergic psychostimulants.[7] Based on a number of experiments, it has been proposed that modafinil promotes waking via an action on noradrenergic neurotransmission. Indeed, the waking effect of modafinil in cats, mice, and monkeys is prevented or attenuated by pretreatment with α_1 or β antagonists.[3,8] Modafinil could also induce waking by its action on dopamine transmission. Indeed, it exhibits weak affinity for the dopamine transporter (DAT)[9] and does not increase waking in mice with deletion of the DAT gene.[10] However, unlike psychostimulants that act on dopamine neurotransmission, such as amphetamine and methylphenidate, modafinil does not induce abnormal behavior modification, tolerance, sensitization, or reinforcing properties.[5,11] In addition, modafinil induces wakefulness without intensifying motor activity or subsequent hypersomnolence rebound.[12,13] Furthermore, it does not stimulate dopamine release in the caudate nucleus of mice[14] or the nucleus accumbens of rats,[15] alter the firing rate of dopaminergic neurons in the ventral tegmental area,[16] or bind to dopamine receptors.[3,8]

In 2 functional studies, c-Fos protein expression following modafinil administration has been observed only in the anterior hypothalamus, while in response to amphetamine and methylphenidate administration, strong c-Fos–like immunoreactivity was found in the striatum and cortical areas.[17,18] In a third study, c-Fos labeling increased following modafinil treatment in histaminergic and orexin-containing neurons,[19] presumably active specifically during waking,[20,21] and decreased in neurons of the ventrolateral preoptic nucleus (VLPO), presumably responsible for sleep onset and maintenance.[19,22] Taken together, these results suggest that modafinil could induce waking by an excitatory action on waking active systems or by an inhibition of VLPO sleep-promoting neurons. Supporting this second hypothesis, modafinil causes a marked decrease in the amount of GABA, as measured by microdialysis in the posterior hypothalamus,[23] known to be a major efferent projection of the VLPO GABAergic neurons[24] and to contain the histaminergic neurons. Modafinil action on VLPO sleep-promoting neurons could be due to a direct inhibition or the potentiation of monoaminergic and cholinergic inhibitions. Besides, we have recently identified, in rat brain slices, the distinctive properties of VLPO neurons presumed to have the capacity to promote sleep.[25] These neurons are GABAergic, triangular- and multipolar-shaped, show powerful low-threshold Ca^{++} spike (LTS), and are inhibited by the major neurotransmitters of wakefulness such as noradrenaline. Taking advantage of this characterization, we studied the effect of modafinil applied alone or in combination with the major arousal neurotransmitters on the membrane potential and firing rate of VLPO sleep-promoting neurons in vitro.

MATERIALS AND METHODS

Slice Preparation

Brain slices containing the VLPO were obtained from 15- to 20-day-old rats (Sprague-Dawley, OFA-SD , IOPS, Charles River, France). Animals were housed under a 12-hour:12-hour light-dark cycle with lights on at 6:00 AM. Standard procedures were followed for slice preparation. The animal was decapitated, the skull opened, and a tissue block containing the VLPO dissected. The block was fixed with

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cyanoacrylate to the stage of a vibrating microtome (WPI, UK) on which, respectively, slices 400-µm and 300-µm thick were cut for intracellular recordings or extracellular recordings in loose cell-attached configuration. The coronal slices were collected and subsequently held in small vials containing artificial cerebrospinal fluid (ACSF) that contained (in µM): 130 NaCl, 20 $NaHCO_3$, 1.25 KH_2PO4, 1.3 $MgSO_4$, 5 KCl, 2.4 $CaCl_2$ and 10 glucose constantly oxygenated (95% O_2/5% CO_2) and held at room temperature. From there, individual slices were transferred to a thermoregulated (32°C) chamber, under either a dissecting microscope for intracellular recordings or an Axioscop 2FS (Zeiss, Germany) equipped with an infrared CCD camera (Hamamatsu, Japan) for extracellular recordings in loose cell-attached configuration of identified cells. Slices were maintained immersed, continuously superfused at 3 to 5 mL per minute with oxygenated ACSF and allowed to equilibrate for at least 1 hour before recording. Recording sites were chosen according to VLPO stereotaxic coordinates published in Paxinos atlas,[26] the localization of VLPO neurons projecting to the noradrenergic locus coeruleus (LC)[27] and histaminergic tuberomammillary nuclei.[24]

Electrophysiology

The data acquisition was done with an Axoclamp 2B amplifier and a Digidata 1200B interface board (Axon Instruments, Foster City, CA). Signals were amplified and collected on an IBM-PIII-600MHz PC by using the data-acquisition software P-Clamp-8.02 (Axon Instruments).

Intracellular Recordings

Blind intracellular recordings were obtained by using sharp electrodes (80-150 MΩ) made of borosilicate glass capillaries (1.2 mm outside diameter [od], 0.69 mm internal diameter [id], Harvard Apparatus, France) that were pulled on a Brown-Flaming micropipette puller (Model P-97, Sutter Instrument, Novato, CA). The micropipette was filled with 3 mol potassium acetate and attached to a piezoelectric microdrive (Burleigh Instrument, France) to advance it vertically every 2 µm through the slice. The intrinsic membrane properties of VLPO neurons were then studied in current-clamp mode by applying current pulses generated by a pulse generator through the recording electrode (Pulsemaster A300, WPI, UK). During drug application, cells were continuously submitted to brief hyperpolarizing current pulses (500 milliseconds, 0.2 Hz) to monitor the input resistance.

Extracellular Recordings in Loose Cell-Attached Configuration

This recording configuration was used to study the effect of multiple drug applications on a single VLPO cell. In contrast to intracellular and patch-clamp approaches, this mode allows stable recordings of healthy neurons for long periods of time necessary to complete pharmacologic experiments. Infrared differential interference videomicroscopy was used to locate the VLPO and to choose the neurons according to their size and triangular or multipolar shape, as we reported previously.[25] Cell-attached recordings were made from the soma with patch micropipettes (5-8 MΩ) pulled from borosilicate glass capillaries (1.5 mm od, 0.86 mm id). The micropipettes, filled with ACSF, were attached to an electric microdrive (Luigs and Neumann, Ratingen, Germany) and placed under visual control in contact with the soma of the cell chosen. During extracellular recordings in loose cell-attached configuration, a seal resistance of 10 to 15 MΩ was kept to avoid damage or mechanical stimulation to the cell. In this mode, cells were classified as noradrenaline-inhibited (NA-inhibited) cells when an application of noradrenaline (0.5-1-10-100 µM, 10 seconds) induced a decrease of their firing rate.

Drugs

A number of reagents were used. These included modafinil (200, 100, 60, and 20 µM, Laboratoire Cephalon France, formerly Laboratoire L. Lafon); noradrenaline (100, 10, 1, 0.5, and 0.1 µM); clonidine (0.05 µM, α_2 agonist); L-phenylephrine (100 µM, α_1 agonist); yohimbine (10 µM, α_2 antagonist); serotonin (100 µM); histamine (100 µM); dopamine (100 and 10 µM); carbachol (5 µM), nisoxetine (10 µM, noradrenaline-reuptake blocker), TTX (1 µM, voltage-dependant Na^+-channel blocker), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 µM, AMPA-receptor antagonist), DL-2-amino-5-phosphonovaleric acid (AP5, 20 µM, NMDA-receptor antagonist) and bicuculline methiodide (10 µM, selective $GABA_A$-receptor antagonist). All drugs were obtained from Sigma (Saint Quentin-Fallavier, France) except TTX (Latoxan, France). They were dissolved in distilled water to a concentration of 10 µM, and the solutions were stored as frozen aliquots at -20°C. Just before bath application, the drugs were diluted in ACSF to their working solution.

Protocol for Drug Applications During Extracellular Recordings in Loose Cell-Attached Configuration

Immediately following the stabilization of the spontaneous firing activity of a VLPO neuron, noradrenaline was applied during 10 seconds to the bath (control condition). When the firing rate of the neuron returned to its basal value, modafinil (200 µM) was perfused continuously (5 minutes) before and after a second 10-second application of noradrenaline. Modafinil application was stopped when the neuron recovered its basal firing rate. The slice was then perfused with ACSF during 6 to 8 minutes before a third 10-second application of noradrenaline (wash condition). A minimum of 6 minutes of perfusion with ACSF was found to be necessary to wash modafinil and obtain the same effect of noradrenaline in wash and control conditions. To test the effect of different concentrations of noradrenaline, modafinil or that of other neurotransmitters, the same protocol was repeated on the same cell either with other concentrations of noradrenaline or modafinil or replacing noradrenaline with clonidine, dopamine, serotonin, carbachol, or histamine. In some experiments of synaptic uncoupling, ACSF containing AP5-CNQX-bicuculline mixture was continuously perfused to block GABAergic and glutamatergic spontaneous or induced postsynaptic events in the recorded cells. In the last set of experiments involving pretreatments with modafinil and nisoxetine, the potentiation of the noradrenaline effect (at 1 µM) by modafinil was checked beforehand. Then, the same protocol was repeated by replacing modafinil first with nisoxetine and second with a solution containing modafinil and nisoxetine.

Data Analysis

Two parameters of the drug responses were systematically quantified: (1) the amplitude corresponding to the maximal change in firing rate induced by the drug application (in Hz or percentage of the basal firing rate) and (2) the duration of the effect (in minutes, time necessary to recover the basal firing rate). Both measures were expressed as mean ± SEM. Amplitude and duration of the effect were compared between control, drug, and wash conditions using a paired Student *t* test, and a *P* value = .05 was considered statistically significant.

RESULTS

In this study, we focused our attention on the sleep-promoting neurons recently identified in the VLPO (N = 94 cells, n = 10 for intracellular recordings, and n = 84 for loose cell-attach experiments).[25] As described previously, this set of neurons, encompassing 80% of the recorded cells in the VLPO, were characterized by the presence of a potent LTS and were systematically hyperpolarized and inhibited by noradrenaline (NA-inhibited cells).

Effect of Modafinil Application on the VLPO NA-inhibited Cells

Intracellular Recordings

A sample of 10 cells endowed with a potent LTS was submitted to modafinil application. Their average resting membrane potential was -

54.3 ± 1.9 mV (mean ± SEM). The LTS was evoked when cells were hyperpolarized from rest and submitted to a depolarizing pulse (asterisk in Figure 1A). When applied to the bath, noradrenaline (100 μM, 30 seconds) produced a membrane hyperpolarization (Figure 1B). The maximal variation of their membrane potential was -4.8 ± 0.5 mV, and the hyperpolarization lasted 3.4 ± 0.2 minutes. The inhibitory action of noradrenaline appeared to be postsynaptic, since it persisted in presence of TTX (n = 5; -9.1 ± 1.4 mV; 3,4 ± 0.8 minutes).

Application of modafinil (200 μM, 1 or 3 minutes) on these cells induced no change in their resting membrane potential and input resistance (Figure 1B).

Extracellular Recordings in Loose Cell-Attached Configuration

The effect of modafinil (200 μM, 3 minutes) was tested on a set of 53 VLPO NA-inhibited cells. They displayed a spontaneous firing rate of 9.3 ± 0.6 Hz. For all neurons, noradrenaline application (100 μM, 10 seconds; Figure 1C) reversibly induced a 93.3% decrease in firing rate for 5.7 ± 0.3 minutes. Application of modafinil (200 μM, 3 minutes) induced no change in the firing rate of 41 of these neurons (Figure 1C). In 8 NA-inhibited cells, modafinil application induced a 40% decrease in firing rate for 6.5 ± 0.9 minutes compared to baseline, while in 4 other cells, it induced a 30% increase in firing rate for 7.1 ± 0.8 minutes (data not shown).

Effect of Noradrenaline Application Following Modafinil Pretreatment

In a first set of NA-inhibited cells, noradrenaline was applied for 10 seconds at 5 concentrations (100, 10, 1, 0.5, and 0.1 μM) following a 5-minute modafinil pretreatment (200 μM). When noradrenaline was applied at 100 μM (n = 19/19, Figure 2A) or 10 μM (n = 8/8, Figure 2B), the decrease in the discharge rate was of the same amplitude but lasted longer (approximately +145% and +50%, respectively, $P = .0003$) following modafinil pretreatment compared to control and wash conditions (Table 1). When noradrenaline was applied at 1 μM, both the amplitude (approximately +94%) and the duration (approximately +42%) of the inhibitory effect were increased following modafinil pretreatment compared to control and wash conditions (n = 38/38, both $P < .0001$, Table 1, Figure 2C). For 7 cells tested with noradrenaline at 0.5 μM, modafinil significantly increased the amplitude (approximately +82%, $P = .004$) but not the duration of the inhibitory effect induced by noradrenaline compared to control and wash conditions (Table 1, Figure 2D). At 0.1

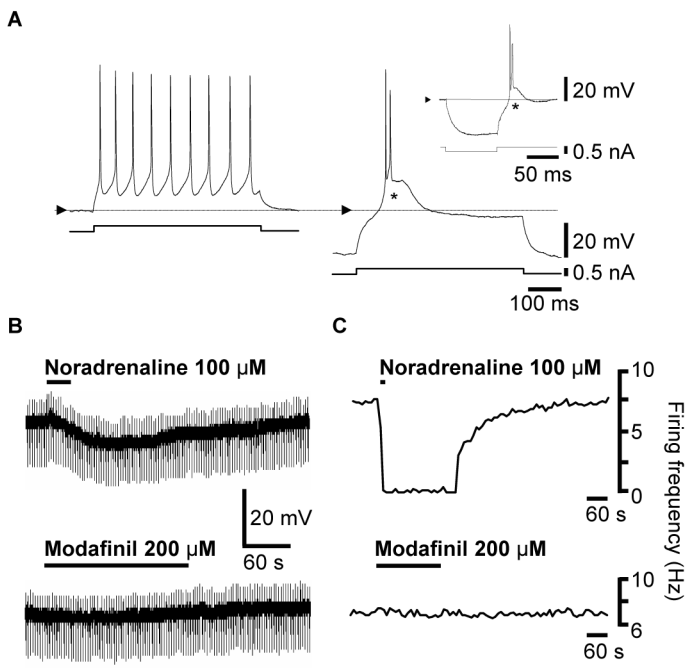

Figure 1—Modafinil applied alone induced no effect on the membrane potential and spontaneous firing rate of sleep-promoting neurons in ventrolateral preoptic nucleus (VLPO) slices. Intracellular recordings in current-clamp mode are illustrated in A-B and extracellular recordings in loose cell-attached configuration in C. **(A)** Sleep-promoting neuron characterized by a low-threshold spike (right panel *) when depolarized from a direct-current hyperpolarized level or when released from an hyperpolarized level (inset). In contrast, from rest, a depolarizing current evoked a tonic discharge in the same cell. Level of resting membrane potential shown by arrowheads: - 56 mV **(B)** Chart paper records showing noradrenaline (NA) and modafinil effects on the membrane potential. Brief bath application of NA (100 μM, 30 seconds) produced a membrane hyperpolarization, while application of modafinil (200 μM, 3 minutes) had no effect on the resting membrane potential of the same cell (resting membrane potential: -55 mV). **(C)** Firing frequency versus time diagram showing the decrease in the spontaneous firing rate of a sleep-promoting neuron induced by a short application of NA (100 μM, 10 seconds). Modafinil (200 μM, 3 minutes) did not modify the spontaneous discharge of the same cell.

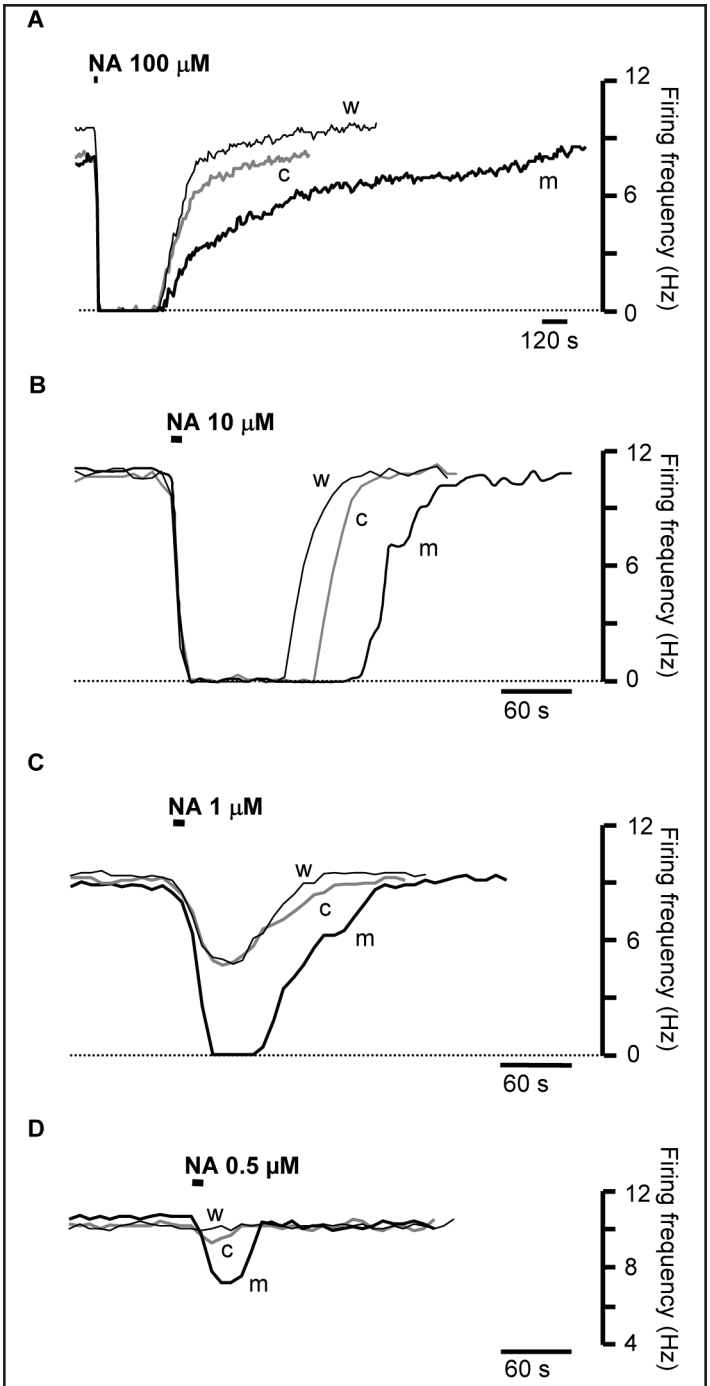

Figure 2—Potentiation by modafinil of the noradrenaline (NA)-induced inhibition of ventrolateral preoptic nucleus sleep-promoting neurons. **(A-D)** Firing frequency versus time diagrams illustrating single experiments on 4 NA-inhibited cells extracellularly recorded in loose cell-attached configuration. After a 5-minute pretreatment with modafinil 200-μM, the inhibitory effect induced by brief NA application at different concentration (100 μM, 10 μM, 1 μM, 0.5 μM) (tracing m) was increased in both amplitude and duration compared to control (tracing c) and wash (tracing w) conditions.

μM, noradrenaline induced no effect when applied alone or following modafinil pretreatment (n = 4/4, data not shown).

In a second set of 8 NA-inhibited cells, 1 μM of noradrenaline was applied for 10 seconds in the control condition and following 5 minutes of modafinil pretreatment at 4 different concentrations (20, 60, 100, and 200 μM). Both the amplitude and duration of the inhibitory effect induced by noradrenaline application were significantly enhanced following modafinil pretreatments at all concentrations (Figure 3A). The effect gradually increased from the lowest to the highest concentration of modafinil (Figure 3B and C).

Long-lasting application of bicuculline (10 μM) induced an increase in the spontaneous discharge rate of the NA-inhibited cells, suggesting that GABA transmission is one of their primary inhibitory synaptic inputs. However, bicuculline did not abolish the enhancement by modafinil of the noradrenaline inhibitory effect (0,5 μM; n = 2/2; Figure 3D). Furthermore, the potentiation of the NA-induced inhibitory effect by modafinil was still observed with the addition of AP5 and CNQX to bicuculline in the ACSF (n = 3/3; Figure 3E).

Effect of L-phenylephrine, Clonidine, and Modafinil Applications

For all cells used in this series of experiments, noradrenaline produced a strong decrease in their discharge rate. The noradrenaline inhibition was antagonized by a 5- to 7-minute pretreatment with yohimbine (10 μM), an α_2 antagonist (n = 3/3). Application of L-phenylephrine (100 μM, 10 seconds), an α_1 agonist, induced no effect (n = 5/5), while that of clonidine (0.05 μM, 10 seconds), a specific α_2 agonist, induced a strong decrease in discharge rate (n = 11/11, Figure 4A and Table 1). The decrease in discharge rate induced by clonidine (0.05 μM, 10 seconds) was not modified by modafinil pretreatment (200 μM, 5 minute) (n = 4/4) (Table 1, Figure 4A).

Effects of Dopamine, Serotonin, Carbachol, and Histamine Applications Following Modafinil Pretreatment

It was first verified for all NA-inhibited cells studied in this series that modafinil pretreatment (200 μM, 5 minutes) reversibly enhances the noradrenaline inhibition of their discharge. Dopamine (100 μM, n = 9/9 or 10 μM, n = 6/6, 10 seconds, Figure 4B) or carbachol (5 μM, 15 seconds, n = 4/4) induced a decrease in their discharge rate. These inhibitory effects were not modified by modafinil pretreatment (200 μM, 5 minutes) (Table 1). Histamine (100 μM, 15 seconds) applied alone or following modafinil pretreatment (200 μM, 5 minutes) induced no change in the firing rate of NA-inhibited cells (n = 6/6). Finally, modafinil pretreatment (200 μM, 5 minutes) did not modify the decrease (n = 6/6) or increase (n = 8/8) in discharge rate induced by serotonin (100 μM, 15 seconds, Table 1).

Table 1—Amplitude and duration of the pharmacologic effects on ventrolateral preoptic nucleus sleep-promoting neurons.

	Amplitude, Hz		Duration, min	
	Control	Modafinil	Control	Modafinil
NA 100 μM (n = 19)	-8.0 ± 0.8	-8.6 ± 0.7	6.2 ± 0.5	15.2 ± 2.4†
NA 10 μM (n = 8)	-6.6 ± 1.0	-7.3 ± 1.1	2.8 ± 0.3	4.2 ± 0.4†
NA 1 μM (n = 38)	-3.5 ± 0.3	-6.8 ± 0.4‡	1.9 ± 0.1	2.7 ± 0.2‡
NA 0.5 μM (n = 7)	-3.3 ± 0.6	-6.0 ± 1.0*	1.4 ± 0.2	2.0 ± 0.3
5-HT inhibitory 100 μM (n = 6)	-4.6 ± 1.7	-4.3 ± 0.9	3.2 ± 0.7	3.7 ± 0.9
5-HT excitatory 100 μM (n = 8)	+2.0 ± 0.3	+2.2 ± 0.4	4.1 ± 0.8	4.2 ± 0.5
Carbachol 5 μM (n = 4)	-2.7 ± 0.5	-2.8 ± 0.3	1.7 ± 0.3	1.6 ± 0.3
Dopamine 100 μM (n = 9)	-5.0 ± 1.3	-4.6 ± 1.4	4.5 ± 0.6	4.8 ± 0.5
Dopamine 10 μM (n = 6)	-3.1 ± 0.6	-3.7 ± 0.8	1.5 ± 0.3	1.7 ± 0.2
Clonidine 0.05 μM (n = 4)	-6.2 ± 1.5	-5.6 ± 1.3	4.2 ± 0.6	4.9 ± 0.8

Data, presented as mean ± SEM, reflect the effects on ventrolateral preoptic nucleus sleep-promoting neurons extracellularly recorded in the loose cell-attached configuration after bath application of each drug in control condition and following a 5-minute pretreatment with modafinil (200 μM). For each drug application, the maximal amplitude and duration of the effect were compared between control and modafinil pretreatment conditions using a paired Student *t* test, and a *P* value ≤ .05 was considered as the level of statistical significance (*$P < 0.01$, †$P < 0.001$ and ‡$P<0.0001$).
NA refers to noradrenaline; 5-HT refers to serotonin.

Effect of Noradrenaline Application Following Modafinil and Nisoxetine Pretreatment

For this set of experiments, the reversible potentiation by modafinil (200 μM, 5 minutes) of the noradrenaline inhibition (1 μM, 10 seconds) was first assessed in 5 NA-inhibited cells (Figure 4C). On the same cells, nisoxetine, a noradrenaline-reuptake blocker, was then perfused alone during 5 minutes at 10 μM, the dose chosen based on previous in vitro studies.[28] This application induced no change in the spontaneous discharge rate of the recorded cells. Noradrenaline application (1 μM, 10 seconds) following this pretreatment induced an inhibition with an amplitude (approximately +246%, $P = .075$) and a duration (approximately +131%, $P = .003$) significantly larger than in control conditions (n = 5/5; Figure 4C). The amplitude of the noradrenaline inhibition following nisoxetine pretreatment was not statistically different from that observed following modafinil pretreatment, while the duration of the

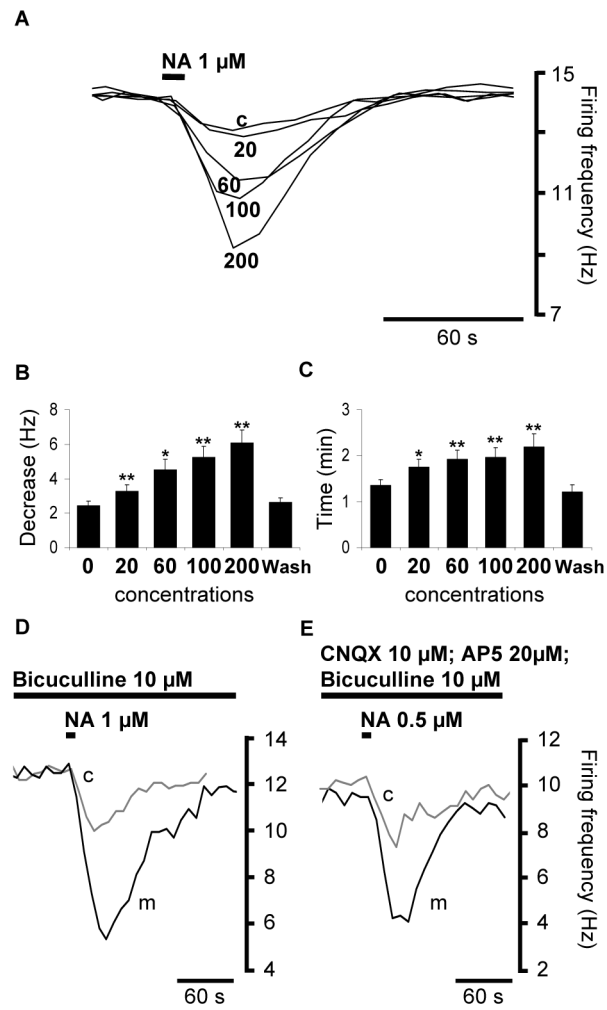

Figure 3—Effect of different concentrations of modafinil on the noradrenaline (NA)-induced inhibition of ventrolateral preoptic nucleus sleep-promoting neurons. **(A)** Firing frequency versus time diagram illustrating 1 NA-inhibited cell extracellularly recorded in loose cell-attached configuration and tested for 4 different modafinil doses. After a 5-minute pretreatment with 20, 60, 100 or 200 μM modafinil, the inhibitory effect of NA (1 μM, 10 seconds) on this neuron was increased compared to control condition (tracing c). **(B and C)** Histograms illustrating the mean ± SEM amplitude (decrease in firing rate in Hz, **B**) and duration (in minutes, **C**) of the inhibitory effects induced by NA in control conditions (0 and wash) and after different modafinil pretreatments (n = 8 complete experiments). Significance levels are indicated by *$P < .01$ and **$P < .001$. **(D and E)** Firing frequency versus time diagrams showing that, in presence of bicuculline alone (**D**) or added to CNQX and AP5 (**E**), brief NA applications produced a decrease of the firing frequency (tracing c) of this sleep-promoting neuron. In the same conditions of synaptic uncoupling, modafinil still potentiated the NA-induced inhibition of this neuron (tracing m).

effect was significantly longer (approximately +57%) (P = .017). After recovery from the noradrenaline effect, nisoxetine and modafinil were applied together during 5 minutes before a 10-second noradrenaline application. The amplitude and the duration of the inhibition induced by this application tended to be weaker but were not significantly different from those obtained when noradrenaline was applied following nisoxetine pretreatment (n = 5/5; Figure 4D). Finally, after a 6-minute wash in ACSF, a last 10-second application of noradrenaline induced an inhibition still significantly larger than in control conditions.

DISCUSSION

Here, we show that modafinil, a wake-promoting agent, potentiates the inhibition induced by noradrenaline of the presumed sleep-promoting neurons recorded in the VLPO. Our data further support the idea that modafinil functions as an inhibitor of the plasma-membrane noradrenaline transporter. As a result, modafinil would facilitate the reinforcement of the inhibition of the VLPO sleep-promoting neurons and thus the promotion of waking.

Postsynaptic Noradrenaline Inhibition of the VLPO Sleep-promoting Neurons

In our initial work, we identified (by combining intracellular and extracellular recordings in loose cell-attached configuration in slices) the distinctive properties of VLPO neurons that have the capacity to promote sleep.[25] These neurons are triangular and multipolar, show powerful LTS, and are almost uniformly inhibited by noradrenaline, a major neurotransmitter of arousal. In agreement with this classification, Matsuo et al[29] recently showed that noradrenaline reversibly hyperpolarized only the multipolar acutely dissociated VLPO neurons. They further demonstrated that the neurons hyperpolarized by noradrenaline are immunoreactive to galanin and thus might encompass the neurons responsible for sleep onset in the VLPO.[24,30] Besides, it has been shown that the sleep-active neurons recorded within the lateral preoptic area in freely moving rats are inhibited following local noradrenaline application or single-pulse stimulation of their noradrenergic brainstem afferents.[31,32] These convergent data highly support that the noradrenaline-induced inhibition is a convincing pharmacologic criterion for identifying the sleep-promoting neurons when extracellularly recorded in VLPO slices.

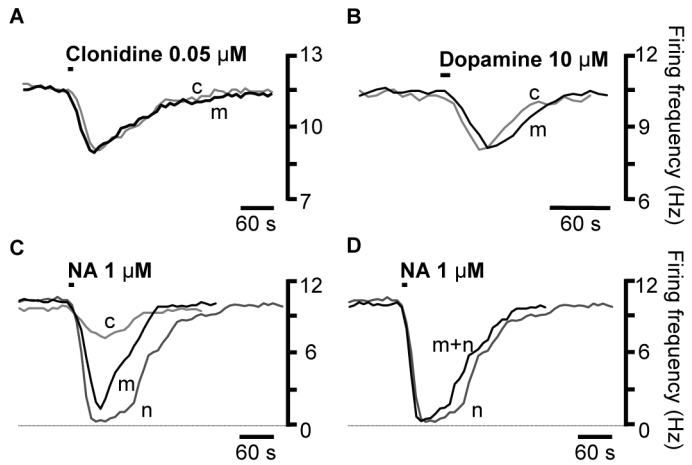

Figure 4—Potentiation by modafinil and nisoxetine of the noradrenaline (NA)-induced inhibition of ventrolateral preoptic nucleus sleep-promoting neurons. (**A and B**) Firing frequency versus time diagrams showing that bath application of clonidine (0.05 μM, 10 seconds, **A**) or dopamine (10 μM, 10 seconds, **B**) induced a decrease in the firing rate of ventrolateral preoptic sleep-promoting neurons (tracing c). These inhibitory effects were not increased by pretreatment with modafinil (tracing m). (**C and D**) Firing frequency versus time diagrams illustrating the decrease of the firing rate of 1 sleep-promoting neuron following NA application (1 μM, 10 seconds) in control condition (tracing c). This effect is clearly potentiated following pretreatment with modafinil (tracing m) and nisoxetine applied alone (tracings m and n, respectively, **C**) or simultaneously (tracing m + n, **D**). For this last experiment, notice the similar kinetics of the effects following applications of nisoxetine alone or in combination with modafinil, suggesting an occlusion by nisoxetine of the potentiation by modafinil of the NA-induced inhibition.

In complement of our original report,[25] the present study clearly establishes that noradrenaline activates postsynaptic α_2 adrenoceptors, since the noradrenaline-induced hyperpolarization of the VLPO sleep-promoting neurons remains in the condition of synaptic uncoupling; is mimicked by the specific α_2-adrenoceptor agonist, clonidine; and is blocked by the specific α_2-adrenoceptor antagonist, yohimbine. In line with our data, previous in vivo and in vitro pharmacologic experiments have provided evidence that noradrenaline activates α_2 adrenoceptors for inhibitory effects and α_1, β, or both α_1 and β adrenoceptors for excitatory effects in the preoptic area.[31-33]

Mechanisms of the Potentiation by Modafinil of the Noradrenaline-induced Inhibition

We observed that modafinil increased both the duration and amplitude of the noradrenaline-induced inhibition of the VLPO sleep-promoting neurons. This action is specific for noradrenaline, since modafinil had no effect when applied in combination with other major arousal neurotransmitters such as carbachol, serotonin, dopamine, and histamine. In addition, the inhibition by clonidine is not enhanced by modafinil pretreatment, eliminating direct or intracellular action of modafinil downstream from the postsynaptic α_2 receptors. Our data thus indicate that modafinil may potentiate the local noradrenergic neurotransmission. Supporting this hypothesis, we further show that pretreatment with nisoxetine also potentiates the inhibition of sleep-promoting neurons induced by noradrenaline in a manner similar to modafinil. Pretreatment with modafinil and nisoxetine together resulted in a potentiation with the same amplitude and duration as that observed with nisoxetine alone. At the concentration used in the present study,[28] nisoxetine is a potent and selective inhibitor of the plasma-membrane noradrenaline transporter (NAT), selectively expressed on noradrenergic nerve terminals where it can exert spatial and temporal control over the noradrenaline action.[34] Nisoxetine blocks the uptake of noradrenaline in synaptosomes with little or no affinity for a range of neurotransmitter receptors[35,36] and strongly increases noradrenaline efflux when perfused locally through a dialysis probe.[37] Our results therefore indicate that NAT is active in VLPO slices. Furthermore, the occlusion of the modafinil effect obtained when nisoxetine and modafinil were applied together highly suggests that both compounds interact with the NAT to prolong the clearance of noradrenaline and increase its local extracellular concentration.

One may propose, however, that the effect of modafinil is not exclusively due to the potentiation of the noradrenergic neurotransmission and reflects the contribution of an additional inhibitory system. Modafinil may facilitate the recruitment of local GABAergic neurons that regulate VLPO neuron activity, in part by presynaptic facilitation of GABA release. In support of this possibility, the GABA transmission is a primary inhibitory synaptic input within the VLPO, since its blockade by bicuculline induced a substantial increase in the spontaneous firing rate of the sleep-promoting neurons (our present data) and a disappearance of miniature inhibitory postsynaptic current recorded in VLPO neurons.[29,38] However, this local GABA modulation is not required for the modafinil effect. First, modafinil, when applied alone, induces no change in the spontaneous activity of the VLPO sleep-promoting neurons. Second, despite the removal of the local GABAergic modulation by application of bicuculline or APV-CNQX-bicuculline (blocking all spontaneous GABA and glutamate synaptic events), modafinil is still able to potentiate the noradrenaline-induced inhibition of the sleep-promoting neurons, in a range similar to that in control experiments. And third, activation by noradrenaline of presynaptic α_2 adrenoceptors on GABAergic terminals decreases the local release of GABA and rather facilitates the excitability of these presumed sleep-promoting neurons.[29]

Although a biochemical demonstration remains to be performed, our present data therefore suggest that the potentiation by modafinil of the noradrenaline-induced inhibition of the sleep-promoting neurons may

reflect a specific increase of the noradrenergic neurotransmission, most probably through interactions between modafinil and NAT in VLPO slices.

Physiologic Significance

The current hypothesis is that during waking, VLPO sleep-promoting cells are tonically inhibited by noradrenergic, serotonergic, and cholinergic wake-active neurons.[25] The progressive removal of these inhibitory inputs is supposed to allow sleep-promoting neurons to start firing and to induce sleep via their direct GABAergic inhibitory projections to the wake-active neurons.[24] The potentiation by modafinil of the noradrenaline-induced inhibition of VLPO sleep-promoting cells could prevent the onset of the activity of these neurons and therefore the onset of sleep. A number of results support this hypothesis. It has indeed been found in 2 studies that c-Fos protein immunoreactivity was only present in a small number of brain structures following modafinil treatment.[17,18] In a third study, the number of c–Fos-stained cells decreased in the VLPO following modafinil treatment compared to control.[19] Moreover, modafinil causes a decrease in concentration of GABA in the posterior hypothalamus, containing histaminergic wake-promoting neurons.[23] In line with our present data, it has been shown that injection in the rat preoptic area of the α_2-adrenoceptor agonist, clonidine, induced arousal, while that of yohimbine, an α_2-adrenoceptor antagonist, induced sleep.[39] Noradrenaline and clonidine, but not the α_1-adrenoceptor agonist methoxamine and the β-adrenoceptor agonist isoproterenol, inhibit sleep-active neurons recorded in the lateral preoptic area.[32] Furthermore, single-pulse stimulation of the noradrenergic cell groups from the LC and ventrolateral medulla induced an inhibition of preoptic sleep-active neurons reversed by the local application of the α_2-antagonist, yohimbine.[31] Besides, it has been shown that a pretreatment with α_1 and β antagonists but not a dopamine antagonist reduces modafinil-induced wakefulness.[3,8] Based upon these convergent results, it is most likely that noradrenaline promotes wakefulness by inhibiting VLPO sleep-promoting neurons and that modafinil increases waking by means of a reinforcing positive loop between VLPO GABAergic and noradrenergic LC neurons. Modafinil would increase the noradrenaline inhibition of VLPO sleep-promoting neurons that in turn would decrease their inhibition of the noradrenergic LC neurons. Therefore, LC cells would simultaneously strengthen the inhibition of the VLPO sleep-promoting neurons via α_2 adrenoceptors and the excitation of cortical cells via α_1 adrenoceptors.[40]

Against this hypothesis, it has also been shown in rats that nisoxetine, a specific NAT blocker, decreases the amount of paradoxical sleep but has only moderate effect on waking quantities.[41,42] Moreover, it has recently been shown that DAT-knockout mice are unresponsive to methamphetamine and modafinil treatment,[10] suggesting that the induction of waking by modafinil is mainly due to the blockade of the DAT. It is hard to reconcile these results with our and numerous previous results indicating that an increase in the noradrenergic rather than in the dopaminergic neurotransmission is responsible for the modafinil effect.[3,5,8,11,14-16]

One possibility is that the distinct pharmacologic profile of modafinil compared to the classic dopamine and noradrenaline reuptake blockers is due to its known low affinity for the DAT and moreover the NAT, compared to these molecules.[9,42] This idea is supported by our findings that the wash-out delay required for the removal of nisoxetine effect is far longer compared to that necessary for modafinil (control vs wash-out values for each compound), while the duration of the noradrenaline-induced inhibition is longer for a concentration of nisoxetine 20 times lower than that of modafinil (10 μM vs 200 μM). Such huge differences in affinity could be responsible for the different pharmacologic profile of modafinil compared to classic noradrenaline- and dopamine-reuptake blockers. Additional in vivo local pharmacologic studies and in vitro experiments on structures other than VLPO in rats and transgenic mice are needed to determine whether modafinil acts as a blocker of the NAT or DAT or both.

It is indeed possible that the effects on VLPO neurons reported here in rat-brain slices play no or a minor role in such induction. The lowest concentration of modafinil we used (20 μM) could be above the cerebral concentration necessary to induce waking in rats. The plasma concentration of modafinil has been determined in healthy male or female volunteers after single oral therapeutic doses of 200 mg (approximately 3-4 mg/kg of modafinil).[43] For this dose, the peak plasma drug concentration is 15 μM. In rats, the dose of modafinil needed to increase waking is 20 to 50 times superior (64-125 mg/kg in 5; 30-300 mg/kg in 12; 150-300 mg/kg in 19) to that in humans. In rats treated with a dose of 100 mg/kg, it was found that the maximal plasma and brain concentration of modafinil is 100 μM.[44] The concentrations of modafinil used in our protocol (20-200 μM) are therefore in the range of those found in the rat brain at the doses that induce waking.

It is also possible that the absence of modafinil effect in DAT-knockout mice is due to the profound neuronal plasticity induced by the absence of the DAT[45] rather than the removal of the transporter itself. Additional studies in these mice are needed in particular to determine whether noradrenergic neurotransmission is impaired, and the effect of modafinil on waking should be determined in other knockout mice, in particular for the NAT.

CONCLUSION

Our study indicates that the awaking effect of modafinil could be due at least in part to the potentiation of the tonic noradrenaline inhibition, present during waking, upon the sleep-promoting neurons from the VLPO, through interactions with NAT. This hypothesis is consistent with clinical data indicating that modafinil could be a novel antidepressant drug,[46,47] while NAT is an established target of many classic antidepressants.

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Modafinil: A Drug in Search of a Mechanism

Comment on Gallopin et al. Effect of the wake-promoting agent modafinil on sleep-promoting neurons from the ventrolateral preoptic nucleus: an in vitro pharmacologic study. *SLEEP* 2004;27(1):19-25.

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IN THE MODERN WORLD OF PHARMACEUTICAL SCIENCE AND DRUG DISCOVERY, MOST DRUGS ARE DEVELOPED FOR THEIR ABILITY TO INTERFERE WITH OR AUGMENT THE FUNCTION OF A PARTICULAR RECEPTOR, TRANSPORTER, OR OTHER KNOWN CHEMICAL MECHANISM. It is becoming increasingly uncommon for a new drug to be developed to the point of clinical application with no known mechanism of action. Modafinil started life in the usual way, as a derivative of a line of compounds with alpha adrenergic agonist properties. However, modafinil was found to have little direct effect on alpha receptors, but instead increased locomotor activity during the normal sleep period in animals.[1] It was soon appreciated by Jouvet and his colleagues that modafinil had arousal-producing properties that were unrelated to any action on, or even much binding to, either adrenergic or dopaminergic receptors.[2] And so, modafinil was developed as a drug for promoting wakefulness in the virtual absence of information on its mechanism of action.

Over the last few years, modafinil has emerged as a first line drug for augmenting wakefulness, without the unwanted side effects of drugs such as amphetamines that have strong dopaminergic effects, but modafinil's mechanism of action remains controversial (see discussion in Scammell et al.[3]). Studies of the pattern of Fos protein expression induced in the brain by modafinil have shown activation of neurons in the anterior hypothalamus in one study each in rat and cat. However, a third study (by the authors of this editorial) could not replicate this finding.[3] Instead, in rats treated with modafinil, we found activation of the hypothalamic waking system, including orexin and histamine neurons, and inhibition of sleep-promoting neurons in the ventrolateral preoptic nucleus (VLPO). Interestingly, we also saw activation of neurons in the striatum, in a pattern typically seen with drugs that promote dopamine signaling. Soon after, Wisor and colleagues[4] showed that modafinil fails to promote wakefulness in mice with disruption of the dopamine transporter gene. Thus, the impact of modafinil on the dopamine system, and the role of that system in its wake-producing effects, appeared to be well established.

Against this background, the new work by Gallopin and colleagues[5] in this issue of *SLEEP* suggests that modafinil inhibits the sleep-promoting neurons of the VLPO by blocking the norepinephrine reuptake transporter (NET). They provide convincing evidence that modafinil potentiates the inhibitory effects of norepinephrine on VLPO neurons in a slice preparation. Surprisingly, modafinil did not potentiate the inhibitory effects of dopamine or serotonin on VLPO neurons. Nisoxetine, a selective NET inhibitor, had a similar effect, and the response to the two drugs was not additive, suggesting they might work through the same biochemical pathways. These observations come as a surprise because modafinil does not bind to the NET,[6] suggesting that modafinil may act downstream on some other aspect of norepinephrine uptake or packaging. Treating NET knockout mice with modafinil could provide a critical test of this hypothesis. To measure the importance of the VLPO in this response, one could also disrupt noradrenergic innervation of the VLPO with 6-hydroxydopamine and then test the response to modafinil.

How can these new observations be reconciled with prior experiments? It is possible that modafinil may enhance wakefulness precisely because it acts on both the noradrenergic and dopaminergic systems. Enhanced signaling through ascending norepinephrine and dopamine pathways could promote wakefulness by activating the cortex and other forebrain targets. Full alertness also requires inhibition of the VLPO, and this local effect may be mediated predominantly by norepinephrine. These mechanisms would be most effective when the dopamine and norepinephrine systems are already active, thus explaining why modafinil promotes arousal during wakefulness and why it does not interfere with sleep when aminergic signaling is minimal.

Modafinil might also promote wakefulness through an interaction of descending and ascending pathways. In a recent editorial in *SLEEP*, Keating and Rye[7] propose that the descending dopaminergic system promotes wakefulness by activating noradrenergic neurons in the locus coeruleus (and probably the ventrolateral medulla, which provides most noradrenergic input to the VLPO). Damage to these and other pathways in Parkinson's disease may cause excessive daytime sleepiness, promote REM behavior disorder, and contribute to the sleepiness of patients who take D2 agonists (which activate inhibitory autoreceptors on remaining dopaminergic neurons). Enhancement of these descending signals by modafinil would thus increase firing in the ascending noradrenergic system. Interestingly, this possibility has been tested by Akaoka and colleagues,[8] who recorded from the locus coeruleus in anesthetized rats and found that modafinil did not increase firing rates. However, the level of activity in the locus coeruleus is usually much greater in wakefulness than it is in sleeping (or anesthetized) animals, and so it would be important to retest this hypothesis in chronically instrumented, awake animals.

Either of these mechanisms could account for the increased wakefulness after modafinil, its absence in animals that lack the dopamine transporter, and the findings that are reported by Gallopin in this issue. These twin actions of modafinil on the transport of both norepinephrine and dopamine would mean that it could increase wakefulness more effectively than would drugs that modulate only one transmitter. This property might explain why modafinil promotes wakefulness at levels of dopamine transporter inhibition that do not cause the unwanted side effects of more potent dopamine reuptake inhibitors.

In the evolution of each drug family, the key event in improving the drug effect is to understand its mechanism of action. Other family members can then be sought that may have similar but perhaps more potent effects, or fewer undesirable side effects. Modafinil is a very good drug, but it is far from perfect. Many patients with either Parkinson's disease or narcolepsy find it useful, but it may not completely alleviate their daytime sleepiness. The road to better, or at least more alert, living will indeed be through improving its chemistry, and that will require us to nail down the mechanism of action of modafinil. The paper by Gallopin and colleagues is a large step down that road, but the next few steps should be decisive.

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