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MODAFINIL MORE EFFECTIVELY INDUCES WAKEFULNESS IN OREXIN-NULL MICE THAN IN WILD-TYPE LITTERMATES

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Abstract—Narcolepsy–cataplexy, a disorder of excessive sleepiness and abnormalities of rapid eye movement (REM) sleep, results from deficiency of the hypothalamic orexin (hypocretin) neuropeptides. Modafinil, an atypical wakefulness-promoting agent with an unknown mechanism of action, is used to treat hypersomnolence in these patients. Fos protein immunohistochemistry has previously demonstrated that orexin neurons are activated after modafinil administration, and it has been hypothesized that the wakefulness-promoting properties of modafinil might therefore be mediated by the neuropeptide. Here we tested this hypothesis by immunohistochemical, electroencephalographic, and behavioral methods using modafinil at doses of 0, 10, 30 and 100 mg/kg i.p. in *orexin*$^{-/-}$ mice and their wild-type littermates. We found that modafinil produced similar patterns of neuronal activation, as indicated by Fos immunohistochemistry, in both genotypes. Surprisingly, modafinil more effectively increased wakefulness time in *orexin*$^{-/-}$ mice than in the wild-type mice. This may reflect compensatory facilitation of components of central arousal in the absence of orexin in the null mice. In contrast, the compound did not suppress direct transitions from wakefulness to REM sleep, a sign of narcolepsy–cataplexy in mice. Spectral analysis of the electroencephalogram in awake *orexin*$^{-/-}$ mice under baseline conditions revealed reduced power in the θ band frequencies (8–9 Hz), an index of alertness or attention during wakefulness in the rodent. Modafinil administration only partly compensated for this attention deficit in the orexin null mice. We conclude that the presence of orexin is not required for the wakefulness-prolonging action of modafinil, but orexin may mediate some of the alerting effects of the compound.

Key words: dopamine, amphetamine, rebound hypersomnolence, arousal, sleep attacks, stimulants.

Modafinil (a diphenylmethyl-sulfinyl-2 acetamide derivative) is a wake-promoting substance used for the treatment of hypersomnolence, in particular that associated with narcolepsy (Bastuji and Jouvet, 1986, 1988; US Modafinil in Narcolepsy Multicenter Study Group, 1998, 2000). It effectively enhances wakefulness in all species tested to date, including *Drosophila*, mice, rats, cats, dogs, monkeys and humans (Lagarde and Milhaud, 1990; Touret et al., 1995; Lin et al., 1992; Shelton et al., 1995; Hermant et al., 1991; Hendricks et al., 2003; Simon et al., 1996; Panckeri et al., 1996). Modafinil is pharmacologically distinct from classical wakefulness-inducing compounds such as the amphetamines, methylphenidate and pemoline (Simon et al., 1995; Mignot et al., 1994), and, in comparison with these agents, the compound is well tolerated and has a relatively low potential for abuse (Jasinski and Kovacevic-Ristanovic, 2000; Malcolm et al., 2002). Despite exhaustive research, the exact mechanism for the wakefulness-promoting action of modafinil remains unknown (Saper and Scammell, 2004).

Significant research has focused on the dopaminergic actions of the compound. Modafinil has been shown to increase extracellular dopamine levels, as measured by microdialysis, in rat nucleus accumbens (Ferraro et al., 1996, 1997) and prefrontal cortex (de Saint Hilaire et al., 2001), as well as in the caudate of narcoleptic dogs (Wisor et al., 2001). Nishino et al. (1998) reported a correlation between the binding affinity at the dopamine transporter (DAT) and the wake-promoting action of several compounds, including modafinil. The most intriguing evidence for a dopaminergic role in modafinil-induced wakefulness, however, comes from a study in DAT knockout mice (Wisor et al., 2001). These mice showed no wakefulness-enhancing response to 300 mg/kg modafinil, a dose much higher than that normally required for this effect in mice. Although the transporter has not been shown to be a direct molecular target of the compound, this result underlines the role that the DAT must play in modafinil-induced wakefulness.

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Abbreviations: DAT, dopamine transporter; EEG, electroencephalogram; EMG, electromyogram; FFT, fast Fourier transform; Fos-IR, Fos-immunoreactive; PBS, phosphate-buffered saline; REM, rapid eye movement.

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Another experimental approach, Fos immunohistochemistry, has been used to determine those brain regions that are activated after modafinil administration (Lin et al., 1996; Engber et al., 1998; Chemelli et al., 1999; Scammell et al., 2000). Modafinil-induced activation was noted in regions such as the tuberomammillary nucleus and the orexin (also called hypocretin) cell group in the perifornical region of the hypothalamus (Scammell et al., 2000) that have been implicated in the promotion of wakefulness. Indeed, the demonstration that modafinil induced Fos in orexin cells, and the putative involvement of these cells in the regulation of wakefulness, together suggest that orexin might be necessary for the wake-promoting effects of the compound. We therefore measured the effectiveness of modafinil at inducing wakefulness in *orexin*$^{-/-}$ mice. We also examined whether the absence of orexin affected the pattern of Fos activation induced by modafinil administration in these mice.

EXPERIMENTAL PROCEDURES

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center and were strictly in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. In particular, the number of mice used in these experiments was the minimum possible for this study, and the animals were treated throughout in such a way that minimized any potential for pain and suffering. F3 and F4 homozygote *orexin*$^{-/-}$ mice and wild-type littermates, on a C57Bl/6-J-129/SvEv background, were derived as previously described (Chemelli et al., 1999).

Modafinil

Modafinil (Cephalon, Inc., West Chester, PA, USA) was freshly prepared before each experiment. The compound was suspended in vehicle (sterile saline with 0.25% carboxy methylcellulose; Sigma, St Louis, MO, USA). Less than 60 s before each injection, modafinil was re-suspended by vortexing and sonication.

Immunohistochemistry

Wild-type and *orexin*$^{-/-}$ mice (N=2–6/group) were injected with vehicle or modafinil (10 or 100 mg/kg, i.p.) at lights-off (19:00 h). This time of injection ensured that the behavioral state of the mice (i.e. spontaneous wakefulness in the vehicle groups and induced wakefulness in the modafinil groups) would be similar. Two hours later, mice were deeply anesthetized by overdose of 5% chloral hydrate in saline (0.8 ml, i.p.) and transcardially perfused with 12–14 ml of saline (4 ml/min) followed by 15 ml of phosphate-buffered 10% formalin (5 ml/min). Brains were removed, post-fixed for 3 h in formalin, and then allowed to equilibrate in 20% sucrose in 0.1 M phosphate-buffered saline (PBS) with 0.02% sodium azide. Brains were then sectioned (30 μm, 1:3 series) on a freezing microtome and stored in PBS–azide at 4 °C. One series was immunostained for Fos as previously described (Scammell et al., 2000). In brief, sections were incubated for 48 h in rabbit anti-Fos antiserum (Ab-5; 1:25,000 dilution; Oncogene Research Products, San Diego, CA, USA), reacted with biotinylated donkey anti-rabbit IgG secondary antiserum (1:1000; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h, and incubated in avidin–biotin complex (Vector Laboratories, Burlingame, CA, USA). A black reaction product was produced in cell nuclei using diaminobenzidine with $NiSO_4$ and $CoCl_2$. The pattern of Fos immunoreactivity was examined throughout the entire brain.

Animal surgery

For chronic electroencephalogram/electromyogram (EEG/EMG) monitoring, 12–14-week-old matched pairs of *orexin*$^{-/-}$ and wild-type male mice (N=10/genotype) were anesthetized with sodium pentobarbital (60 mg/kg i.p.; Nembutal; Abbott Laboratories, North Chicago, IL, USA) and chronically implanted with custom manufactured electrodes as previously described (Chemelli et al., 1999). Mice were also implanted with indwelling i.p. catheters to allow administration of modafinil or vehicle without handling of the mice, so minimizing disturbance during the experimental procedure. Catheters were fabricated from PE50 polyethylene tubing with PE90 cuffs, so that the total dead space was less than 0.1 ml. The sites for insertion of the catheters were shaved and prepared with Betadine solution (Baxter Health Care Corporation, Deerfield, IL, USA) and 70% ethanol. Using a large-bore blunted 18 g needle, a s.c. tunnel between the dermis and underlying musculature was made, starting at the nape of the neck, following down the dorsum of the back, and around to the lower right abdomen. A 1 cm midline incision exposed the abdominal wall. A puncture hole was then created in the abdominal musculature with a 22 g needle, and the catheter was threaded down the s.c. tunnel and its cuff placed through the puncture. The catheter was fastened to the muscle wall by a small figure-eight stitch of 6–0 silk, and secured above the neck to the EEG/EMG implant through a stainless steel loop screw. The exposed extent of the catheter was affixed to the EEG/EMG tether with surgical tape. A resistance-free injection of saline through the exposed end of the catheter, positioned 10 cm above the head, insured patency of the cannula. The cannula was then sealed with an airtight stainless steel stylet, and the surgical sites were closed with 6–0 silk suture and treated with Betadine ointment (Baxter Health Care Corporation).

Behavioral studies

All mice were housed singly and allowed to recover and habituate to tethers, catheters, and the experimental procedures for 14–15 days prior to EEG/EMG recording. Exposed catheter ends were swabbed externally with 70% ethanol and flushed with sterile saline every 48 h to maintain patency. Food and water were made available *ad libitum* throughout the study and mice were housed under a 12h light/dark cycle (lights on at 07:00 h) and a constant temperature of 24±1 °C.

The time of injection was chosen as lights off (19:00 h) for the behavioral study so that the data would be compatible with results from the immunohistochemical study. Furthermore, this time of administration is most consistent with the clinical use of modafinil, which is usually taken by narcoleptic patients at the onset of the active phase of the diurnal cycle. Hence, just before the onset of the dark phase at 19:00 h, mice were administered modafinil (0, 10, 30, or 100 mg/kg; 10 ml volume/kg body weight; 0.12–0.17 ml absolute volume) through the indwelling cannulae. Each injection was immediately followed by a flush of the same volume of sterile saline. Simultaneous EEG/EMG/video recording was initiated at the onset of the dark phase and continued for 24 h. Each mouse received all doses of modafinil or vehicle in a randomized crossover design over the experimental sessions. Each session was separated by 72 h to ensure washout of the compound. After completion of all experimental sessions, animals were killed by sodium pentobarbital overdose (Nembutal; 100 mg/kg i.p.) and cervical dislocation. Cannulae were examined postmortem to confirm correct placement and continued patency during the study.

Data analysis

EEG/EMG signals were amplified and filtered (EEG 0.3–100 Hz; EMG 30–300 Hz; Grass Model 78; Astro-Med Inc., West Warwick, RI, USA), digitized at 250 Hz and recorded on-line using custom