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MICRODIALYSIS PERFUSION OF OREXIN-A IN THE BASAL FOREBRAIN INCREASES WAKEFULNESS IN FREELY BEHAVING RATS

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INTRODUCTION

The discovery of rapid eye movement (REM) phase of sleep (2) combined with increasing knowledge of neurophysiology and neuroanatomy, ushered in a new era of research into the control mechanisms of both REM and nonREM sleep. With respect to REM sleep, current theory suggests REM-sleep promoting brainstem mesopontine laterodorsal and the pendunculopontine tegmental (LDT/PPT) cholinergic neurons supply cholinergic innervation to the pontine and bulbar reticular formation. These reticular neurons, in turn, likely act as effectors for many REM sleep phenomena, including the muscle atonia, rapid eye movements, muscle twitches, and initiation of ponto-geniculo-occipital (PGO) waves. The cholinergic LDT/PPT neurons are under inhibitory control of the serotonergic neurons of the dorsal raphe (DRN) and the noradrenergic neurons of the locus coeruleus (LC). In the forebrain, the basal forebrain (BF) has been identified as an area important in maintaining EEG arousal and wakefulness, and adenosine has been identified as a factor mediating the sleepiness following prolonged wakefulness through inhibitory actions on wakefulness-promoting BF neurons. (For reviews see: 12, 14, 19, 20, 35).

However, there have been few brain neurotransmitter/neuromodulator systems identified that might regulate and coordinate all behavioral states – REM sleep, nonREM sleep, and wakefulness. Hence the excitement over the recent identification of orexins (alternatively known as hypocretins) and their receptors. Orexin peptide-containing neurons are exclusively localized in the lateral hypothalamus and send out diffuse projections to a number of distinct brain regions known to be involved in the regulation of both REM sleep and wakefulness (W)/nonREM sleep, including the cholinergic LDT/PPT, the LC, the DRN, the pontine brainstem reticular formation and the cholinergic neurons of the BF (6, 8, 11, 21).

With respect to REM sleep and REM phenomena, Lin et al. (16) reported that an abnormality in the orexin type II receptor gene was the basis of inherited narcolepsy in dogs. Chemelli et al. (5) created constitutive knockout mice which lacked the orexin gene. These orexin knockout mice (-/-) had increased REM sleep and also cataplexy-like episodes that were entered directly from states of active movement. Recent confirmation in man of orexin's importance has been provided

by Nishino et al. (22) who reported that narcoleptic humans often have undetectable levels of orexin in cerebrospinal fluid (CSF). Finally, Thakkar et al. (37) reported that microdialysis perfusion of antisense oligonucleotides against orexin type II receptor mRNA into the subcoeruleus (SubC) reticular region of the brainstem increased REM sleep and induced cataplexy-like episodes in freely behaving rats. These combined reports suggested that the absence, or reduction, of orexin peptide or orexin receptors decreases W and enhances REM sleep, whereas endogenous orexin promotes W and suppresses the appearance of REM sleep.

However, it has remained unclear at which (if any) specific forebrain sites orexin might act to control behavioral state. Two studies have found that a single intraventricular injection of orexin-A (10 μg) produced an increase in wakefulness and a decrease in REM sleep, with no significant change in nonREM sleep (9,25). However, the intraventricular route of injection meant that the site of action could not be specified. We noted that orexin projections densely innervate the field of cholinergic neurons in the BF (5 and David Rye, personal communication), an area involved in the regulation of cortical activation and wakefulness, and previously studied by us in terms of adenosine effects.

Several lines of evidence suggest that the cholinergic magnocellular regions of the BF are critical in the regulation of nonREM sleep and wakefulness (reviewed in 13, 31, 35). Lesions of the BF cholinergic cells are associated with a decrease in cortical acetylcholine release and a parallel decrease in cortical activation (4, 17, 33). Reciprocally, electrical or chemical stimulation of the cholinergic basalis neurons in anesthetized or brainstem-transected animals leads to a parallel increase in cortical acetylcholine release and cortical activation (29). Pharmacological increases in acetylcholine concentration induce cortical EEG desynchronization, an effect that is antagonized by atropine, an acetylcholine antagonist (e.g., 15). Single unit recording in unanesthetized, naturally sleeping animals have shown that the majority of the BF neurons have highest discharge activity during active W (35, 36). It has been suggested that these cholinergic wake-active neurons are important mediators of cortical activation, EEG desynchronization, and W/arousal (31, 35). Our own work has documented the wakefulness-reducing effects of adenosine in the BF (26, 34). It must be cautioned that non- cholinergic neurons in the BF also project to cortex and may also play an important role in sleep- wakefulness control, as pointed out by Jones et al. (14). However, recent work by this group has emphasized that it is the cholinergic neurons (juxtacellularly labeled) that have the requisite discharge pattern to promote EEG activation (18).

Based on these data, we decided to test the hypothesis that orexin-A enhances wakefulness via a local action in the BF, and consequently examined the behavioral state effects of local unilateral microdialysis perfusion of orexin-A in the BF. We here report that microdialysis perfusion of orexin-A in the cholinergic region of horizontal band of Broca and the substantia innominata increased wakefulness in a dose-dependent manner with a concomitant decrease in nonREM sleep and REM sleep.

METHODS

Adult male Sprague-Dawley rats (Charles River Breeding Laboratories), weighing between 300 and 400 gms were housed under constant temperature (23 ± 1°C) and 12:12 light dark cycle (light-on period from 0700 h to 1900 h) with food and water available ad libitum. Under general anesthesia (i.p. sodium pentobarbital 2.5 mg/100 gm body weight) and a combination i.m.-injected ketamine (3.75 mg/100 gm), xylazine (0.19 mg/100 gm) and acepromazine (0.038/100 gm), bilateral screw electrodes were fixed onto the skull above the frontal area (2 mm anterior to the bregma and 4 mm lateral to the mid sagittal suture) for recording EEG. EMG electrodes consisting of flexible wires with stainless steel loops were fixed onto the superior nuchal muscles. Intracerebral guide cannulas (for later insertion of the microdialysis probes) were targeted to the field of cholinergic neurons in the horizontal diagonal band (HDB) at coordinates: AP - 0.40, ML ± 1.8 and DV -8.0 (1 mm above the target) (23). All animals (n = 10) were treated in accordance with the American Association for Accreditation of Laboratory Animal Care's policy on care and use of laboratory animals.

Recordings were carried out in a sound-attenuated chamber after the rats had completely recovered from the post-operative trauma. The microdialysis probe (CMA 11, 1 mm membrane length, 0.24 mm diameter; CMA/ Microdialysis, Stockholm) was lowered through one of the guide cannulas and the animals were attached to the recording cables for behavioral state recording and then to the probe inlet and outlet tubing (1 m pieces of low dead volume FEP tubing; CMA/ Microdialysis). After at least 12 h of recovery from probe insertion, the experiment was begun. Artificial cerebrospinal fluid (ACSF = NaCl 147 mM, KCl 3 mM, CaCl2 1.2 mM, MgCl2 1.0 mM, pH 7.2) or orexin-A was perfused with simultaneous electrographic recording of the behavioral states. During control days, ACSF was continuously perfused, at the flow rate of 1.5 µl/min, during the light period for 6 h from 11.00 h to 17.00 h (see Table 1). During the orexin perfusion days, ACSF was perfused from 11.00 h to 13.00 h (used to allow full adaptation to experimental apparatus) followed by 2 hours of orexin-A perfusion (13.00 h to 15.00 h). This was followed by 2 hours of ACSF perfusion (15.00 h to 17.00 h); during this last epoch the time course of decrease of orexin-A perfusion effects was evaluated, enabling determination of whether there were any persisting effects after the perfusion period ("hangover effects"). Because of the possibility of long-duration effects of orexin, initial experiments were run with the experimental day always following the control day. After determining that the behavioral effects of orexin-A BF perfusion were of short duration, the order of presentation of the control and experimental day conditions (Table 1) was counterbalanced for the last two animals.

Three different concentrations (0.1, 1, and 10 µM) of orexin-A were administered in a counterbalanced order.

After completion of the experiment, the animals were deeply anaesthetized with sodium pentobarbital and then perfused with 100 ml of cold saline followed by 150 ml of 4% formaldehyde in 0.1 M phosphate buffer. The brain stem was isolated, blocked, and placed overnight in the same solution. It was then transferred to 20% sucrose-0.1 M phosphate buffer until the tissue block sank. Then 40 µm coronal sections were cut on a freezing microtome. A series of one-in-four sections were subsequently processed for ChAT immunohistochemical staining to visualize the cholinergic neurons, or with 0.5% cresyl violet to verify the perfusion site. Stained coronal sections which showed the tip of the probe were used to reconstruct the perfusion zone.

Table 1. - *Experimental protocol.*

Time of Day	Control Day Condition	Experimental Day Condition
11-13 hr (Time 1)	ACSF	ACSF
13-15 hr (Time 2)	ACSF	Orexin-A (0.1, 1.0 or 10 nM)
15-17 hr (Time 3)	ACSF	ACSF

Behavior was classified into 3 different states: wakefulness (W), nonREM sleep (nonREM), and rapid eye movement sleep (REM) (37). Data analysis was carried out in 6 s epochs and scored visually as described earlier (37).

Statistical analysis. - The main hypothesis of the study was that behavioral state would be found to be dependent on the dose of orexin, and, more specifically, that W would be increased in a dose- dependent manner with increasing doses of orexin.

Use of a mixed, repeated measures MANOVA model allowed us to fully assess the possible interaction of the effects of the drug on 3 different behavioral states, given the design of the study shown in Table 1. The particular model (32) was chosen to reflect the fact that, for technical reasons, slightly different numbers of rats were used as subjects for different dose levels of orexin. Hence dose was used as a between subject grouping variable, while rat was used as a covariate to take account of the fact that almost all of the animals were used in all the experiments. The dependent variable was percentage of the different behavioral states, W, nonREM and REM. State was thus used as a grouping variable, which appeared in the SPSS 10 analysis as a between factor with three levels.

The MANOVA model thus had:

Two within factors:

1) Condition (2 levels: control day, and experimental day).

2) Time of day (3 levels: 11-13 hr, Time 1 (two hours prior to the drug perfusion on experimental day); 13-15 hr, Time 2 (two hours of drug perfusion on the experimental day); 15-17 hr, Time 3, (two hours following drug perfusion on the experimental day).

Two between factors:

1) State (3 levels: W, nonREM, and REM sleep).

2) Drug dosage (3 levels: 0.1 μM, 1.0 μM, 10 μM).

Rat was a covariate in all analyses and all F statistics are reported using Wilks' Lamba.

RESULTS

Histology. - All the microdialysis probe tips were located in the cholinergic zone of the BF between the AP levels P 0.26. and P 0.92 (23) as shown in Figure 1. In the rat this cholinergic zone includes the horizontal limb of the diagonal band, HDB, the magnocellular preoptic nucleus, MCPO, and the substantia innominata, SI. All (n = 10) of the probe locations were in or near the HDB (Figure 1).

MANOVA analysis. - Behavioral state was found to vary with time of day and condition (control or experimental[orexin]day), as reflected in a two-way interaction of time of day and state [$F(4,98) = 22.3$, $p < .001$], and of condition and state [$F(2,50) = 36.8$; $p < .001$], as well as a three-way interaction of time of day x condition x state [$F(4,98) = 47.5$; $p < .001$]. Furthermore, the four way interaction of time of day x condition x state x drug dosage [$F(8,98) = 9.85$; $p < .001$) indicated that drug dosage also played a role in determining state.

Hence, our post-hoc tests focused on comparing the effect of drug dosage on the three different behavioral states for the two comparable time of day windows across conditions, e.g. 11-13 hr for both control and experimental conditions, and 13-15 hr in both control and experimental conditions (the only time window with orexin administration); and 15-17 hr in both control and experimental conditions (Table 2; Table 3 lists the mean values ± SEM). For this follow-up analysis, we

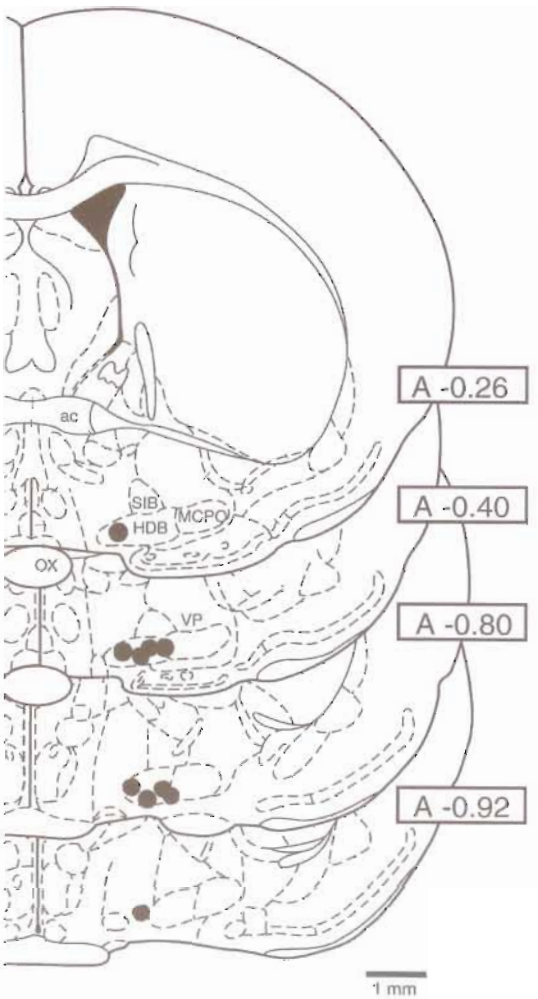

Fig. 1. - *Schematic representation of the anatomical location of the histologically identified probe tip sites in all 10 animals (black dots).*

Probes were placed unilaterally in each animal. All the target sites were located between AP-0.26 and-0.92 and all are mapped onto the four coronal basal forebrain sections, including homotopic mapping for contralateral sites (anatomic diagram adapted from 23). Abbreviations: ac, anterior commissure; HDB, nucleus horizontal limb of the diagonal band; ox, optic chiasm; SIB, substantia innominata basal; MCPO, magnocellular preoptic nucleus; VP, ventral pallidum. For identification of unlabeled structures see Paxinos and Watson (23).

used a mixed model, repeated measures MANOVA with condition as a within factor (2 levels: control and experimental) and dose as a between factor. Only the time window with ACSF-Orexin perfusion showed a significant interaction of condition and orexin dose level (Table 2) for W and nonREM, with REM showing a trend level significance. This means that, as predicted, orexin produced dose-dependent alterations in W. Furthermore, nonREM also showed a dose-dependent effect, while REM showed a trend-level dose responsiveness.

Of interest also is the 2 h sampling period after the removal of orexin from the perfusate to determine when the sleep-wakefulness behavior returned to baseline (Time 3 in Table 2). Note that none of the states showed any significant effects, indicating the behavioral state effects of orexin reversed shortly after cessation of orexin perfusion.

Table 2. - *Orexin dose-dependent behavioral state differences between control and experimental conditions.*
Significance level of interaction of condition x orexin dose level (and, in parentheses, Wilks lambda). All values had 2, 16 df.

Time of day window and control-experimental day perfusates	W	nonREM Sleep	REM Sleep
11-13 hr, Time 1, ACSF-ACSF	NS	NS	NS
13-15 hr, Time 2, ACSF-Orexin	0.002 (10.00)	0.003 (8.387)	0.079 (2.982)
15-17 hr, Time 3, ACSF-ACSF	NS	NS	NS

We next turned to follow-up tests to determine the source of the condition x orexin dose level interaction reported for Time 2, and to a graphical presentation of the dose-response data.

Wakefulness. - The group means (± SEM) of W (Table 3) during control and orexin-A are plotted in Figure 2. There was a significant increase in W during orexin perfusion (see Table 2) as compared with control perfusion. Regression analysis indicated a linear dose response effect on this semi-log plot (slope = 0.678, $t = 3.915$, $p < 0.001$). Note that perfusion of 10 µM orexin-A produced a 5-fold increase in W, with this state occupying about 44% of the two hour sample during the light (inactive) period, compared with less than 10% during the control day perfusion. Microdialysis perfusion of 1 µM showed, as compared with control

Table 3. - Mean ± SEM of % time in state for Control Day and Experimental Day.
The rows in italic (Time 2) indicate the time of perfusion of orexin-A on the experimental day. Note that Time 2 shows increased W and decreased nonREM and REM. See text.

	Control Day (ACSF perfusion)			Experimental Day		
	Control for 0.1 µM	Control for 1.0 µM	Control for 10 µM	Orexin-A 0.1 µM	Orexin-A 1.0 µM	Orexin-A 10 µM
			Wakefulness			
Time 1	15.5 ± 0.6	18.3 ± 0.7	17.4 ± 1.0	18.9 ± 1.6	14.5 ± 2.5	16.1 ± 2.3
Time 2	*12.7 ± 0.7*	*11.2 ± 1.6*	*8.0 ± 1.7*	*25.5 ± 1.5*	*30.6 ± 2.9*	*44.4 ± 4.8*
Time 3	14.5 ± 1.4	14.2 ± 2.1	18.0 ± 1.8	12.8 ± 2.4	14.4 ± 2.5	12.7 ± 1.5
			nonREM Sleep			
Time 1	79.5 ± 0.6	77.4 ± 0.8	78.4 ± 0.9	76.7 ± 1.5	81.7 ± 2.7	79.5 ± 2.4
Time 2	*81.9 ± 0.8*	*84.2 ± 1.3*	*86.3 ± 1.6*	*71.1 ± 1.9*	*67.7 ± 3.1*	*54.4 ± 5.1*
Time 3	80.7 ± 1.7	81.7 ± 1.6	77.3 ± 1.9	82.3 ± 3.0	81.5 ± 2.8	82.4 ± 1.7
			REM Sleep			
Time 1	5.1 ± 0.6	4.3 ± 0.6	4.1 ± 0.2	4.4 ± 0.7	3.8 ± 0.8	4.5 ± 0.1
Time 2	*5.5 ± 0.3*	*4.6 ± 0.4*	*5.8 ± 0.6*	*3.5 ± 0.9*	*1.7 ± 0.3*	*1.5 ± 0.5*
Time 3	4.9 ± 0.3	3.9 ± 0.9	4.7 ± 0.8	4.8 ± 0.7	4.2 ± 0.9	5.0 ± 0.4

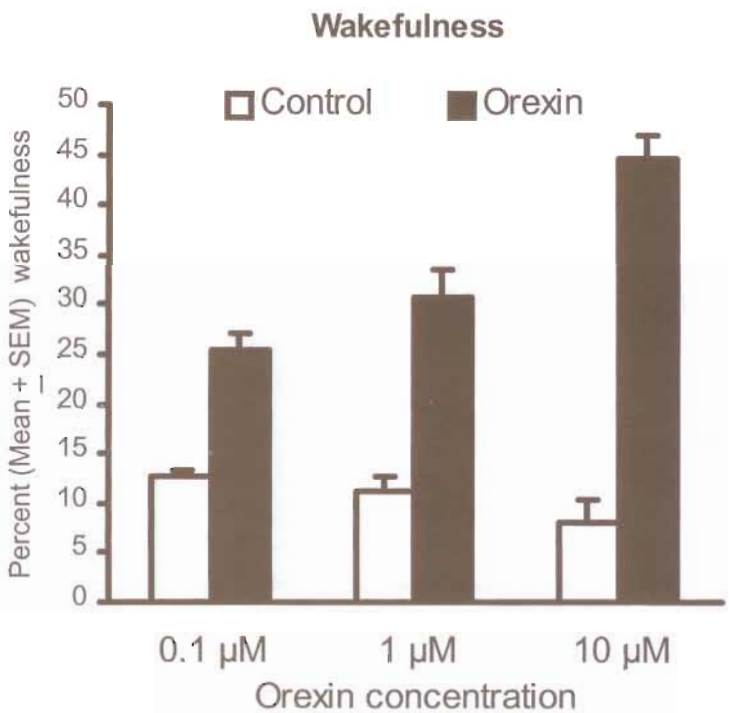

Fig. 2. - *Unilateral perfusion of orexin-A in the BF produced an increase in W.*

The graph shows the group mean values of W (± SEM) during the control period (ACSF; open bars) and during the perfusion of three doses of orexin-A (0.1, 1 and 10 µM; black bars). Note that the three doses of orexin-A perfusion in the BF produced a linear dose-dependent increase, in this semi-log plot, in the time spent in W during the two hours of orexin-A perfusion (see text for details).

day perfusion, a nearly twofold (173%) increase in W to occupy 31% of the two hour sample period during the light period, while the time spent in W was doubled with 0.1 µM orexin-A perfusion. In all cases the orexin-induced increase in W returned to control values during the two hours of post orexin ACSF perfusion.

NonREM sleep. - Microdialysis perfusion of orexin-A in the BF decreased nonREM sleep (Table 2, Figure 3), with a significant linear dose-dependent effect on this semi-log plot (slope = -0.620, $t = -3.54$, $p < 0.004$). 10 µM orexin-A showed the maximum decrease of 38% in nonREM sleep (Table 3) followed by 1 µM (a decrease of 19.5%) and 0.1 µM (a decrease of 13%) as compared to control values. Note also that nonREM sleep returned to baseline/control level during the two hours of post-orexin ACSF perfusion.

REM sleep. - Microdialysis perfusion of orexin-A produced a trend-level decrease in REM sleep (Table 2, Figure 4). The time spent in REM sleep decreased from 5.8% during control ACSF to 1.5% during 10 µM of orexin-A perfusion (Table 3), whereas, with 1 µM of orexin-A perfusion the time spent in REM sleep decreased from 4.63 % (control value) to 1.7 %. With the lowest dose of 0.1 µM the time spent in REM sleep was reduced to 3.5% from 5.5% during control day perfusion.

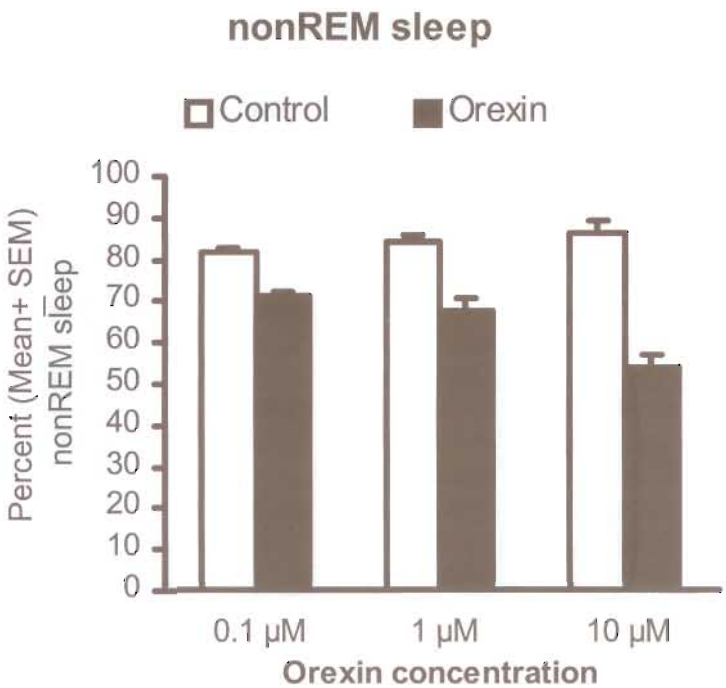

Fig. 3. - *The graph shows the group mean values (± SEM) of the percent of total time spent in non-REM sleep during ACSF (open bars), or orexin-A perfusion (three doses; black bars).*

The percent of total time spent in nonREM sleep was significantly decreased during orexin-A perfusion compared to the control/ACSF perfusion. The effect was log-linear across the three doses of orexin-A.

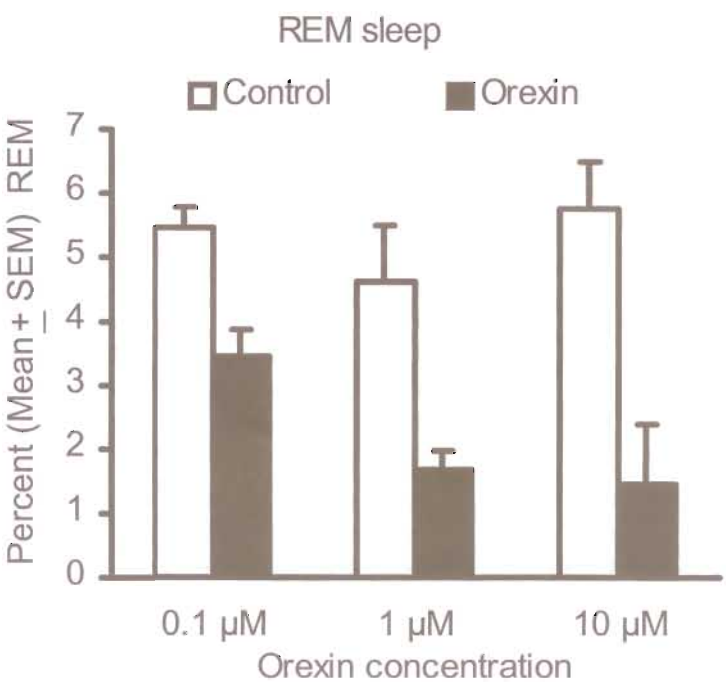

Fig. 4. - *The percent of time spent in REM sleep showed a trend level decrease following perfusion with the three doses of orexin-A in the BF (0.1, 1 and 10 μM), as compared with the control ACSF perfusion.*

The highest dose of 10 μM produced maximal reduction in REM sleep (about 73% (for details see Table 3), followed by 1 μM of orexin-A, (a reduction of 64% in REM sleep). The lowest dose (0.1 μM) showed a decrease of about 34% in REM sleep relative to the control ACSF perfusion.

DISCUSSION

The main finding of this study was that orexin-A, applied locally by microdialysis perfusion in the cholinergic zone of the rat BF, increased W, decreased nonREM sleep and, at a trend level, decreased REM sleep. These behavioral state effects were dose-dependent, with the highest dose (10 μM) producing a 5-fold increase in W. Following the cessation of orexin-A perfusion the distribution of behavioral states returned rapidly to baseline, indicating the BF contains efficient mechanisms for the removal or degradation of orexin.

Microdialysis delivery of neuropeptides. - With respect to orexin-A delivery, certain technical features of the present study seem important and worthy of emphasis. The introduction of microdialysis as a means of drug delivery represents a fundamental improvement in drug delivery protocols (28). Microdialysis uses a semipermeable membrane probe and makes it possible to perfuse small molecules or peptides of interest to the extracellular space. The dialysis membrane forms a sterile barrier between perfusate and brain tissue and is known to minimize tissue damage. In the present study, the use of this technique allowed for site-specific, rate controlled, perfusion of orexin-A into the extracellular space of the target zones. It should be noted that the extracellular concentration achieved is only a fraction of the one present in the perfusate due to the diffusion gradient established across the microdialysis membrane. A more specific consideration of the gradient effect and the "*in vitro*" evaluation of the amount of drug delivery has been reported in previous studies (27, 38). We have used similar considerations to predict that, in this study, the concentration of exogenous orexin-A delivered extracelluarly would be less than 10% of the concentration added to the perfusate. Further, the microdialysis probes delivering orexin-A had a length and width of the dialyzing membrane designed to deliver orexin-A within the dimensions of the cholinergic HDB/MCPO and were histologically localized to lie within or very close to this nucleus and within a field of immunohistochemically identified cholinergic neurons.

Experimental design and statistical analysis. - A discussion of the experimental design and statistical analysis chosen is warranted. MANOVA analysis was used because only a multivariate approach appeared reasonable for the complex experimental design (32). While common in epidemiological and behavioral studies, such an analysis is less often used in biology, but appears to us to be useful for studies of drug application. It also appears useful to comment on our experimental design. When we began our studies, we did not know if orexin-A would have short or long duration effects and therefore we did not counterbalance ACSF and orexin-A perfusion, but we did counterbalance the administration of the three orexin-A doses. Once we were convinced that the W inducing effects of orexin-A did not last for more than two hours after the cessation of orexin-A perfusion, in the last two animals we did counterbalance the administration of ACSF and orexin-A.

However, the multivariate statistical analysis controlled for the noncounterbalanced days, since all perfusions, ACSF as well as orexin, were compared to the same experimental day.

For the day of orexin-A perfusion, it appeared useful to have three conditions (see experimental design in Table 1). The first two hours were ACSF perfusion, an adaptation period to allow for the dissipation of any behavioral state-disruptive effects of placing the animals in the experimental cage and attaching the perfusion/recording connections. The control day condition of perfusion of ACSF alone would allow determination of whether the first two hours showed indications of disruption or not. As can be seen from Table 2, the first two hour period of ACSF perfusion shows more W than subsequent two hour periods. However, it was not surprising that the MANOVA statistical analysis indicated a significant time of day effect. While some circadian effects might be present, the existence of a large effect in the first two hours suggests that the experimental manipulation was the major variable. The second two hours were the time of orexin-A perfusion, while the third two hour perfusion period allowed for determination of whether orexin-A effects persisted beyond the time of perfusion.

One of the questions raised by a pharmacological treatment is whether the treatment is mimicking a physiological condition, or whether the results are purely pharmacological. An important traditional measure of the physiological nature of the response, is the presence of a dose-response relationship. In the case of orexin-A, this dose-response relationship was present. There was a log-linear dose dependent increase in time spent in W with orexin-A perfusion (log linear, since one log unit increase in dose yielded a linear response). The size of the orexin response was remarkable, with the highest dose of 10 μM orexin-A showing a five fold increase in W, with 44.4% of the usually inactive light period being occupied by W, compared with 8% in the ACSF control day perfusion.

To our knowledge, this is the first report of an experiment using localized microdialysis perfusion of exogenous orexin-A to increase the extracellular concentration of orexin-A in the BF, and induce W. A previous study by Hagan et al. (9) found that a single intraventricular injection of orexin-A (10 μg) produced an increase in arousal/W (2.6-fold increase, less than in the present study) and a decrease in REM sleep, with no significant change in nonREM sleep. These behavioral state effects returned to baseline within 4 h post-injection (9). Intraventricular injection of a neuropeptide takes advantage of the rapid diffusion through the ventricular system allowing orexin to act throughout the brain. In contrast, an important feature of the present study is the use of localized microdialysis perfusion to identify specific brain regions that may mediate the behavioral state effects of orexin-A. Thus, despite the limited area of diffusion produced by local unilateral perfusion in the present study, orexin-A perfusion in the BF produced a robust increase in W (up to 5-fold), indicating that the BF is a potent site for the W-promoting effects of orexin-A. In contrast, the absence of a robust effect on REM sleep is consistent with our hypothesis that the primary effect of endogenous orexin in the BF is to enhance W, and the effects on nonREM and REM sleep are

secondary and possibly indirect. The findings also suggest that the ability of endogenous orexin to inhibit REM sleep expression may be mediated by orexin projections to other brain regions. The next section provides both a general review and a specific review of evidence supporting our hypothesis that orexin's actions on brainstem sites are important in REM sleep control, while BF projections are especially important in control of cortical activation and wakefulness.

Orexin Neurons: Projections, Receptors, and Function.

Orexin neurons of the lateral hypothalamus. - In late 1997, two independent groups identified the orexins/hypocretins as two related peptides derived from a single precursor protein. De Lecea et al. (7) referred to these neuropeptides as hypocretin-1 and -2, whereas Sakurai et al. (30) using a different approach, identified these same two peptides, which they termed orexin-A (= hypocretin-1) and orexin-B (= hypocretin-2); these terms are used in the present paper. These first two reports indicated that neurons containing the orexins are found exclusively in the dorsal and lateral hypothalamic areas (7, 30), and that the orexins may function as neurotransmitters since they were localized in synaptic vesicles and had neuroexcitatory effects on hypothalamic neurons (7).

The discrete localization of orexinergic neurons to the dorsolateral hypothalamus, an area involved in the control of homeostatic drives such as feeding behavior (3), suggested early on that orexins might be involved in energy homeostasis. Indeed, Sakurai et al. (30) found that intracerebroventricular injections of the orexin-A or -B increased food consumption, and fasting increased the expression of prepro-orexin mRNA, suggesting a physiological role for orexins in the regulation of feeding behavior. However, subsequent work revealed that, although orexins stimulate short term food intake, they do not alter total consumption over a 24 hr period, and feeding effects differ with the circadian time of administration (12). Furthermore, as pointed out by various authors (12, 24) the widespread distribution of orexin projections to all levels of the brain and spinal cord suggests that orexin is likely to play a role in multiple physiological functions, or has a broader role than the regulation of feeding and energy balance. Thus, it has been postulated that orexins have a neuromodulatory role in several neuroendocrine/homeostatic functions such as food intake, body temperature regulation, and blood pressure regulation, as well as the control of wakefulness and sleep (5, 16, 24, 40).

Projections of orexin neurons. - As mentioned above, immunohistochemical studies reveal a distribution of orexin projections that is remarkable for the targeting of a number of distinct brain regions known to be involved in the regulation of sleep and wakefulness, including both brainstem and forebrain systems (6, 8, 11, 24, 26). Orexin projections to forebrain include the cholinergic BF (in the rat this includes the HDB, the MCPO and the SI) and the histaminergic tuberomammillary nucleus (TMN). Furthermore, Chemelli et al. (5) as well as others (David Rye,

personal communication) have noted a heavy concentration of orexin-containing fibers around the somata of cholinergic neurons of the BF. The target site of the microdialysis perfusion in the present study was in, or near, the field of cholinergic neurons in the HDB/MCPO. Brainstem targets of orexin projections include the pontine and medullary brainstem reticular formation, the cholinergic mesopontine LDT/PPT, the LC, and the DRN.

Cellular action of orexin. - Orexin A has been shown to excite the noradrenergic neurons of the locus coeruleus, providing at least one documented mechanism by which orexin can promote wakefulness (9, 10) and suppress REM sleep. However, orexin is not always excitatory and the work by van den Pol et al. (41) reveals the variety and complexity of orexinergic effects at the cellular level. Both excitatory and inhibitory neurons express orexin receptors, and orexin has potent effects at both presynaptic and postsynaptically localized receptors (41). Acting at presynaptic receptors on axon terminals orexin can increase the release of either the inhibitory neurotransmitter GABA, or the excitatory transmitter glutamate (41). Hence, orexin is capable of a variety of complex cellular actions and the net effect of these actions on a particular brain circuit system physiology and consequent behavioral effects needs to be determined at the systems level for each brain region. Based on the present results orexin is predicted to have an excitatory effect on the discharge activity of the wake-active neurons of the BF region.

Orexin receptors and their distribution. - Two orexin receptors have been identified (30). Orexin-A is a high-affinity ligand for the orexin receptor type I (Orexin-I) whereas the orexin receptor type II (Orexin II) exhibits equally high affinity for both peptides. Due to unavailability of sufficiently specific ligands for orexin I and II receptors, the distribution of the receptors is unknown. In situ hybridization studies of orexin receptor mRNAs (5, 39) citing their unpublished data) have shown a diffuse pattern, consistent with the widespread nature of orexin projections, although there was a marked differential distribution of the orexin type I and II mRNAs. Trivedi et al. (39) suggest the cholinergic BF has only type II receptors, whereas the locus coeruleus contains abundant orexin 1 mRNA but no orexin II mRNA. Thakkar et al. (37) recently demonstrated the usefulness of microdialysis perfusion of antisense directed against orexin type II receptor mRNA to elucidate the function of the two orexin receptors. This antisense perfusion in the sub-coerulean region of the brainstem increased REM sleep and induced cataplexy-like episodes (37).

Hypotheses about sites of orexin effects on behavioral state. - Combined with the present study, the findings of Thakkar et al. (37) support the hypotheses that 1) orexin regulates REM sleep and cataplexy via selective action on brainstem neurons, primarily those in the nucleus sub-coeruleus alpha (SubCa), with mediation by the orexin II receptor; and 2) that orexin influences W through forebrain sites such as the cholinergic BF, and possibly the TMN, also with mediation by the

orexin II receptor. Thus, it is not difficult to propose that the diffuse orexin projections act on multiple brain sites via two different receptors to regulate and control various aspects of behavioral states. Additional studies are needed to more precisely define the physiological and behavioral roles of the two orexin receptors and ligands in different brain regions.

Conclusion. - The results of this study clearly demonstrate a potent capacity of orexin-A to induce W via a local action in the BF. The results are consistent with previous work suggesting that the BF cholinergic neurons have a critical, although not exclusive, role in the regulation of EEG activation and W. This data suggests that orexin-A has a significant role in the regulation of arousal/wakefulness, in addition to the previously described role of orexin in the regulation and expression of REM sleep and REM sleep-related phenomena.

SUMMARY

Recent work indicates that the orexin/hypocretin-containing neurons of the lateral hypothalamus are involved in control of REM sleep phenomena, but site-specific actions in control of wakefulness have been less studied. Orexin-containing neurons project to both brainstem and forebrain regions that are known to regulate sleep and wakefulness, including the field of cholinergic neurons in the basal forebrain (BF) that is implicated in regulation of wakefulness, and includes, in the rat, the horizontal limb of the diagonal band, the substantia innominata, and the magnocellular preoptic region. The present study used microdialysis perfusion of orexin-A directly in the cholinergic BF region of rat to test the hypothesis that orexin-A enhances W via a local action in the BF. A significant dose-dependent increase in W was produced by the perfusion of three doses of orexin-A in the BF (0.1, 1.0, and 10.0 μM), with 10.0 μM producing more than a 5-fold increase in wakefulness, which occupied 44% of the light (inactive) phase recording period. Orexin-A perfusion also produced a significant dose-dependent decrease in nonREM sleep, and a trend-level decrease in REM sleep. The results clearly demonstrate a potent capacity of orexin-A to induce wakefulness via a local action in the BF, and are consistent with previous work indicating that the BF cholinergic zone neurons have a critical role in the regulation of EEG activation and W. The data suggest further that orexin-A has a significant role in the regulation of arousal/wakefulness, in addition to the previously described role of orexin in the regulation and expression of REM sleep and REM sleep-related phenomena.

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