Induction of Active (REM) Sleep and Motor Inhibition by Hypocretin in the Nucleus Pontis Oralis of the Cat

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Xi, Ming-Chu, Simon J. Fung, Jack Yamuy, Francisco R. Morales, and Michael H. Chase. Induction of active (REM) sleep and motor inhibition by hypocretin in the nucleus pontis oralis of the cat. J Neurophysiol 87: 2880–2888, 2002; 10.1152/jn.00505.2001. Hypocretin (orexin)-containing neurons in the hypothalamus, which have been implicated in the pathology of narcolepsy, project to nuclei in the brain stem reticular formation that are involved in the control of the behavioral states of sleep and wakefulness. Among these nuclei is the nucleus pontis oralis (NPO). Consequently, the present study was undertaken to determine if the hypocretinergic system provides regulatory input to neurons in the NPO with respect to the generation of the states of sleep and wakefulness. Accordingly, polygraphic recordings and behavioral observations were obtained before and after hypocretin-1 and -2 were microinjected into the NPO in chronic, unanesthetized cats. Microinjections of either hypocretin-1 or -2 elicited, with a short latency, a state of active sleep [rapid eye movement (REM)] sleep that appeared identical to naturally occurring active sleep. The percentage of time spent in active sleep was significantly increased. Dissociated states, which are characterized by the presence of muscle atonia without one or more of the electrophysiological correlates of active sleep, also arose following the injection. The effect of juxtacellular application of hypocretin-1 on the electrical activity of intracellularly recorded NPO neurons was then examined in the anesthetized cat. In this preparation, the application of hypocretin-1 resulted in the depolarization of NPO neurons, an increase in the frequency of their discharge and an increase in their excitability. These latter data represent the first description of the in vivo action of hypocretin-1 on intracellularly recorded neuronal activity and provide evidence that the active sleep-inducing effects of hypocretin are due to a direct excitatory action on NPO neurons. Therefore we suggest that hypocretinergic processes in the NPO may play a role in the generation of active sleep, particularly muscle atonia and therefore are likely to be involved in the pathology of narcolepsy.

INTRODUCTION

Hypocretin (orexin)-containing neurons are located exclusively in the hypothalamus (Peyron et al. 1998; Sakurai et al. 1998; Zhao et al. 2001). These neurons send diffuse projections throughout the brain and spinal cord, and the targets of these projections suggest that they have a neuromodulatory role in energy homeostasis, food intake, temperature regulation, and motor control as well as participating in the regulation of sleep and wakefulness (Date et al. 1999; Fung et al. 2001; Peyron et al. 1998; Nambu et al. 1999; van den Pol 1999). Of particular interest with respect to the present study are the findings that hypocretin-containing axon terminals are located in brain stem areas such as the laterodorsal tegmental and pedunculopontine tegmental nuclei (LDT/PPT), locus coeruleus, dorsal raphe, and the pontine reticular formation (Chemelli et al. 1999; Date et al. 1999; Hagan et al. 1999; Nambu et al. 1999; Peyron et al. 1998). In addition, both hypocretin-1 and -2 receptors have been identified in the same areas (Greco and Shiromani 2001; Hervieu et al. 2001; Marcus et al. 2001). All of these areas are known to be involved in the control of the behavioral states of sleep and wakefulness (Chase and Morales 1990; Jones 1991; Rye 1997; Siegel 2000; Steriade and McCarley 1990; Xi et al. 1999b, 2001a).

Recent molecular, anatomical, and behavioral studies have linked the dysfunction of the hypocretinergic system to narcolepsy, a sleep disorder characterized by a number of symptoms including sleepiness and a loss of muscle tone (i.e., cataplexy) triggered by sudden strong emotions. Lin et al. (1999) reported that the mutation of hypocretin type II receptor gene is the genetic cause of canine narcolepsy. Chemelli et al. (1999) found that knockout mice lacking hypocretin exhibit sleep abnormalities similar to those observed in narcoleptics. In addition, human narcoleptics have shown a reduced number of hypocretinergic neurons in the hypothalamus (Peyron et al. 2000; Thannickal et al. 2000) and a reduced level of hypocretin-1 in their cerebrospinal fluid (Nishino et al. 2000). Based on these data, it has been suggested that the hypocretinergic system is involved in the regulation of sleep and wakefulness and in the pathology of narcolepsy (Kilduff and Peyron 2000; Sutcliffe and de Lecea 2000).

At present, neither the site of action vis-à-vis sleep and wakefulness nor the mechanisms of action of the hypocretinergic system is clear. One possibility is that hypocretin acts by modulating the neuronal activity of nuclei in the pontine tegmentum that are known to play a key role in the generation of active sleep [also referred to as rapid eye movement (REM) sleep] (Chase and Morales 1990; Jones 1991; Rye 1997; Siegel 2000; Steriade and McCarley 1990; Xi et al. 2001a). For example, cholinergic neurons in the LDT/PPT and noncholinergic, cholinceptive neuron in the nucleus pontis oralis (NPO) are innervated by hypocretin-1 and -2 terminals (Chemelli et al. 1999; Nambu et al. 1999; Peyron et al. 1998). Recently, we...
found that the microinjection of hypocretin-1 into the LDT of the cat produces a significant increase in wakefulness and a decrease in active sleep (Xi et al. 2001b). Thakkar et al. (1999) reported that bilateral microdialysis perfusion of antisense to the hypocretin-2 receptor mRNA into the rat pontine reticular formation increases active sleep and produces cataplexy. We hypothesize that the pontine tegmentum is a site of action for certain functions of the hypocretinergic system that are involved in the control of sleep and wakefulness and motor activity that occurs during these states. This hypothesis predicts specific actions of hypocretinergic projections on neurons in the NPO vis-à-vis these behavioral states. To test this hypothesis, we examined the behavioral responses of chronic, unanesthetized cats following the microinjection of hypocretin-1 and -2 into the NPO and the effects of juxtacellular application of hypocretin-1 on the electrical activity of NPO neurons in acute, anesthetized cats.

METH ODS

Behavioral studies in the chronic preparation

ANIMALS AND SURGICAL PROCEDURES. Five adult cats (3.0–5.5 kg) were used in the chronic behavioral study. The animals were prepared for monitoring the states of sleep and wakefulness and for drug administration as previously described (Yamuy et al. 1993). All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press 1996) and approved by the Animal Research Committee of the UCLA Office for the Protection of Research Subjects. Briefly, under halothane anesthesia, using sterile surgical procedures, the cats were implanted with electrodes for recording the electroencephalogram (EEG), electrooculogram (EOG), and electromyogram (EMG). Electrodes for recording ponto-genicul-occipital (PGO) waves were also implanted, as detailed in López-Rodríguez et al. (1992). A Winchester plug, connected to these implanted electrodes, and a chronic head-restraining device were bonded to the skull with acrylic cement. A hole 4–5 mm in diameter, made in the calvarium overlying the cerebellar cortex, was covered with bonewax; it provided for subsequent access for a cannula that was used for drug microinjection. Following surgery, antibiotics were administrated both systemically and topically.

After recovery from surgery, all cats were adapted to the recording conditions by placing them in a head-restraining apparatus each day for 2 wk. Thereafter whenever they were placed in the head-restraining apparatus, the animals exhibited spontaneous periods of wakefulness, quiet sleep, and active sleep.

DRUG ADMINISTRATION. Following the adaptation period, carbachol (0.25 μL, 22 mM in saline) was injected into the rostral pontine reticular formation (L: 2; P: 3, and H: −4) (Berman 1968) of each animal to determine the optimal stereotaxic coordinates for the effective site in the NPO using a 2-μL Hamilton syringe. The injection syringe was connected to a remote-controlled hydraulic micromanipulator so that the animals were not disturbed by the injection procedure. The effective NPO site was defined as the stereotaxic coordinates at which an injection of carbachol-induced active sleep with a latency shorter than 4 min. In experimental sessions, all of which were conducted between 10:00 and 16:00 h, hypocretin-1 (0.25 μL, 5–500 μM in saline; American Peptide, Sunnyvale, CA) or hypocretin-2 (0.25 μL, 500 μM in saline; American Peptide) were microinjected, unilaterally, into the NPO. In all cats, control solutions of saline (0.25 μL) were injected into the same site that received the injection of drugs (saline sessions). Injections were made more than 30 min after the cats were placed in the recording apparatus and after they had exhibited at least one episode of quiet sleep. Transition to active sleep is most likely if pharmacological agents are injected during quiet sleep; therefore hypocretin was delivered during a period of 1 min while the animals were in quiet sleep. A single injection was carried out during each experimental session. A maximum of six microinjections were made into the same site on either the left or the right side of each animal. No injections were carried out during control sessions.

POLYGRAPHIC RECORDING AND DATA ANALYSIS. Polygraphic records were digitized and recorded using a Power Macintosh computer running SuperScope II software (GW Instruments, Somerville, MA). The behavioral states of wakefulness (W), quiet (non-REM) sleep (QS), and active sleep (AS) were scored according to standard polygraphic and behavioral criteria (Ursin and Sterman 1981). The following dependent variables were determined during each recording session based on polygraphic and behavioral observations: percentage of time spent in wakefulness, quiet sleep, active sleep, and dissociated state during the first hour following the injection; latency to the onset of the first episode of active sleep or dissociated states, as measured from the time of the beginning of the injection; and duration of each behavioral state. Experimental data are expressed as means ± SE. The statistical significance of the difference between sample means was evaluated using the two-tailed paired or unpaired Student’s t-test and an ANOVA. The criterion chosen to discard the null hypothesis was P < 0.05.

After the completion of all microinjections in each animal, the site of drug injection was marked with 0.5 μL of a 2% solution of Chicago sky blue dye in 0.5 M Na-acetate. The animal was then killed with an overdose of pentobarbital sodium (Nembutal) and perfused with saline followed by a solution of 10% formaldehyde. Serial sections of brain stem tissue were examined to verify the injection sites. The histological studies revealed that the effective injection sites were located within the NPO (Fig. 1).

Electrophysiological studies in the acute preparation

ANIMALS AND SURGICAL PROCEDURES. Two adult cats (3.6–5.0 kg) were used in the electrophysiological experiments. Surgical procedures, which were carried out under halothane anesthesia, have been described in detail in previous papers (Morales et al. 1987). Briefly, the carotid artery and jugular vein were cannulated for blood pressure monitoring and for fluid and drug administration, respectively.

Hypocretin Injection Sites

FIG. 1. Schematic representation of the anatomical location of hypocretin injection sites in the rostral pons of 5 cats. All injection sites were located between P 2.5 and 3.0 and are all mapped onto this 1 coronal brain stem section at level P 3.0. ▫, effective sites for which active sleep-like state was induced following an injection of hypocretin-1 or -2. □, sites for which injections of hypocretin did not induce active sleep but an increase in the time spent in wakefulness and a decrease in the time spent in active sleep. 5n, 5th nerve; bc, brachium conjunctivum; bp, brachium pontis; cp, cerebral peduncle; Lc, locus coeruleus; ml, medial lemniscus; PBN, parabrachial nucleus; TR, tegmental reticular nucleus.
INTRACELLULAR RECORDINGS AND JUXTACELLULAR APPLICATION OF DRUG. The recording sessions commenced 2 h after the cessation of halothane administration to provide for the systemic clearance of halothane (Cowles et al. 1968; Yanagida et al. 1975). To test the effects of hypocretin on NPO neurons, hypocretin-1 was ejected juxtacelularly onto a neuron that was simultaneously being recorded intracellularly. For this purpose, a composite micropipette fabricated for the pressure and/or microiontophoretic ejection of test substances was directed to the pontine tegmentum (L: 2, P: 3, and H: −4) (Berman 1968). The composite pipettes, which we have developed and used in our laboratory, were constructed as previously described (Chase et al. 1989; Kohlmeier et al. 1997; Soja et al. 1991; Xi et al. 1999a). Briefly, three- to five-barrel micropipette assemblies (1.5 mm OD) were pulled on a vertical pipette puller using the “glue and twist method” (Chase et al. 1989; Kohlmeier et al. 1997; Soja et al. 1991; Xi et al. 1999a). Their tips were broken back to a diameter of ~15 µm/barrel, and the distal 12–14 mm of the barrel shank was bent at an angle of 15°. Under microscopic control, the shank was positioned parallel to that of another pulled micropipette capillary tube (2.0 mm OD), which was used for intracellular recording. The tip of the recording barrel protruded 60–100 µm; the entire assembly was bonded together using cyanoacrylate and dental cement; it was then coated with water-insoluble dental sealant. The intracellular recording pipette was filled with either 2 M K-citrate or 3 M KCl (tip resistances: 50–90 MΩ and 30–60 MΩ, respectively). One barrel of the micropipette assembly was filled with hypocretin-1 (100 µM in saline; American Peptide) and another barrel was filled with saline. Hypocretin-1 was ejected by pressure using a two-channel picoinjector (PLI-100; Medical Systems). The injection pressure was varied from 5 to 30 psi for a duration of 5–30 s. Hypocretin-1 was selected to examine the effects of hypocretin on the electrical activity of NPO neurons because it has a high affinity for both hypocretin-1 and hypocretin-2 receptors (Sakurai et al. 1998; Chemelli et al. 1999) and is pharmaceutically stable (Kastin and Akestrom 1999).

The recording electrodes were connected to a high-input impedance preamplifier (Axoclamp 2A). High-gain (×100) DC and low-gain (×10) DC intracellular activity was displayed on an oscilloscope and stored on a video cassette recorder by means of a PCM recording adapter (Vetter, model 4000). The data were digitized off-line at 20 kHz and analyzed with a microcomputer (Power Macintosh) using specially designed software.

DATA ANALYSIS. Experimental data values are expressed as means ± SE of measurements. The statistical level of significance of the difference between sample means was evaluated using the two-tailed, unpaired or paired Student’s t-test (P < 0.05).

At the end of experiments, the site of recordings was marked with 0.5 µl of a 2% solution of Chicago sky blue dye in 0.5 M Na-acetate. The animal was then killed with an overdose of Nembutal and perfused with saline followed by a solution of 10% formaldehyde.
The mean latency of the hypocretin-induced active sleep was significantly shorter than that observed following the injection of saline (Fig. 3A). Both injections of hypocretin were carried out during quiet sleep. The microinjection of hypocretin-1 immediately induced an active sleep-like state that was indistinguishable from a spontaneous episode of active sleep. A dissociated state was characterized by the presence of muscle atonia, a synchronized EEG, a few ponto-geniculo-occipital (PGO) waves, but no rapid eye movements. EOG, electrooculogram; LGN, lateral geniculate nucleus; EMG, electromyogram. All vertical bars: 100 µV.

FIG. 2. Microinjections into the nucleus pontis oralis (NPO) of hypocretin induced an active sleep-like state or a dissociated state. Representative polygraphic recordings of an episode of active sleep-like state that was induced following an injection of hypocretin-1 (A) and an episode of a dissociated state which occurred following an injection of hypocretin-2 (B). Both injections of hypocretin were carried out during quiet sleep. The microinjection of hypocretin-1 [horizontal bar above electroencephalogram (EEG) of polygraphic recordings] immediately induced an active sleep-like state that was indistinguishable from a spontaneous episode of active sleep (C). A dissociated state occurred with a latency of 3.3 min following the injection of hypocretin-2. This dissociated state was characterized by the presence of muscle atonia, a synchronized EEG, a few ponto-geniculo-occipital (PGO) waves, but no rapid eye movements. EOG, electrooculogram; LGN, lateral geniculate nucleus; EMG, electromyogram. All vertical bars: 100 µV.

The mean latency of the hypocretin-induced active sleep was significantly shorter than that observed following the injection of saline (Fig. 3A; df = 2, F = 18.34, P = 0.0003, ANOVA). In addition, dissociated states were not observed following control injections of saline. These differences between hypocretin injections and saline control injections indicate that the effects of hypocretin injections were produced by pharmacological actions of these drugs and were not due to the microinjection procedures.

The effects of hypocretin on the percentage of time that the cats spent in sleep and waking states during the first hour following the injection of hypocretin are presented in Table 1. Compared to the percentage observed following saline injections, injections of hypocretin-1 significantly increased the time spent in active sleep (64.2%, df = 12, t = 2.72, P = 0.019, unpaired t-test). Injections of hypocretin-2 also significantly increased the time spent in active sleep (47.8%, df = 11, t = 2.30, P = 0.042, unpaired t-test). To determine the effect of injections of hypocretin for a longer period of time, an examination was made of the percentages of time spent in sleep and waking states for a 3-h recording period following each injection. The percentages of time that cats spent in wakefulness, quiet sleep, and active sleep during the 3-h recording period following an injection of hypocretin-1 or hypocretin-2 were not significantly different from those observed following injection of saline. These results indicate that the response to hypocretin was mainly due to changes that occurred during the first hour following the injection.

It is interesting to note that, unlike microinjections of the cholinergic agonist, carbachol, microinjections of hypocretin-1 or hypocretin-2 induced episodes of active sleep that were of a relatively short duration. The mean duration of the active sleep-like episodes induced following the injection of hypocretin-1 or hypocretin-2 was not significantly different from that observed following the injection of saline (Fig. 3B; hypocretin-1: 10.22 ± 0.32 min, n = 7; hypocretin-2: 5.0 ± 1.0 min, n = 6).

FIG. 3. Effects of hypocretin-1 and -2 on the latency of active sleep or dissociated state and the duration of active sleep. A: graph shows latency to the onset of the 1st episode of active sleep or a dissociated state following the injection of hypocretin [hypocretin-1 (500 µM), n = 7; hypocretin-2 (500 µM), n = 6] or saline (n = 7) and during control sessions (no injection, n = 7). Each bar represents the mean latency; error bars indicate the SE of each population. Injections of hypocretin-1 and -2 significantly reduced the mean latency of active sleep. B: graph shows the mean duration of active sleep following injections [hypocretin-1 (500 µM), n = 5; hypocretin-2 (500 µM), n = 4; saline, n = 7] or during control sessions (n = 7). Note that the mean duration of active sleep episodes induced by the injections of hypocretin was not significantly different from that observed following the injection of saline. Asterisks indicate the levels of statistical significance of the difference between means: **P < 0.01.
Hypocretin has a direct excitatory action on NPO neurons

To determine whether the behavioral responses induced by hypocretin were due to direct effects of this substance on neurons in the NPO, intracellular recordings were obtained from 16 neurons in this region while hypocretin-1 was applied, juxtacellulary, to the recorded cells. Recordings in 12 of these neurons in the NPO were maintained long enough for us to examine the effect of hypocretin-1 for more than 5 min. Following the application of hypocretin-1, 9 of these 12 NPO neurons exhibited membrane depolarization (i.e., a reduction in the resting membrane potential) and an increase in the frequency of spontaneous discharge. Figure 5 is a sample record from an NPO neuron, showing the reversible effects of hypocretin on the resting membrane potential and spontaneous spike activity. Hypocretin-1 (100 μM, 15 psi, 30 s) produced a depolarization of 10.2 mV in the resting membrane potential that occurred 3 s after the beginning of the application of hypocretin. The frequency of spontaneous discharge increased from 0.9 spikes/s before the application of hypocretin to 39.6 spikes/s during the application of hypocretin. These effects of hypocretin were dose dependent.

Hypocretin-induced active sleep is dose dependent

Hypocretin-1 was also injected into the NPO at seven different concentrations, ranging from 5 to 500 μM. Figure 4A demonstrates that microinjections of hypocretin-1 into the NPO produced a dose-dependent increase in the percentage of active sleep observed during the first hour following the injection. The percentage of time spent in active sleep increased steadily with increasing concentrations of hypocretin-1 and reached the highest value with a concentration of 500 μM. A positive correlation was found between the dose of hypocretin-1 and the mean percentage of time spent in active sleep during the first post-injection hour (5 μM: 11.5 ± 2.3%, n = 4; 10 μM: 10.3 ± 3.1%, n = 3; 50 μM: 11.0 ± 2.3%, n = 3; 100 μM: 15.2 ± 2.9%, n = 3; 200 μM: 16.1 ± 3.3%, n = 4; 400 μM: 19.2 ± 3.4%, n = 3; 500 μM: 19.7 ± 2.6%, n = 7; r = 0.95, P = 0.029, Spearman rank correlation coefficient).

Increasing the dosage of hypocretin-1 also produced a dose-dependent decrease in the latency to the onset of the first episode of active sleep (Fig. 4B). Injections of the lowest dosages of hypocretin-1 (5 and 10 μM) did not produce a significant decrease in the latency to active sleep (5 μM hypocretin-1: 37.3 ± 4.1 min, n = 4; 10 μM hypocretin-1: 34.0 ± 6.0 min, n = 3; saline: 47.1 ± 7.8 min, n = 7; df = 2, F = 0.77, P = 0.487, ANOVA). However, following the injection of a 50 μM solution of hypocretin-1, the mean latency to active sleep was 12.6 ± 4.6 min (n = 3), which was significantly shorter than that observed following saline injections (df = 8, t = 2.29, P = 0.037, unpaired t-test). The mean latency of active sleep fell sharply following the injection of a 100 μM solution of hypocretin-1. The 500 μM dosage of hypocretin-1 produced the shortest latency to active sleep.

(Data are means ± SE. n are from control, saline, hypocretin-1, and hypocretin-2 recording sessions that were obtained from four adult cats. Hypocretin data were obtained following an injection of 0.25 μl of solution of hypocretin-1 (500 μM in saline) or hypocretin-2 (500 μM in saline). Asterisks indicate the levels of statistical significance between the means: * P < 0.05, F = 3.97, df = 3 and ** P < 0.01, F = 5.74, df = 3, according to the ANOVA test, which was used to compare data from control, saline, hypocretin-1, and hypocretin-2 sessions.)

**TABLE 1.** Effects of maximal dose (500 μM) of hypocretin-1 and -2 injections on sleep and wakefulness during the first hour post-injection recording period

<table>
<thead>
<tr>
<th>Control</th>
<th>Saline</th>
<th>Hypocretin-1</th>
<th>Hypocretin-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>% wakefulness</td>
<td>31.9 ± 3.3</td>
<td>32.7 ± 3.6</td>
<td>25.4 ± 3.0</td>
</tr>
<tr>
<td>% quiet sleep</td>
<td>56.7 ± 3.3</td>
<td>55.3 ± 3.5</td>
<td>46.1 ± 6.1</td>
</tr>
<tr>
<td>% active sleep</td>
<td>11.4 ± 2.3</td>
<td>12.0 ± 1.6</td>
<td>19.7 ± 2.6*</td>
</tr>
<tr>
<td>% dissociated state</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>8.8 ± 5.2**</td>
</tr>
</tbody>
</table>

FIG. 4. Dose-dependent effects of hypocretin-1 on the mean percentage of time spent in active sleep (A) and the latency to the 1st episode of active sleep (B) during the 1st hour after a microinjection. All 7 dose of hypocretin-1 were injected in a saline solution of 0.25 μl. Data were obtained from 27 injections (5 μM, n = 4; 10 μM, n = 3; 50 μM, n = 3; 100 μM, n = 3; 200 μM, n = 4; 400 μM, n = 3; 500 μM n = 7). Each point presents the mean for each dose of hypocretin-1; error bars indicate the SE.

FIG. 5. Hypocretin-induced membrane depolarization and spontaneous activity increase in an NPO neuron. A: Depolarization and increased frequency (39.6 spikes/s) of spontaneous discharges following the systemic administration of 100 μM hypocretin-1 in a saline solution. B: Control time course of active sleep in the same neuron (56.1% of time spent in active sleep during the first hour following the saline injection).
Hypocretin on the resting membrane potential and the frequency of discharge were dose-dependent and usually lasted throughout the period of application; they began to decay and ended within 2 min after the termination of ejection (Fig. 5B).

In all NPO neurons examined, the application of hypocretin-1 (10–12.5 psi, 30 s) produced a depolarization in the resting membrane potential (6.1 ± 1.2 mV, 9 cells) and a significant increase in the mean frequency of discharge (control: 0.7 ± 0.2 spikes/s vs. hypocretin: 20.8 ± 4.4 spikes/s, 9 cells, df = 8, t = 4.73, P = 0.001, paired t-test). The application of hypocretin-1 produced an increase in the excitability of NPO neurons. Figure 6 are sample records showing the voltage response to intracellularly applied depolarizing current pulses before and during the application of hypocretin to a neuron in the NPO. Prior to hypocretin application, this NPO neuron responded to a current pulse with a single action potential. After the application of hypocretin-1 for 20 s, this neuron responded to an identical current pulse with a train of six action potentials. Rheobase (Rh, which is defined as the threshold stimulus intensity of a 50-ms duration intracellular depolarizing current pulse to consistently elicit an action potential and reflects the excitability of a neuron) was significantly reduced by 43.8% from 1.6 ± 0.4 nA before to 0.9 ± 0.3 nA during the application of hypocretin (7 cells, df = 6, t = 3.54, P = 0.012, paired t-test). These data suggest that hypocretin acts directly on NPO neurons as an excitatory neurotransmitter.

**DISCUSSION**

The present study demonstrated that the application of hypocretin-1, as well as hypocretin-2, within the NPO results in the generation of active sleep with a short latency. In addition, dissociated states, which were characterized by the presence of muscle atonia, but not all of the other electrophysiological correlates of active sleep, were also induced following the application of hypocretin-1. The resting membrane potential before the application of hypocretin-1 was −57.0 mV (---); it depolarized by 10.2 mV after the application of hypocretin-1 for 30 s (100 μM, 15 psi, 30 s; A). Note that the frequency of spontaneous discharge greatly increased following the application of hypocretin in conjunction with depolarization in the membrane potential. The latency to the onset of hypocretin-induced effects was approximately 3 s. Increasing the intensity of the pressure pulse (from 7.5 to 15 psi) used to eject hypocretin produced a dose-dependent increase in the frequency of spontaneous discharge of this NPO neuron (B).

**FIG. 5.** The application of hypocretin produced a depolarization of the resting membrane potential and an increase in the frequency of discharge in this representative NPO neuron, which was recorded intracellularly. The resting membrane potential before the application of hypocretin-1 was −57.0 mV (---); it depolarized by 10.2 mV after the application of hypocretin-1 for 30 s (100 μM, 15 psi, 30 s; A). Note that the frequency of spontaneous discharge greatly increased following the application of hypocretin in conjunction with depolarization in the membrane potential. The latency to the onset of hypocretin-induced effects was approximately 3 s. Increasing the intensity of the pressure pulse (from 7.5 to 15 psi) used to eject hypocretin produced a dose-dependent increase in the frequency of spontaneous discharge of this NPO neuron (B).

**FIG. 6.** The juxtacellular application of hypocretin-1 produced an increase in the excitability of an intracellularly recorded NPO neuron. A: this representative NPO neuron responded to an intracellular applied current pulse (bottom) with a single action potential during control conditions (prior to hypocretin application). The magnitude of current injection was 1.6 nA, which was the rheobase of this neuron (resting membrane potential: −60.5 mV). B: after 20 s of hypocretin-1 application, this neuron responded to a current pulse of the same magnitude and duration with a train of 6 action potentials (hypocretin: 100 μM, 12.5 psi, 30 s; resting membrane potential: −53.0 mV).
shown that hypocretin-containing fibers are presented within the NPO (Chenelli et al. 1999; Peyron et al. 1998). Both hypocretin-1 and -2 receptors have also been identified in the same area of the rat (Greco and Shiromani 2001; Hervieu et al. 2001; Marcus et al. 2001). In particular, Zhang et al. (J.-H. Zhang, S. Sampogna, F. R. Morales, and M. H. Chase, unpublished data) in our laboratory have found that hypocretin immunopositive fibers lie in close apposition to neuronal perikarya in the NPO of the cat and that both hypocretin-1 and -2 receptors are distributed in this region of the cat brain stem.

In support of the preceding anatomical data, our electrophysiological results present the first in vivo electrophysiological description of the cellular actions of hypocretin on neurons of the NPO, indicating that hypocretin influences the activity of NPO neurons. The present results are also consistent with data from recent electrophysiological studies that show that hypocretin has an excitatory action on cells in the hypothalamus, locus coeruleus, and spinal cord (Bourgin et al. 2000; de Lecea et al. 1998; Hagan et al. 1999; Horvath et al. 1999; van den Pol 1999). However, using whole cell patch recording and calcium-imaging techniques, van den Pol et al. (1998) demonstrated that hypocretin increases Ca\(^{2+}\) influx via a G-protein-mediated enhancement in hypothalamic cells, resulting in postsynaptic excitatory effects and that hypocretin also has a neuromodulatory effect at presynaptic receptors by acting on both glutamate- and GABA-releasing axon terminals in the hypothalamus. An excitatory effect on locus coeruleus neurons has been attributed to a reduction of K\(^{+}\) conductance (Horvath et al. 1999). Further study will be needed to determine the precise mechanisms of the excitatory actions of hypocretin on NPO neurons.

The present data provide additional support for the hypothesis that the NPO is one of the control sites of action for the hypocretinergic system with respect to the regulation of active sleep (see reviews of Chase and Morales 1990, 2000; Jones 1991; Siegel 2000; Steriade and McCleary 1990). Specifically, anatomical evidence has shown that the NPO receives cholinergic innervation from the LDT/PPT (Mitani et al. 1988; Shiromani et al. 1988). Microinjections of cholinergic agonists, GABAergic antagonists, and nerve growth factor into the NPO reliably induce an active sleep-like state that is indistinguishable from naturally occurring active sleep (Baghdoyan et al. 1984, 1989, 1993; George et al. 1964; Xi et al. 1999b, 2001a; Yamamoto et al. 1990; Yamuy et al. 1993, 1995). In addition, neurons in the NPO increase their discharge rate during active sleep (McCarley and Hobson 1971; McCarley et al. 1995). The NPO neurons are, therefore most likely effector cells for many active sleep phenomena, including the initiation of PGO waves and rapid eye movements. In particular, NPO neurons are a key component of the brain stem-spinal cord inhibitory system that is responsible for the muscle atonia during active sleep. They are also part of the “neuronal gate” that converts excitatory drives to motoneurons into a powerful inhibitory input during active sleep, which is the basis for the phenomenon of reticular response-reversal (Chase and Morales 1990, 2000). Because the present electrophysiological data clearly demonstrate that hypocretin excites neurons of the NPO, we suggest that the change in behavioral state induced by the injection of hypocretin into the NPO results from the effects of this substance on the same population of NPO neurons that is involved in the generation of active sleep and the epiphenomena which comprise this state.

Recently, Thakkar et al. (1999) examined the effects on behavioral states of microperfusion of hypocretin-2 receptor antisense into the pontine reticular formation of rats. Interestingly, they found that hypocretin-2 receptor antisense perfusion increases active sleep and also produces cataplexy. While their data, with respect to the production of cataplexy (but not active sleep), appear to contradict the conclusions that we have drawn from our results, the discrepancy may be due to anatomical differences between species (rat vs. cat) and sites of investigations (the ventral part of the pontine reticular formation versus the NPO). On the other hand, a recent study by Kiyashchenko et al. (2001) has shown that the microinjection of hypocretin into the pontine reticular formation (at a site just ventral to the LC) produced motor inhibition in decerebrated rats, a result that is consistent with our observation that the microinjection of hypocretin into the NPO elicits an active sleep-like state.

The present findings have led us to hypothesize that the hypocretinergic system acts by exciting, through hypocretinergic synaptic contacts on NPO neurons, the brain stem-spinal cord inhibitory system that is responsible for the glycinergic inhibition of motoneurons during active sleep. Therefore in the absence or the reduction of hypocretinergic excitatory drives on neurons of the NPO, we would hypothesize that a decrease in motor inhibition would be expected to occur during active sleep in narcoleptics. This hypothesis is supported by a large body of data demonstrating that narcoleptic humans and canines exhibit a decrease in the degree of motor inhibition that occurs during active (REM) sleep. Individuals with narcolepsy also have more frequent arousals than control subjects (Browman et al. 1986) and a large number of narcoleptic patients have periodic movements in sleep (PMS) (Wittig et al. 1983). In fact, the occurrence of nocturnal myoclonus accounts for longer night-time REM latency in patients with narcolepsy (Mosko et al. 1984). Disrupted night-time sleep with frequent awakenings and increased body movements are common in patients with narcolepsy and, in some, may be a prominent complaint (Aldrich 1992). A relative lack of muscle inhibition during cataleptic attacks has also been observed in narcoleptic dogs (Miller and Dement 1977). In addition, there is very poor motor suppression during active sleep in narcoleptic dogs (J. M. Siegel, personal communication).

We therefore believe that when pathological circumstances arise and active sleep-related motor disorders occur, such as narcolepsy, and when hypocretin is not present or is present but in a greatly reduced concentration (Nishino et al. 2000), and/or there is malfunction of hypocretin receptors (Lin et al. 1999), there is a corresponding reduction in the activity of NPO neurons. This decrease in the activity of NPO neurons, which
are a key component of the inhibitory system that mediates atonia during active sleep, results in a “lessening” or reduction in the degree of motor inhibition during this state. This results in a decrease in the degree of atonia that normally occurs during active sleep in narcoleptics. To recapitulate, this decrease in atonia, we suggest, is due to the withdrawal of powerful hypocretinergic excitatory drives that normally excite neurons in the NPO during active sleep. On the other hand, when hypocretin is injected into the NPO, active sleep occurs, which is a state composed of a confluence of specific patterns of activity of a number of physiological systems, including motor inhibition, REMs, and PGO waves.

The present data also highlight the importance of site specificity for understanding the actions of hypocretin within the brain stem. Hypocretin clearly has different effects on the regulation of active sleep and wakefulness that depend on which brain stem nucleus receives the peptide. For example, Bourgin et al. (2000) reported that the injection of hypocretin-1 into the LC suppresses active sleep and increases wakefulness, and we recently observed the same effects following the injection of hypocretin-1 into the LDT (Xi et al. 2001b). However, in the present study, we have observed exactly the opposite effect when hypocretin was applied into the NPO. Thus it appears that the hypocretinergic system exerts dual functions that are state dependent, i.e., hypocretin acts at different levels of the neuraxis not only to promote wakefulness and patterns of motor activation that occur during this state but also to generate active sleep and its accompanying pattern of motor inhibition.

It is interesting to note that in the present study, microinjection of hypocretin into the NPO also induced a dissociated state, which was characterized by the presence of muscle atonia but not all of the other components of active sleep. Although we do not know exactly why injections of hypocretin in some experimental sessions induced dissociated states instead of all of the components of active sleep, it is possible that those injections were not located at the most efficacious site in the NPO to induce active sleep. Further studies will be needed to determine the mechanisms that are responsible for the induction of a dissociated state.

In conclusion, the present findings demonstrate that active sleep as well as muscle atonia can be induced with a short latency following the microinjection of hypocretin-1 or -2 into the NPO and that the juxtacellular application of hypocretin-1 produces an increase in excitability and discharge of NPO neurons. These data suggest that hypocretin plays a key role in the mechanisms that are resident within the NPO that generate active sleep and its accompanying pattern of muscle atonia.

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