Distribution of Hypocretin-Containing Neurons in the Lateral Hypothalamus and c-Fos-Immunoreactive Neurons in the VLPO

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The present study investigated the distribution of neurons implicated in the regulation of sleep in three species generally used in sleep research, i.e., mice, rats and cats. We focused on sleep active neurons in the ventral lateral preoptic (VLPO) area and the hypocretin/orexin-containing neurons in the lateral hypothalamus. The latter groups of neurons were found recently to play an important role in the regulation of REM sleep. The expression of the transcription factor, c-Fos, was used to identify the VLPO. In mice and rats, in response to sleep, a discrete cluster of c-Fos positive cells was found in the VLPO. In mice, this cluster was located more medially compared to the rat, and as in the rat, galanin immunostained neurons were found in the VLPO. In the cat, c-Fos positive cells did not segregate to a specific location but were more diffusely represented in the preoptic area. In all three species, orexin/hypocretin-containing neurons were located only in the lateral hypothalamus with the distribution being more diffuse in the cat. The grouping of sleep-active cells in rodents makes it feasible to extract these cells for tissue culture and molecular analysis. Moreover, given that rodents have a distinct circadian distribution of sleep-wakefulness, the connectivity with the suprachiasmatic nucleus can also be determined.

CURRENT CLAIM: The VLPO is a discrete cluster in rodents but is diffuse in cats.

Sleep is composed of slow-wave sleep (SWS) and REM sleep. SWS precedes REM sleep and occupies roughly 80% of total time spent asleep. Much converging evidence indicates that hypothalamic mechanisms are responsible for the initiation of SWS whereas REM sleep originates from the pontine brainstem (Shiromani, 1998). Recent reports have now further identified the specific neuronal populations, neurotransmitters and receptors involved in regulating SWS and REM sleep. For instance, a group of cells in the ventral lateral preoptic (VLPO) that is important for SWS have been identified. Neurons in the VLPO are active during sleep (both SWS and REM sleep), as determined by electrophysiology (Szymusiak et al., 1998) and c-Fos studies (Sherin et al., 1996), and they project to the monoaminergic neurons implicated in waking (Sherin et al., 1996, 1998). The VLPO neurons contain galanin and GABA (Sherin et al., 1998) and are hypothesized to initiate sleep via their inhibitory influence on waking neurons (Shiromani et al., 1998). Lesions of the VLPO produce a long-lasting and severe insomnia (Lu et al., 2000).

Neurons important for REM sleep onset have been identified to be located in the pontine tegmentum. Recently, our understanding of the mechanisms regulating REM sleep was further advanced with the finding that a mutation in the hypocretin (orexin) 2 receptor gene was associated with canine narcolepsy (Lin et al., 1999). Mice lacking the hypocretin (orexin) peptide were found to display narcoleptic-like behavior (Chemelli et al., 1999), further reinforcing the role of this peptidergic system in REM sleep. The hypocretin (orexin) containing neurons are localized only in the lateral hypothalamus (Nambu et al., 1999; Peyron et al., 1998) and project to major arousal centers in the brainstem and forebrain (Peyron et al., 1998). The projections to the pons, especially the locus coeruleus and dorsal raphe, are noteworthy given the extensive research implicating these two pontine neuronal populations in REM sleep generation.

In the present study, we identify the distribution of neurons implicated in sleep in three species that have generally been used in sleep research, i.e., mice, rats and cats. We hypothesized that in mice, as has been found in rats, c-Fos immunoreactive neurons will be found in the preoptic area. In support of our hypothesis, we found in mice, a discrete cluster of c-Fos positive cells in a region that also contained galanin immunostained neurons. In the cat, c-Fos positive cells did not segregate to a specific location but were more diffusely represented in the preoptic area. In all three species, orexin/hypocretin-containing neurons were located only in the lateral hypothalamus with the distribution being more diffuse in the cat. The grouping of sleep-active cells in rodents makes it feasible to extract these cells for tissue culture and molecular analysis. Moreover, given that rodents have a distinct circadian distribution of sleep-wakefulness, the connectivity with the suprachiasmatic nucleus can also be determined.

METHODS

Animals

Sixteen male mice (~3 months old, 25-30 grams, Jackson Lab, C57BL/6j) were implanted under anesthesia (acepromazine,
ketamine and xylazine, IM) with chronically indwelling sleep recording electrodes. Two cortical screw electrodes (one frontal cortex and the second in the contralateral parietal cortex) recorded the electroencephalogram, and two flexible multistranded wires in the nuchal muscles recorded the muscle activity. All electrodes were inserted into an amphenol plug and secured onto the skull with dental cement. The animals were housed singly in plastic cages with wood-shavings; food and water were available ad libitum. The temperature in the room was 25°C and an entrained light-dark cycle was maintained (0700-1900 h, lights on; 75 lux). For visualization of galanin-containing cells, two mice were given colchicine (ICV, 2 ug/1 ul; stereotaxic surgery under anesthesia), and 24 h later perfused and the brains extracted for immunohistochemistry.

**Prolonged Wakefulness/Recovery Sleep Protocol**

The mice were allowed at least two weeks to recover from surgery followed by a three day adaptation to the recording environment. The mice were kept awake for three hours (1000-1300 h) by lightly tapping the cage when the mice showed EEG or behavioral signs of sleep. At 1300 h some of the mice (n=9) were killed, while the remaining animals (n=7) were permitted sleep for at least 2 h and then killed. EEG/EMG recordings were obtained throughout the period of enforced wakefulness and sleep periods. The mice were sacrificed by deeply anesthetizing with Nembutal (200 mg/kg) followed by transcardial perfusion with 0.9% saline (20 ml) and 4% formaldehyde in 0.1M PBS (~40 ml). The brains were then placed in 20% sucrose (4°C) until they sank.

**Analysis of EEG/EMG Recordings**

The EEG/EMG signals were recorded on a Grass model 9 polygraph and stored on a computer using the ICELUS program (M. Opp, UTMB, Texas). The EEG/EMG recordings were scored manually in 12 second epochs for waking, slow wave sleep (SWS) and rapid eye movement (REM) sleep. Wakefulness was identified by the presence of desynchronized EEG and high EMG activity. SWS consisted of high amplitude slow waves and a low EMG activity relative to waking. REM sleep was identified by the presence of regular theta activity coupled with low EMG activity relative to SWS. The EEG data were analyzed for changes in the percentage of total sleep time (TST, the sum of the time spent in SWS and REM sleep). Each mouse was designated by letter; the analysis was therefore blind.

**Immunohistochemistry**

The brains were cut on a sliding microtome (30 um, coronal sections). Tissue sections were processed for c-Fos immunohistochemistry, using rabbit anti-c-Fos (AB 5; 1:100,000 dilution; Oncogene Sci, NY), rabbit anti-galanin (1:1000; Chemicon), or rabbit anti-orexin A (Chemicon, 1:20,000), an avidin-biotin secondary antibody and an avidin-biotinylated peroxidase complex. The peroxidase reaction was developed with 3,3’ diaminobenzidine (DAB) and hydrogen peroxide as the chromagen (Vector Laboratories, CA). C-Fos positive nuclei are black as a result of the nickel chloride intensifier. Prior to analysis, concentrations of primary antibodies were optimized to permit clear visualization of each product. The drawings of labeled somata were made using a camera-lucida drawing program (Neurolucida, Vermont).

**Quantification of Immunoreactive Neurons in the VLPO**

In mice, bilateral counts were obtained from a one-in-four series of sections, and at least three sections bridging the middle portion of the VLPO were counted. Briefly, the VLPO was identified in the coronal plane beginning at the rostral pole of the supraoptic nucleus and extending rostrally until the decussation of the anterior commissure. C-Fos-immunoreactive cells within an area 350 um from the midline and extending 150 um laterally, and 100 um dorsally were counted. Counts were obtained bilaterally from at least three sections (in a one-in-four series) and the numbers represent the average counts from one section from one side. All cells were counted using a Leitz microscope (magnification=10x). A multiple linear regression was used to assess the number of single-labeled cells as a function of percent total sleep time during the 1 h period before sacrifice.

**Rats**

To compare the location of the VLPO in rats with the location in mice, we examined tissue from young (3.5 months) Sprague-Dawley rats (n=10) that had been processed for visualization of c-Fos immunohistochemistry in previous studies (Shiromani et al., 2000). Briefly, in that study young rats were implanted under anesthesia (acepromazine, ketamine and xylazine, IM) with electrodes to record sleep. Two weeks after recovery from surgery the rats were adapted to the recording environment for three days. Subsequently, the rats were kept awake for 12 h (1300-1900 h), and then some rats were killed (n=3) (Nembutal overdose, IP) whereas other rats were allowed 1 h of sleep (n=7) and then killed. The brains were perfused and removed for histological examination.

For comparison of the distribution of the orexin-containing cells, two Sprague-Dawley rats were killed (by overdose of Nembutal, IP) and then perfused. The brains were extracted for immunohistochemistry. For visualization of galanin-containing cells, two rats were given colchicine (ICV, 5 ug/5 ul; stereotaxic surgery under anesthesia), and 24 h later perfused and the brains extracted for immunohistochemistry.

**Cats**

To identify the location of the VLPO in cats, slides from previous studies (Shiromani et al., 1994) were examined. To identify orexin-containing cells in the cat, frozen tissue from previous studies was used. The tissue had been placed in cryoprotectorant solution and stored at –80°C. Tissue of the brains from two cats that was previously frozen was used.
RESULTS

C-Fos Labeled Cells in VLPO

Mice that were killed at the end of the 3 h period of wakefulness (n=9) had numerous c-Fos containing cells throughout the brain compared to mice that were killed following sleep (n=7). The sleep-induced reduction in c-Fos has been reported previously by others (Grassi-Zucconi et al., 1994; Cirelli et al., 1993) and us (Basheer et al., 1997). In the VLPO, there was a significant increase in the number of c-Fos positive cells in animals that were asleep (n=7) compared to animals that were awake (n=9) (mean number of cells in the VLPO in awake mice=3.9±0.86; mean number in asleep mice=12.34±1.9; independent t-test=4.25; df=14; p<0.0001). There was also a significant relationship between total sleep time and the number of c-Fos labeled cells in the VLPO (r=0.78; df=15; p<0.001) (see Figure 1). Figure 1 also has schematic figures depicting the location of c-Fos labeled cells in representative awake and asleep mice. Photomicrographs of c-Fos labeled cells are presented in Figure 2. Figure 2C shows that the c-Fos positive cells in asleep animals corresponds with the location of galanin-positive cells, thus indicating that this region represents the VLPO in mice.

When we compared the location of the VLPO in mice with the location in rats (Figure 3), we noticed that in mice, the VLPO (c-Fos and/or galanin cells) is more medial compared to the rat. Moreover, in both species there is a clear clustering of c-Fos labeled cells in the region that also contains galanin cells. In rats, many of the c-Fos immunoreactive cells in the VLPO also contain galanin and project to the tuberomammillary nucleus (Sherin et al., 1998).

We then proceeded to determine whether in the cat, a cluster of cells analogous to the VLPO might also be found. However, as depicted in Figure 4, we cannot identify a collection of c-Fos labeled cells in the cat in response to sleep even after 24 h prolonged wakefulness (n=3) followed by 1 h sleep (n=5) (Shiromani et al., 1994; Shiromani, 1998). We have not administered colchicine to cats to determine the distribution of galanin-positive cells in the cat, but based on our observations that the c-Fos labeled cells are diffusely represented, we anticipate the distribution of galanin-labeled cells will likewise be diffuse and scattered.

Orexin/Hypocretin Containing Cells

The distribution of orexin-immunoreactive cells are depicted in Figure 5 (for mice), Figure 6 (rats) and Figure 7 (cats). Previous reports have detailed the distribution of these neurons in mice (3) and rats (9, 10). We now identify these neurons in cats. To facilitate comparison, we have also included our results in mice and rats. In all three species, orexin-containing cells were found only in the lateral hypothalamus. The distribution of these cells ranged from the perifornical area of the hypothalamus, medially into the dorsomedial hypothalamic nucleus, and laterally into the lateral hypothalamic nucleus. In cats, the distribution of these neurons was diffuse with neurons extending medially to the third ventricle and ventrally to the arcuate nucleus. Proper stereometric measurements are required to identify the actual numbers of neurons in the three species.

Figure 1. With sleep, there is an increase in the number of c-Fos labeled cells in the ventral lateral preoptic (VLPO) area in mice. The top panel indicates a statistically significant relationship (r=0.78; p<0.001; df=15) between the number of c-Fos labeled cells in the VLPO and the amount of sleep during the 1 h period before sacrifice. The middle and bottom panels depict a coronal section through the mouse brain at the level of the anterior commissure (ac) and identify c-Fos labeled cells (black dots) in representative animals that were killed after being awake (middle panel) or asleep (bottom panel). In awake animals there are numerous c-Fos labeled cells in many regions of the brain, except in the VLPO (identified by arrow), where c-Fos labeled cells are seen only in response to sleep. Abbreviations: ac=anterior commissure; CAU=caudate nucleus; oc=optic chiasm; VLPO=ventral lateral preoptic nucleus.
Figure 2. Photomicrographs depict c-Fos labeled cells in the preoptic area of mice that were awake (Panel A) or asleep (Panel B). Panel C depicts galanin-immunoreactive neurons. Note that increased numbers of c-Fos labeled cells in animals that are asleep identifies the ventral lateral preoptic nucleus (VLPO). We suggest that this represents the VLPO since galanin-immunoreactive cells are also present in this region (Panel C). The VLPO in rats is slightly more lateral (see Figure 3), but as in mice it is found at the level of the decussation of the anterior commissure. Black bar=100 um; oc=optic chiasm.

Figure 3. Photomicrographs depict c-Fos labeled cells in the preoptic area of rats that were awake (Panel A) or asleep (Panel B). Panel C identifies galanin-immunoreactive cells in the same region. In the rat, the VLPO is identified by the presence of c-Fos labeled cells in asleep rats and where galanin-immunoreactive cells are also present. Black bar=100 um.

Figure 4. Photomicrograph depicts c-Fos labeled cells in the preoptic area of cats that were awake (Panel A) or asleep (Panel B). Many more c-Fos labeled cells are evident in awake cats compared to asleep cats, a finding which is consistent with data in mice (see Figure 2A, and Basheer et al., 1997) and rats (see Figure 3A). However, unlike rodents, in cats in association with sleep a cluster of c-Fos cells denoting the VLPO is not evident, but instead the few c-Fos labeled cells that are present are diffusely represented. Black bar=100 um.
Figure 5. Distribution of orexin/hypocretin-immunoreactive neurons in the lateral hypothalamus of mice. Black dots denote orexin/hypocretin-immunoreactive somata. The figures are camera lucida drawings of coronal sections (30 μm thick) through the mouse posterior hypothalamus (bregma –1.34 to –2.06 according to Franklin and Paxinos, 1997). Abbreviations: Arc=arcuate nucleus; BLA=basolateral nucleus of the amygdala; DG=dentate gyrus; DM=dorsomedial nucleus of the thalamus; LH=lateral hypothalamus; MHb=medial habenula; mt=mammillothalamic tract; PeF=perifornial nucleus; Rt=reticular thalamic nucleus; VMH=ventral medial hypothalamus; ZI=zona incerta.

Figure 6. Distribution of orexin/hypocretin-immunoreactive neurons in the lateral hypothalamus of rats. Schematic sections from the rat atlas of Paxinos and Watson identify the location of the camera lucida drawings relative to bregma. Black dots denote orexin/hypocretin-immunoreactive somata. The figures are camera lucida drawings of coronal sections (30 μm thick) through the rat posterior hypothalamus. Abbreviations: Arc=arcuate nucleus; BLA=basolateral nucleus of the amygdala; DG=dentate gyrus; DM=dorsomedial nucleus of the thalamus; ic=internal capsule; LH=lateral hypothalamus; MHb=medial habenula; mt=mammillothalamic tract; PeF=perifornial nucleus; PV=paraventricular nucleus of the thalamus; Rt=reticular thalamic nucleus; VMH=ventral medial hypothalamus; ZI=zona incerta.
DISCUSSION

The principal finding of this study was that in mice, in response to sleep, c-Fos labeled cells are present in a region that also contains galanin-positive cells. We conclude that this cluster of c-Fos/galanin labeled cells represents the VLPO, a region with c-Fos immunoreactivity first related to sleep in rats (Sherin et al., 1996). This cluster in mice is slightly more medially located compared to the rat but, as in the rat, it is found at the level of the decussation of the anterior commissure, anterior to the suprachiasmatic nucleus and the supraoptic nucleus. When this preoptic area is examined in cats that were asleep (n=5), we identified scattered c-Fos labeled cells which cannot be grouped into a cluster. In awake cats (n=3), this portion of the brain, like most other brain areas, contains more c-Fos labeled cells. Thus, in rodents, the VLPO segregates as a discrete collection of neurons, but in the cat the VLPO is more diffuse. The grouping of these sleep-active cells in rodents makes it feasible to extract these cells for tissue culture and molecular analysis. Moreover, the grouping in rodents facilitates connections to other brain regions such as the suprachiasmatic nucleus (SCN), as was done recently by Novak and Nunez (2000).

We examined the distribution of orexin-containing neurons because of the finding that canine narcolepsy is associated with a mutation in the orexin (hypocretin-2) receptor (Lin et al., 1999). The distribution of orexin-containing neurons has been made in mice, rats and humans (Peyron et al., 1998; Sakurai et al., 1998; De Lecea et al., 1998; Elias et al., 1998; Nambu et al., 1999). We have now shown that in the cat, orexin-immunoreactive neurons are also located in the lateral hypothalamus. Because of the location of the orexin neurons in a region that has been implicated in feeding, this neuropeptide has been implicated in controlling appetite and energy metabolism. Application of orexin stimulates feeding (Dube et al., 1999). Two orexin receptors have been identified and the distribution of the receptor mRNA levels has been determined (Trivedi et al., 1998). Hypocretin-1/orexin-1 receptor mRNA is more abundant in ventromedial hypothalamic nucleus, hippocampal formation, dorsal raphe and locus coeruleus. In the rat, hypocretin-2/orexin-2 receptor mRNA is mainly expressed in cerebral cortex, nucleus accumbens, subthalamic and paraventricular thalamic nuclei, and posterior pretemporal nuclei (Trivedi et al., 1998; Date et al., 1999). The locus coeruleus receives the heaviest projection of orexin-containing fibers, and intraventricular administration of orexin A or hypocretin-2/orexin B excites LC neurons (Horvath et al., 1999; Hagan et al., 1999). Orexin-containing terminals are also found in areas implicated in wakefulness such as the tuberomammillary nucleus (TMN), the dorsal raphe, and the basal forebrain (Peyron et al., 1998). Because of these projections to neuronal populations implicated in wakefulness, some have proposed that orexin promotes wakefulness (Peyron et al., 1998).

A comparative analysis of the distribution of the orexin/hypocretin and the VLPO neurons should be undertaken in other species, considering that sleep occurs in virtually all mammals and birds that have been examined. In those animals

Figure 7. Distribution of orexin/hypocretin-immunoreactive somata in the hypothalamus of cats. The figures represent camera lucida drawings of the posterior hypothalamus in cats. The figures represent an area that spans 10.2 to 12.6 mm anterior to the interaural line (based on Berman’s atlas of the cat forebrain). In contrast to the rats and mice, the cells extended medially to the third ventricle and ventrally to the median eminence. Abbreviations: F=fornix; ic=internal capsule; ot=olfactory tubercle; SOC=supraoptic chiasm; VMH=ventral medial hypothalamus; 3V=third ventricle.
that have been investigated for signs of sleep, one finds that the changes in EEG, behavioral quiescence and the alternation between wake to non-REM to REM sleep are quite similar across mammals and birds. Moreover, disruption of sleep leads to increased sleepiness, and there is an increase in sleep when the animal has an opportunity to sleep (Borbely, 1994). Cholinergic stimulation increases REM sleep in rats, cats, dogs and humans (Shiromani, 1998). There is a rapid decline in c-Fos with sleep in mice (Basheer et al., 1997), rats (Grassi-Zucconi et al., 1994; Cirelli et al., 1993) and cats (Shiromani et al., 1994), perhaps indicating a similar process of protein degradation across species (Basheer et al., 1997). Electrophysiology studies have identified sleep-active neurons in the same regions of the brain in rats, cats, dogs and monkeys (for review see Szymusiak, 1995; Szymusiak et al., 1998). C-Fos labeled cells also indicate that sleep-active cells are located in the VLPO in mice (this study), rats (Sherin et al., 1996) and degus (Novak et al., 1999). Sleep active cells, based on electrophysiology (Szymusiak and McGinty, 1986) (see also Szymusiak, 1995, for review) and c-Fos labeling, are also located in this region in the cat, but they are more diffusely represented compared to rodents. There is now clear evidence that there are similarities in the influence of specific genes on sleep across species. For instance, a defect in the orexin system is associated with cataplexy in both dogs (Lin et al., 1999) and mice (Chemelli et al., 1999). Recently, Tafti et al., 1997, identified different loci for REM sleep amounts, which also indicates the contribution of specific genes to REM sleep. These findings suggest that at the genetic level there is also a conservation of mechanism underlying sleep across species.

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