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THE HYPOCRETIN NEURON SYSTEM: AN AROUSAL SYSTEM IN THE HUMAN BRAIN

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INTRODUCTION

Hypothalamic participation in behavioral state regulation has been recognized since early demonstrations of diminished sleep after anterior hypothalamic lesions and increased sleep after posterior hypothalamic lesions (cf. ref. 7 for review). At one level, these observations imply that there is a sleep-promoting region in the anterior hypothalamus, with the ventral lateral preoptic area now the principal candidate (19), and a wake-promoting, or arousal, system in the posterior hypothalamus. A hypothalamic arousal system could act in two ways, through projections into other subcortical systems which enhance cortical activity or by direct activating projections to the cerebral cortex. Both of these mechanisms probably participate in promoting wakefulness, but recent data have emphasized the magnitude of direct hypothalamocortical projections (15, 17, 18).

Importantly, very recent data indicate that posterior and lateral hypothalamic neurons producing a newly-discovered peptide class, the hypocretins (orexins), project widely over the neuraxis in the rat (1, 3, 4, 11, 14, 16, 20). Neurons producing hypocretins are found exclusively in the posterior and lateral hypothalamus and project both widely to cerebral cortex and to a series of subcortical areas, brainstem cholinergic nuclei, locus coeruleus, midbrain raphe nuclei, midline thalamus and nucleus basalis, thought to be involved in arousal (11). The significance of the hypocretin system is emphasized by the discovery in the last year of a defect in the hypocretin 2 receptor in canine narcolepsy (9), the production of a murine model of narcolepsy by removal of the hypocretin gene (2) and the observation that CSF hypocretin content is markedly reduced in most narcoleptic patients (13), further suggesting a role for this system in arousal and the maintenance of waking behavior. In this study, we examined the hypocretin neuron system in the human brain to test the hypothesis that it is organized in a manner similar to that in rodents to serve as a substrate for behavioral arousal.

METHODS

Human brains.

Human brain material was obtained from routine autopsies performed at the University of Pittsburgh Medical Center. Brains of both sexes from adults, ages 27-74, without known

neurological disease, were removed after a postmortem interval of 4-24 hours, fixed for 14 days in buffered 4% paraformaldehyde (pH 7.4) and dissected into blocks which were placed into graded sucrose solution prior to sectioning for immunocytochemistry. Brain tissue was obtained under a protocol approved by the University of Pittsburgh Institutional Review Board.

Immunocytochemistry.

Tissue blocks containing hypothalamus, basal forebrain, midline thalamus, prefrontal cortex (area 46), anterior cingulate cortex (area 24), primary motor (area 4) and sensory cortex (areas 3, 1, 2), cortex of the superior temporal gyrus (area 22), visual cortex (area 17), midbrain raphe nuclei and locus coeruleus were cut at 30 μm in the coronal plane on a freezing microtome and collected in phosphate buffered saline (pH 7.4). Sections were processed for immunocytochemistry using a polyclonal antiserum raised in rabbit against a synthetic hypocretin 17 mer (3) with the ABC technique (6), as described previously (10). Sections were analyzed with an Olympus Vanox microscope and images were captured using a SPOT camera (Diagnostic Images, Inc.). Images have been manipulated to maximize sharpness and contrast and were printed using the camera software and NIH Image.

RESULTS

Perikarya.

Perikarya exhibiting hypocretin (HYP+) immunoreactivity are found exclusively in the perifornical area and posterior hypothalamus of the human brain (Figure 1 C-G). In a rostrocaudal sequence, the first HYP+ perikarya appear just ventral to the fornix at the level of the posterior part of the ventromedial nucleus. In subsequent sections, the number of HYP+ perikarya increases markedly and the group extends laterally into the lateral hypothalamic area. At its greatest extent, the HYP+ neuron population forms a dense band of cells extending medially, dorsally and laterally around the fornix. The majority of HYP+ neurons at this level are located just dorsomedial to the fornix in the posterior hypothalamic area. At this level, a small number of HYP+ neurons are also observed in the dorsomedial hypothalamic nucleus and very rarely in the periventricular zone. In addition, HYP+ neurons are observed in a region dorsolateral to the fornix and ventral to the zona incerta. Caudally, the HYP+ neuron population narrows, is bisected by the mammillothalamic tract and ends at the level of the mid-portion of the medial mammillary nucleus. Overall, HYP+ neuron perikarya extend in a rostrocaudal direction over approximately 8.5 mm of the hypothalamus. Photomicrographs of hypocretin neurons are shown in Figure 2.

Fig. 1 - *Schematics of hypocretin-immunoreactive (HYP+) fibers and cell bodies in the human hypothalamus, from rostral (A) to caudal (G).*

Note that these drawings are intended to illustrate the location and relative densities of immunoreactive structures. In actuality, the numbers of cells and fibers are much greater (cf. Fig. 2). The numbers above each level indicate the distance (in mm) from the middle of the anterior commissure. Axons are shown as irregular wavy lines and HYP+ neurons as dots.

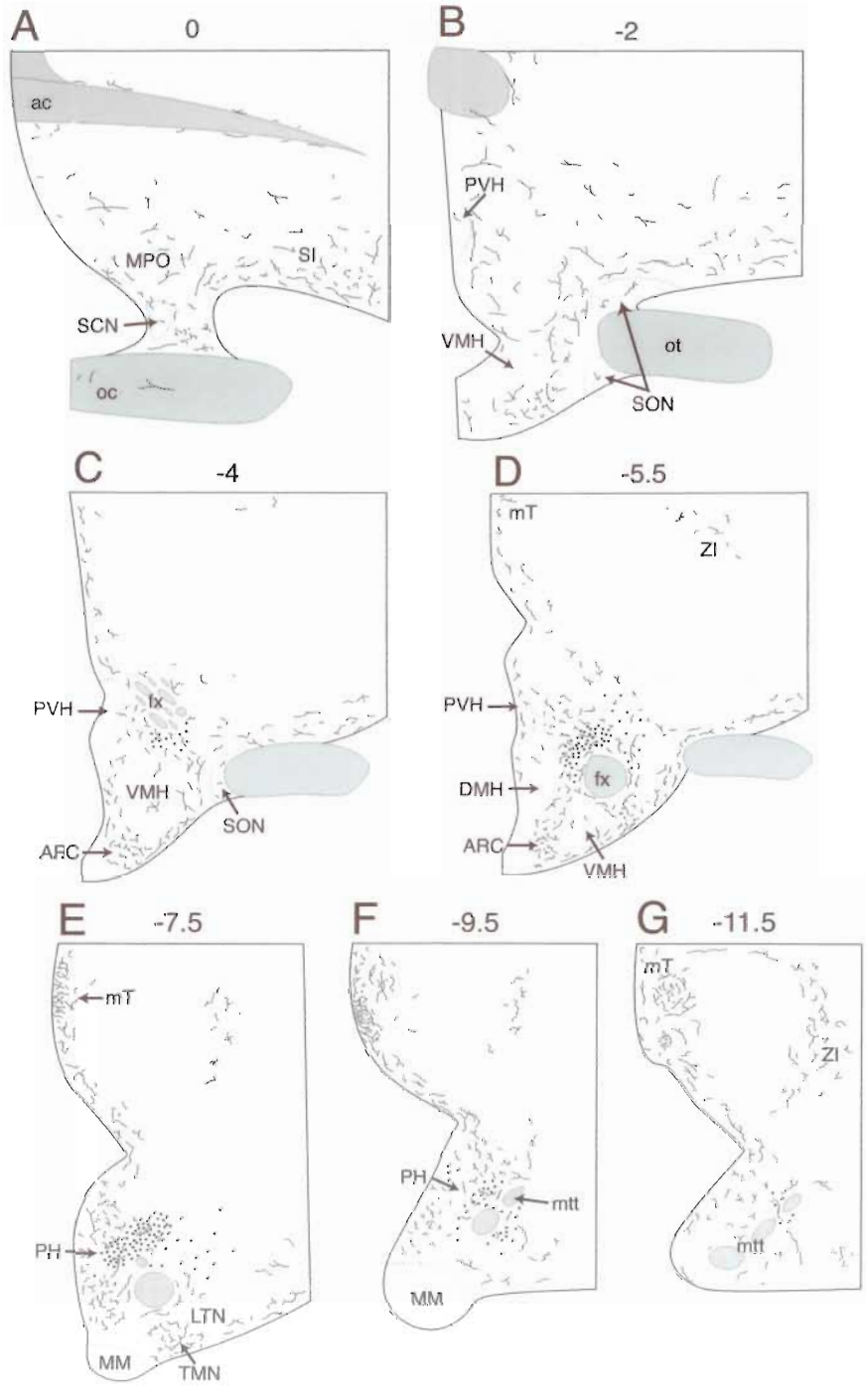
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Fig. 1

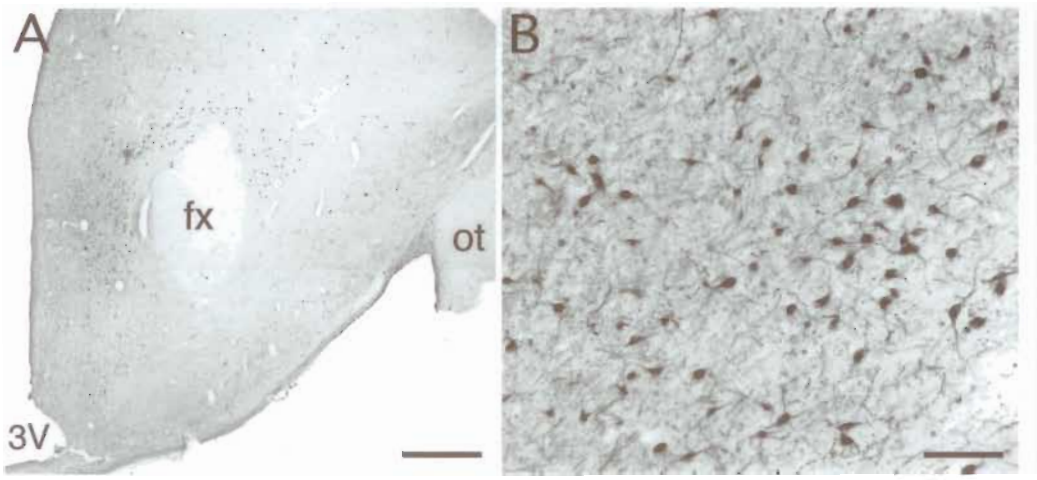

Fig. 2. - *Brightfield digitized image montage (A) and high power image (B) of (HYP+) neurons in the human hypothalamus.*

The HYP+ neurons are located in a dense band just dorsomedial to the fornix in the posterior hypothalamic area. Image (A) corresponds approximately to level (D) in Fig. 1. The asterisk in (A) marks the center of image (B). Marker bars (A = 625 μm), (B = 125 μm).

Terminal fields.

Hypothalamus. Plexuses of varicose axons are found throughout the preoptic, anterior and tuberal hypothalamus (Figure 1 A-G). Ascending projections from the main population of HYP+ neurons follow three pathways. HYP+ fibers innervating the medial hypothalamus, preoptic area and SCN follow a ventral pathway which begins at the mid-rostrocaudal extent of the HYP+ neuron population, courses ventrolaterally around the fornix, and continues rostrally along the base of the brain, lateral to the ventromedial hypothalamic nucleus. A large number of fibers branching off from this pathway is observed in the arcuate nucleus and retrochiasmatic area, a moderate number in the periventricular zone, paraventricular nucleus, anterior hypothalamic nucleus and preoptic area and a small number in the dorsomedial and ventromedial hypothalamic nuclei, the supraoptic nucleus and the suprachiasmatic nuclei. HYP+ fibers projecting toward the midline thalamus follow the second pathway which begins at the mid-rostrocaudal extent of the HYP+ neuron population, courses medially and rostrally through the paraventricular nucleus (Figure 3) and ascends dorsally, adjacent to the third ventricle, to innervate the midline thalamic nuclei. HYP+ fibers following the third pathway project laterally from the main HYP+ neuron population to distribute throughout the lateral hypothalamic area and zona incerta. HYP+ fibers continue rostrally within the lateral hypothalamic area to provide dense innervation of the substantia innominata and the nucleus basalis of Meynert (Figure 3). HYP+ fibers are particularly prominent throughout

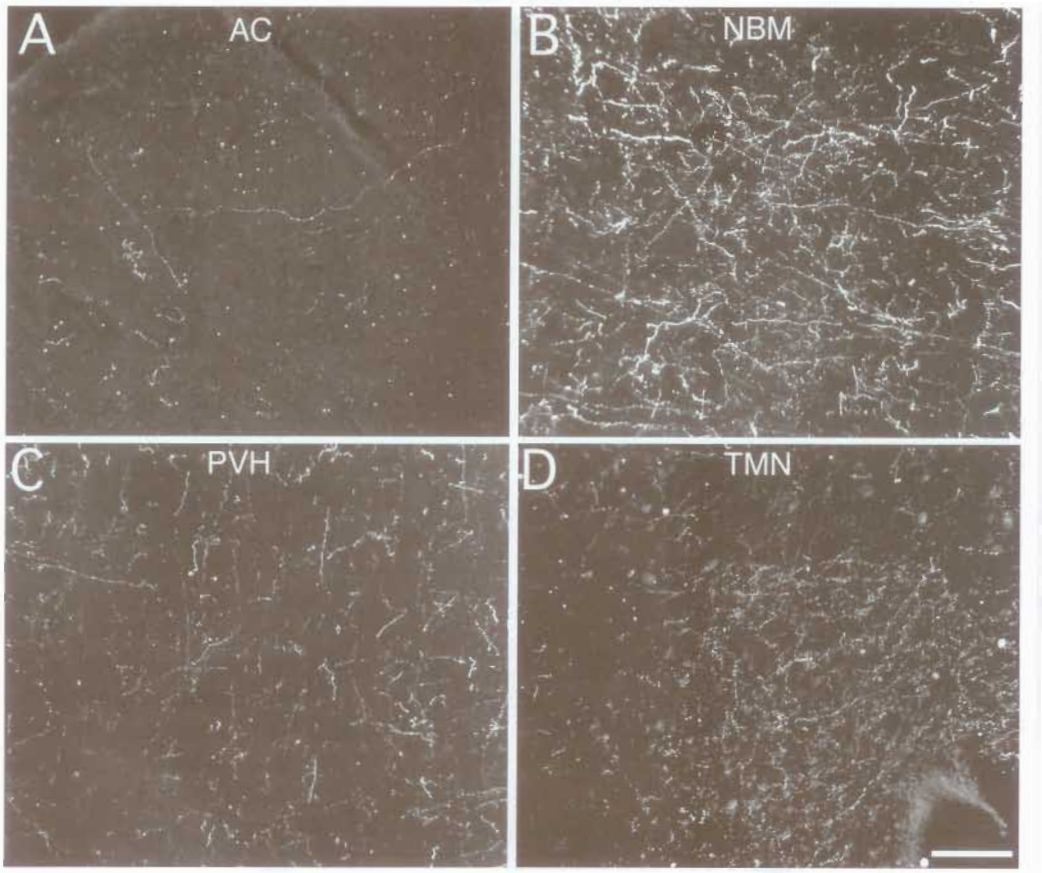

Fig. 3. - *Darkfield digitized images of HYP+ fibers in the anterior cingulate cortex (A: AC), nucleus basalis of Meyert (B: NBM), paraventricular hypothalamic nucleus (C: PVH), and tuberomammillary nucleus (D: TMN).*

Marker bar = 125μm.

the zona incerta. Fibers projecting through the zona incerta also appear to curve dorsally and medially to contribute to the innervation of the dorsal midline thalamic nuclei.

Midbrain raphe. A dense plexus of varicose axons extends throughout the dorsal raphe nucleus, both in the medial portion of the nucleus and the lateral wings, and extends ventrally into the median raphe. The plexus appears to make predominantly axodendritic contacts as there is no particular concentration around raphe perikarya.

Locus coeruleus. A dense plexus of axons is present over the entire locus coeruleus extending into the immediate subcoeruleus region (Figure 4). Like that in the raphe, it appears to be predominantly axodendritic.

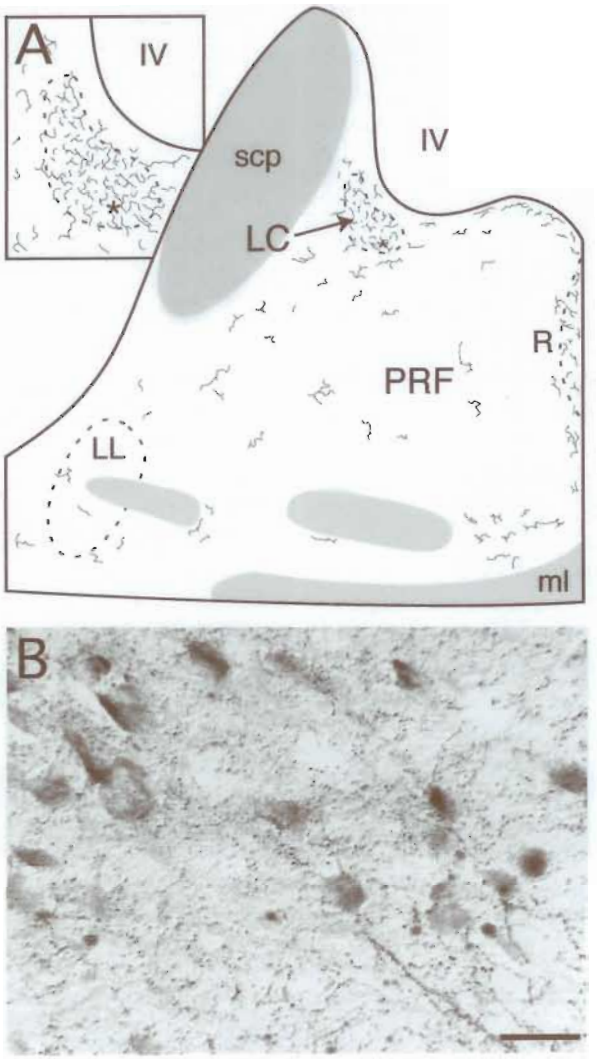

Fig. 4. - *Brainstem distribution of (HYP+) fibers illustrated schematically (A) and with a brightfield digitized high magnification image (B). HYP+ fibers are most numerous in the median raphe nucleus (R) and the locus coeruleus (LC).*

The inset in (A) serves to emphasize the dense innervation of the LC by HYP+ fibers. Asterisks in (A) indicate the center of the image in (B). The arrows in (B) point to a large HYP+ fiber entering the caudomedial border of the nucleus. Marker bar (B = 12.5 μm).

Thalamus. There is a dense plexus of HYP+ axons in the paraventricular thalamic nucleus. HYP+ fibers are present in moderate numbers throughout the remainder of the midline thalamus, but there is no evident innervation of adjacent relay nuclei.

Nucleus basalis. A dense plexus of axons, more dense than that in the adjacent anterior hypothalamus, extends over the horizontal portion of the nucleus of the diagonal band and entire nucleus basalis. This also is a predominantly axodendritic plexus (Figure 3).

Cerebral cortex. The innervation of the cortical areas is quite variable with the densest array of axons and varicose segments occurring in frontal (area 46) and temporal (area 22) association cortex and in anterior cingulate cortex (area 24). The density of innervation is less in motor cortex (area 4) and sparse in somatosensory (areas 3, 1, 2) and visual (area 17) cortex (Figure 5).

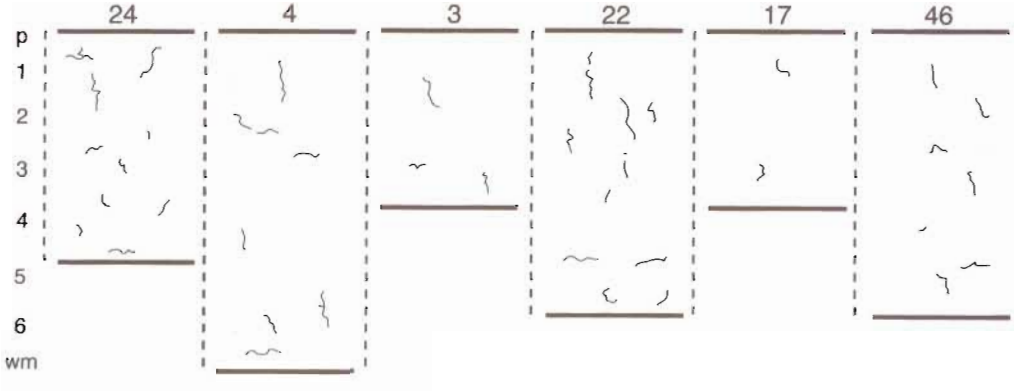

Fig. 5. - *Hypocretin-immunoreactive (HYP+) fibers in several cortical areas illustrated schematically.*

Note that fibers in the deep layers are oriented both vertically and horizontally, whereas fibers in the more superficial layers are oriented primarily vertically. Each cortical area examined is indicated by a Brodman area number and a schematic illustrating relative density and orientation of HYP+ fibers.

Varicose axons enter cortex from the subcortical white matter in a loose plexus which produces both horizontal and vertically organized axons in the deep layers and predominantly vertically-organized fibers in the superficial layers. The density of the cortical innervation is significantly less than that in the innervated subcortical areas.

The number and distribution of hypocretin neurons in the hypothalamus, and the pattern and density of terminal field innervation, did not appear to vary with either sex or age.

DISCUSSION

The objective of this study was to determine whether the pattern of organization of the HYP neurons system in the human brain is similar to that described previ-

ously in the rat (11, 14). Our data indicate that it is and that the similarities are striking. First, the HYP+ perikarya are present exclusively in the posterior and lateral hypothalamus in a distribution very similar to that in the rat, extending from the perifornical region into the lateral hypothalamic area and the medial hypothalamic area. As has been noted previously, the HYP+ perikarya are distinct from a similarly distributed group containing melanin concentrating hormone (4). Second, although we did not examine all of the areas studied by the groups working on the rodent brain (11, 14), the similarity in patterns of terminal plexuses in the rat and human brains is striking in most of the areas where comparisons can be made. For example, perhaps the densest area of innervation in the rat brain is the locus coeruleus (5, 14) and this is also true in the human. Another area of particularly dense innervation in the rat and human is the nucleus of the diagonal band and nucleus basalis. Other areas where the density is similar include the hypothalamus, raphe nuclei, zona incerta, midline thalamus and periaqueductal gray. One area of discordance is in innervation of cerebral cortex. In the human, the innervation appears more sparse than in the rat and it appears distributed rather evenly across the laminar architecture. In the rat, the HYP+ fiber plexus generally appears more dense in deep than in superficial layers and all cortical areas appear to have a similar density of innervation. In all areas innervated, the pattern of HYP+ axons indicates an axodendritic synaptic organization and this is consistent with studies indicating that the hypocretins are excitatory (3, 5). There is a relative gradient of density of innervation in human cortical areas with the densest plexuses in anterior cingulate cortex (area 24), superior temporal cortex (area 22) and prefrontal cortex (area 46), an intermediate level in motor cortex (area 4), and the least dense plexuses in somatosensory cortex (area 3) and, particularly, visual cortex (area 17).

It is difficult, of course, to compare density of innervation in material obtained with optimal fixation in experimental animals with that obtained from postmortem human brain. Most of our human material is from elderly subjects and we do not know the effect of age on this system. Also, although peptides generally survive fairly well in postmortem brain, we do not know specifically about HYP. Thus, subject age and postmortem interval prior to fixation could affect the apparent density of innervation in ways we would not appreciate. Nevertheless, the similarities between the rat and human material are generally consistent except that there are differences between rat and human in the density of innervation across cortical areas.

The initial studies of the HYP neuron system emphasized a function in the regulation of feeding behavior (1, 4, 11, 16). The dense innervation by HYP+ fibers in the arcuate nucleus, paraventricular nucleus, ventromedial nucleus and lateral hypothalamic area are consistent with a putative involvement of the HYP+ neuron system in the regulation of feeding behavior. More importantly, though, the location of the HYP+ perikarya and the pattern of distribution of the terminal plexuses produced by these neurons suggests a function in arousal and the maintenance of waking behavior. This is consistent with the observation that HYP has an excitatory synaptic function (20), particularly in the locus coeruleus (5). If we consider the areas that participate in cortical activation, upper brainstem reticular

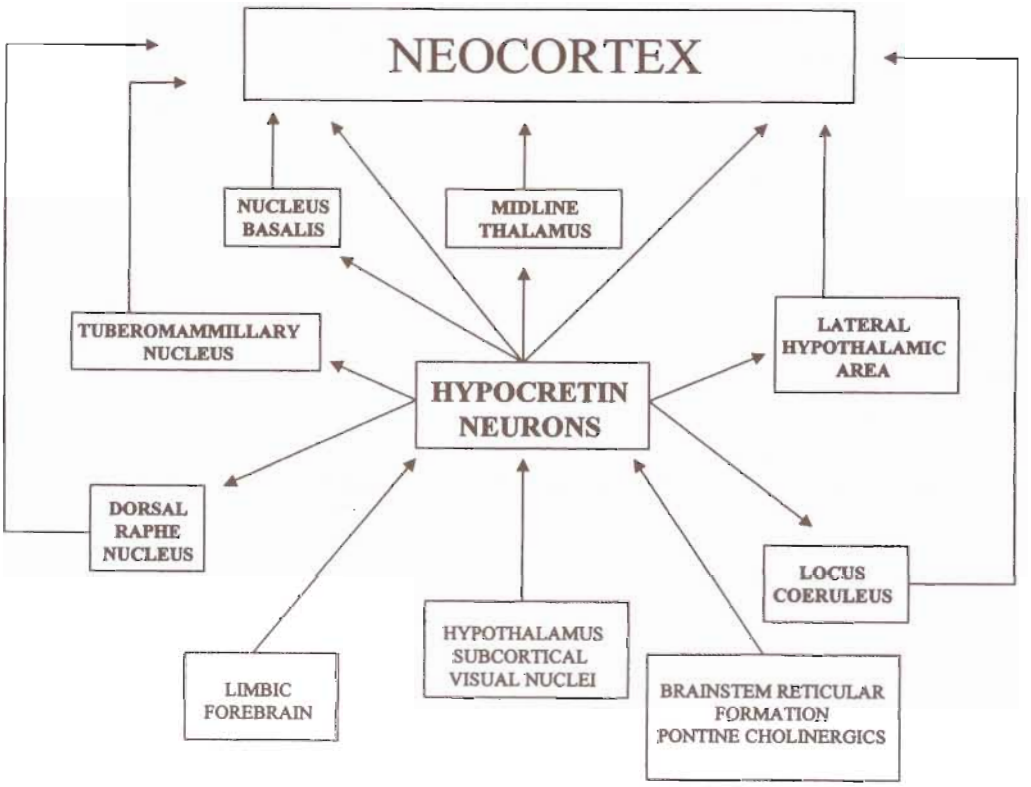

Fig. 6. - *Diagram showing the pattern of afferent and efferent connections of the hypocretin neurons of the posterior hypothalamus.*

See text for further description.

formation, locus coeruleus, midbrain raphe nuclei, posterior hypothalamus, midline thalamus and nucleus basalis, all receive a dense input from the HYP system (Figure 6). In studies in the rat (E.E. Abrahamson and R.Y. Moore, unpublished), we have shown that HYP is colocalized with glutamate in the posterior hypothalamic neurons, a further indication of an excitatory role. Indeed, the nature of the transmitter phenotype, glutamate colocalized with hypocretin, with the extraordinary pattern of innervation of both the entire neocortex and a series of subcortical structures which participate in arousal and attention, would appear to make this system one that is involved in behavioral activation, perhaps in concert with the tuberomammillary histamine neurons which also provide a wide innervation of cortex and are known to fire during waking (8). The view that the HYP system is involved in arousal and maintenance of waking is certainly buttressed by the recent association with narcolepsy (2, 9, 13).

Since the early pathological observations of von Economo (21), and the classic experimental study of Nauta (12), we have recognized that the posterior hypothalamic area is a critical component of the neural mechanisms of arousal and maintenance

of the behavioral state of waking, the essential foundation for the elaboration of adaptive behavior. The discovery of the HYP neuron system provides a new set of hypothalamic neurons ideally organized to mediate this function.

SUMMARY

Hypocretins are recently discovered neuropeptides produced by a small group of posterior hypothalamic neurons which project widely over the neuroaxis. In this study, we note that hypocretin neuron perikarya in the human brain are localized to the perifornical region of the posterior hypothalamus, extending into the lateral hypothalamus. These neurons lightly innervate all areas of cerebral cortex studied in a variable pattern with denser innervation of association cortex than primary motor or sensory cortex. There is a dense innervation of hypothalamus, locus coeruleus, raphe nuclei, midline thalamus and nucleus of the diagonal band-nucleus basalis complex of the forebrain. This pattern of projections from the hypocretin neurons is compatible with an important role in arousal and the maintenance of the waking state.

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It is a privilege to contribute to this volume in memory of Professor Nathaniel Kleitman, who contributed so much to our understanding of sleep and who also taught me neurophysiology in medical school.

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