Neurons Containing Hypocretin (Orexin) Project to Multiple Neuronal Systems

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The novel neuropeptides called hypocretins (orexins) have recently been identified as being localized exclusively in cell bodies in a subregion of the tuberal part of the hypothalamus. The structure of the hypocretins, their accumulation in vesicles of axon terminals, and their excitatory effect on cultured hypothalamic neurons suggest that the hypocretins function in intercellular communication. To characterize these peptides further and to help understand what physiological functions they may serve, we undertook an immunohistochemical study to examine the distribution of preprohypocretin-immunoreactive neurons and fibers in the rat brain. Preprohypocretin-positive neurons were found in the perifornical nucleus and in the dorsal and lateral hypothalamic areas. These cells were distinct from those that express melanin-concentrating hormone. Although they represent a restricted group of cells, their projections were widely distributed in the brain. We observed labeled fibers

throughout the hypothalamus. The densest extrahypothalamic projection was found in the locus coeruleus. Fibers were also seen in the septal nuclei, the bed nucleus of the stria terminalis, the paraventricular and reuniens nuclei of the thalamus, the zona incerta, the subthalamic nucleus, the central gray, the substantia nigra, the raphe nuclei, the parabrachial area, the medullary reticular formation, and the nucleus of the solitary tract. Less prominent projections were found in cortical regions, central and anterior amygdaloid nuclei, and the olfactory bulb. These results suggest that hypocretins are likely to have a role in physiological functions in addition to food intake such as regulation of blood pressure, the neuroendocrine system, body temperature, and the sleep–waking cycle.

Key words: neuropeptide; hypothalamus; immunohistochemistry; blood pressure; feeding; autonomic functions; hypocretin; orexin; melanin-concentrating hormone; neuroendocrine

The hypothalamus is an essential interface between endocrine, autonomic, and somatomotor systems. In mammals, the hypothalamus is a hub of central regulatory centers for autonomic and endocrine homeostatic systems such as cardiovascular, temperature, and abdominal visceral regulation, as well as ingestive behaviors (Swanson, 1987). For some of these systems, particular peptides have been identified as major products of individual nuclei; the localization of vasopressin and oxytocin to the paraventricular and supraoptic nuclei is a prime example. It is likely that other hypothalamic peptides are yet to be identified that may be similarly localized and that may contribute to the physiological functions regulated by the hypothalamus.

To identify novel hypothalamic peptides, an analysis of the mRNAs whose expression is restricted to or enriched in the rat hypothalamus was undertaken using directional tag-PCR subtraction (Gautvik et al., 1996). A nucleotide sequence encoding a 130 residue protein called preprohypocretin (hcrt) was isolated from

this hypothalamus-enriched cDNA library (Sutcliffe et al., 1997; de Lecea et al., 1998). *In situ* hybridization revealed that neurons expressing hcrt mRNA were located exclusively in the tuberal region of the hypothalamus (Gautvik et al., 1996). Sequence analysis indicated that hcrt yields two peptides, hcrt-1 (residues 28–66) and hcrt-2 (residues 69–97). The structure of hcrt, its expression in hypothalamic neurons, and its accumulation in vesicles of axon terminals suggested that the hcrt peptides may have intercellular signaling activity, and synthetic hcrt-2 was excitatory when applied to synaptically coupled rat hypothalamic neurons *in vitro* (de Lecea et al., 1998).

More recently, screening of high-resolution HPLC fractions on cell lines expressing orphan G-protein-coupled receptors resulted in the isolation of two peptides called orexin A and B (Sakurai et al., 1998) that are identical to hypocretin 1 and 2, respectively. Chemical analyses confirmed the identity of orexin B and hcrt-2, defined the N terminal of orexin A (hcrt-1) as residue 33, and verified that both peptides are amidated at their C terminals. These two peptides activate two distinct G-protein-coupled receptors, OX1 and OX2 (Sakurai et al., 1998).

To characterize further these new peptides and to obtain clues about their potential physiological functions, we undertook an immunohistochemical study to examine the distribution of hcrt-immunoreactive neurons and fibers in the brain. Sakurai et al. (1998) reported that intracerebroventricular injection of hcrt stimulates food intake. However, the widespread distribution of hcrt fibers observed in the present study suggests that hcrt is likely to play a role in other physiological functions as well.

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MATERIALS AND METHODS

Perfusion, fixation

Adult male Wistar rats were deeply anesthetized with a lethal dose of Nembutal (80 mg/kg) and perfused transcardially with 0.9% saline followed by an ice-cold fixative solution containing 4% paraformaldehyde and 0 or 0.25% glutaraldehyde in 0.1 M phosphate buffer (PB). Brains were removed, post-fixed overnight by immersion in the same fixative without glutaraldehyde, and cryoprotected with 30% sucrose for 2–3 d at 4°C. Brains were rapidly frozen in dry ice and sliced into 20- μ m-thick coronal sections on a cryostat (–23°C). Free-floating sections were rinsed several times and stored in 0.1 M PB containing 0.9% NaCl and 0.3% Triton X-100 plus 0.1% sodium azide (PBST-Az) at 4°C until use.

Immunohistochemistry

Immunohistochemical detection of hcr was done by sequential incubations of free-floating sections in (1) hcr antiserum raised in rabbit (1:5000 in PBST-Az) for 4 d at 4°C or overnight at room temperature (RT), (2) biotinylated goat anti-rabbit IgG (1:2000 in PBST; Vector Laboratories, Burlingame, CA) for 90 min at RT, and finally (3) avidin–biotin–HRP complex (1:1000 in PBST; Vector Elite Kit; Vectastain) for 90 min at RT. After each incubation, the sections were rinsed twice for 15 min in PBST. The sections were then immersed in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.025% 3,3'-diaminobenzidine-4HCl (DAB; Sigma, St. Louis, MO), 0.6% ammonium nickel (II) sulfate hexahydrate (Nacalai Tesque, Kyoto, Japan), and 0.003% H₂O₂, for 30 min at RT. The histochemical reaction was stopped by two rinses in PBST-Az. After this procedure, the hcr staining appeared as black punctuate granules in somata and processes of hcr neurons (see Fig. 1*A,C*). To visualize the precise location of labeled neurons and fibers in the brain, we counterstained some sections with neutral red (Sigma).

Ultrastructural immunohistochemistry

For electron microscopy sections, brains were fixed by transcardial perfusion with physiological saline followed by 4% paraformaldehyde and 0.2% glutaraldehyde, cut in 50- μ m-thick sections, frozen in liquid nitrogen to increase reagent penetration, and immunostained with hcr antisera as described above but without nickel intensification. After treatment with 1% osmium tetroxide for 45 min, sections were dehydrated in an ascending series of ethanol and embedded in Epon. Ultrathin sections were cut on a Reichert Ultracut microtome and studied in a Jeol 1200 EXII electron microscope at an accelerating voltage of 60,000 V.

Antisera and controls

Antisera were raised in rabbits by immunization with the 17 C-terminal amino acids of hcr (CPTATATALAPRGGSRV, antiserum #2050) conjugated to keyhole limpet hemocyanin (Sigma) or with the bacterially expressed histidine-tagged preprohypocretin (residues 28–130-amide, antiserum #2123) following the procedures described by Sutcliffe et al. (1983). The antiserum was purified as a polyclonal antibody using the E-Z-SEP kit (Pharmacia, Piscataway, NJ).

Immunohistochemical controls

To confirm the specificity of both antisera for immunohistochemical use, we preincubated each antiserum (1:5000) for 24 hr at 4°C with an excess (10 μ g/ml) of five different peptides: the 17 C-terminal amino acid residues of hcr (113–130-amide, CPTATATALAPRGGSRV), the bacterially expressed preproprotein (residues 28–130-amide), hcr-2 (residues 69–97-amide, PGPPGLQGRLLQRLQANGNHAAGILTM-NH₂), orexin A (residues 33–66-amide, QPLPDCCRQKTCSCRLYL-LHGAGNHAAGILTL-NH₂), or rat secretin (Peninsula Laboratories, Belmont, CA). Sections were then incubated with those solutions overnight at RT. The immunostaining was performed following the procedure described above.

In situ hybridization procedures

Preparation of hcr and melanin-concentrating hormone riboprobes. Hcr cDNA was inserted into the pBCSK⁺ vector (Stratagene, La Jolla, CA) at *NotI* and *EcoRI* sites. Melanin-concentrating hormone (MCH) cDNA was inserted into the pBCSK⁺ vector at *BamHI* and *EcoRI* sites. Both hcr and MCH plasmids were linearized using *EcoRI* (Life Technologies, Gaithersburg, MD). Radiolabeled hcr riboprobes were synthesized by *in*

Figure 1. Photomicrographs of adjacent frontal sections counterstained with neutral red at the level of the perifornical nucleus of the hypothalamus. *A*, Hcr neurons were labeled using antiserum #2050 against the C-terminal 17 aa portion of the preproprotein. *B*, Hypocretin immunoreactivity was absent after preincubation of antiserum #2050 with the whole preproprotein, showing that this antibody specifically recognized hcr. *C*, Hcr neurons were labeled with antibody #2123. Identical staining was obtained with both antisera. *f*, Fornix. Scale bars, 65 μ m.

vitro transcription of a full-length (569 nucleotides) rat hcr probe using T3 polymerase (Ambion, Austin, TX) and [³⁵S]UTP (DuPont NEN, Boston, MA). For the colorimetric procedure, the hcr and MCH riboprobes were transcribed *in vitro* following the standard digoxigenin (DIG)-labeling reaction protocol from Boehringer Mannheim (Indianapolis, IN) using 10 $\times$ transcription buffer and T3 RNA polymerase (Life Technologies). Riboprobes were then purified by ethanol precipitation and stored at –70°C.

Figure 2. Photomicrographs illustrating hcrt neurons in the perifornical nucleus of the hypothalamus. *A*, Neurons containing mRNA for hcrt visualized with a homogeneous red coloration of the cytoplasm with an *in situ* hybridization technique using Fast red as a substrate for alkaline phosphatase. *B*, Enlargement of *A*. *C*, Photomicrograph showing that all neurons that stained red after *in situ* hybridization (recognizing hcrt mRNA using Fast red) are labeled black by immunohistochemistry (recognizing the protein with the antiserum #2050) for hcrt. This result indicates that the antiserum #2050 is specific for hcrt. *D*, High magnification of double-labeled cells after *in situ* hybridization (red) and immunohistochemistry (black). *E*, Photomicrograph of neurons containing mRNA for the melanin-concentrating hormone (labeled in red by *in situ* hybridization using Vector red as substrate) and of hcrt neurons (labeled in black by immunohistochemistry using DAB with nickel as the substrate). Note that no double-labeled cells are present, indicating that MCH and hcrt are found in two distinct populations of neurons. *F*, High magnification of the MCH (red) and hcrt (black) neurons in the perifornical nucleus. Scale bars: *A*, *C*, *E*, 65 μ m; *B*, *D*, *F*, 36 μ m.

Estimation of DIG-labeled riboprobe yields. The yield of the DIG-labeled hcr and MCH riboprobes was approximated by comparison with DIG-labeled control RNA (Boehringer Mannheim). Serial dilutions of DIG-labeled control RNA and hcr and MCH riboprobes were spotted onto a nylon membrane (Nytran Plus; Schleicher & Schuell, Keene, NH). The membrane was then incubated in 0.1 M PBS solution containing 1% Triton X-100 and 4% bovine serum albumin (PBST-BSA) for 15 min at RT, followed by an incubation with a sheep antibody against DIG conjugated with alkaline phosphatase (1:5000 in PBST-BSA; Boehringer Mannheim) for 30 min at RT. The membrane was rinsed in 0.1 M PBS for 15 min followed by a 3 min rinse in a development buffer (100 mM Tris buffer, 50 mM MgCl₂, and 150 mM NaCl, pH 9). Finally, the reaction was developed with nitroblue tetrazolium (4.5 μ l/ml) and 5-bromo-4-chloro-3-indolyl phosphate (3.5 μ l/ml) in the development buffer (Bio-Rad, Hercules, CA) for 30 min at RT.

Hybridization. Twenty-five micrometer thick brain sections preserved in a cryoprotectant solution of 0.1 M PBS with 30% glycerol and 30% ethylene glycol were stored at -70°C . After thawing of frozen sections, free-floating sections were washed in 0.1 M PBS and then incubated in PBS with 0.5% Triton X-100 for 10 min, deproteinized with 0.1N HCl for 10 min, and acetylated with acetic anhydride (0.25% in 0.1 M triethanolamine hydrochloride, pH 8) for 10 min. The sections were then post-fixed in 4% paraformaldehyde for 10 min. Five minute washes in PBS were done after each of the above steps. The sections were then prehybridized in a solution containing 4 $\times$ PIPES, 10% (w/v) dextran sulfate, 50% deionized formamide, 5 $\times$ Denhardt's, 50 mM DTT, 0.2% (w/v) SDS, 250 mg/ml denatured salmon sperm DNA, and 250 mg/ml yeast tRNA for 3 hr at 55°C . Labeled antisense hcr or MCH riboprobes (10^6 cpm/ μ l for autoradiographic localization; ~ 600 ng/ml for colorimetric procedures) were heated at 68°C for 10 min and added to the sections in the prehybridization solution for an overnight incubation at 55°C . The sections were then (1) washed in 2 $\times$ SSC with 10 mM β -mercaptoethanol (β -ME) for 30 min at RT; (2) digested with 4 μ g/ml RNase A in 5 $\times$ Tris buffer (50 mM Tris-HCl, 5 mM EDTA, and 0.5 M NaCl) at 37°C for 1 hr; (3) rinsed in 1 $\times$ SSC, 50% formamide, and 5 mM β -ME at 55°C for 2 hr; (4) incubated in 0.2 $\times$ SSC, 10% formamide, 1 mM β -ME, and 0.1% Sarkosyl at 68°C for 1 hr; and (5) washed three times in PBS at RT.

For autoradiographic localization, sections were mounted on slides, dehydrated in a series of 50–100% ethanol, defatted in 50%/50% ethanol–chloroform followed by 100% ethanol, air dried, and then exposed to Kodak X-Omat AR film for 1–7 d. Sections were subsequently dipped in Ilford K5 liquid photographic emulsion and exposed for 2–4 weeks at 4°C . The resultant autoradiographs were developed in Kodak D-19 and counterstained with Richardson's blue.

For the colorimetric procedure, sections were washed in PBST-BSA for 2 hr at RT and then were incubated with a sheep anti-DIG-alkaline phosphatase antiserum (1:3000 in PBST-BSA) overnight at RT. For color development, sections were first rinsed twice for 30 min in 0.1 M Tris-HCl buffer, pH 8.2, and then immersed in a Tris-HCl buffer containing Fast red (Sigma Fast; Sigma) (see Fig. 2*A–D*) or Vector red (Vector Laboratories) (see Fig. 2*E,F*) as a substrate for alkaline phosphatase for 5 hr at RT. To increase the intensity of the signal, we added 0.3 M NaCl in the Fast red solution as suggested by Chiu et al. (1996). Stained neurons have an homogeneous red color of the cytoplasm with both substrates.

In situ/immunostaining

After *in situ* hybridization, the sections were subjected to the same immunohistochemistry procedure as described above but with minor changes. Anti-hcr (#2050) antiserum was used at a 1:1000 dilution, and successive incubations were done in PBST-BSA. Double-labeled cells were identified by the presence of black granulations over a red coloration of the cytoplasm (see Fig. 2*C,D*). To test whether there was cross-reactivity between the sheep anti-DIG-alkaline phosphatase and the secondary anti-rabbit IgG, we conducted the procedure without the primary antibodies (#2050 and #2123).

Data analysis

The sections were mounted on gelatin-coated glass slides, dried, dehydrated, and coverslipped with Mounting medium (Harleco; Diagnostic Systems, Gibbstown, NJ) or rinsed with water and coverslipped with aqueous mounting medium (Scytek Laboratories, Logan, UT). They were later observed with an Olympus BH-2 microscope. Mapping of neurons and fibers immunoreactive to hcr in rat brain was done using a Nikon light microscope equipped with a motorized X/Y stage, position

Figure 3. Distribution of hcr-labeled neurons on frontal sections at three rostrocaudal levels of the tuberal region of the hypothalamus (*A* to *B* to *C*) determined using #2123 antiserum. Sections were counterstained with neutral red (pink staining of all cells). The neutral red staining allowed us to determine the exact location of hcr neurons in the brain that are localized exclusively in the tuberal region of the hypothalamus ventral to the zona incerta and that extend 1 mm rostrocaudally, beginning caudal to the paraventricular nucleus of the hypothalamus. 3V, 3rd ventricle; Arc, arcuate nucleus; DMH, DM, dorsomedial hypothalamic nucleus; f, fornix; ic, internal capsule; opt, optic tract; SOR, retrochiasmatic part of the supraoptic nucleus; VMH, ventromedial hypothalamic nucleus; ZI, zona incerta. Scale bars, 275 μ m.

Figure 4. *A*, Large cells in the lateral hypothalamus are immunoreactive for hypocretin. Staining was found in the cytoplasm and dendrites (*short arrow*) but not in the nucleus (*long arrow*). Scale bar, 15 μ m. *B*, Electron microscopic examination of immunoreactive neurons showed punctate staining in the cytoplasm, often associated with dense core granules and parts of the Golgi apparatus (*thin arrow*). Random organelles near dense core granules sometimes showed peroxidase label, probably because of diffusion during the process of immunocytochemistry. *GA*, Golgi apparatus; *HCRT*, hypocretin; *NCL*, nucleole (*thick arrow*); *NU*, nucleus.

encoders, and a video camera connected to a computerized image data analysis system (Neurolucida; MicroBrightField, Colchester, VT). Outlines of sections and major structures were drawn at a low magnification (4 $\times$), whereas labeled neurons and fibers were plotted at higher magnification (20–40 $\times$). Finally, the drawings were assembled with Adobe Illustrator 7.0 to obtain the figures depicting the distribution of hcrt

projections. Counting of hcrt neurons was done bilaterally on 15- μ m-thick coronal sections taken every 120 μ m (one-eighth of the brain). The number of labeled cells in the brain was estimated using the method of Abercrombie (1946).

Photomicrographs were taken on the Olympus microscope, and the films were scanned with a Kodak slide scanner. To obtain optimal

Figure 5. Schematic drawings of 20 μ m rostrocaudal coronal sections illustrating the distribution and relative density of hcrt fibers in the prefrontal cortex and the olfactory bulb after immunohistochemistry for hcrt using antibody #2050. *aci*, Anterior commissure, intrabulbar part; *AI*, agranular insular cortex; *AOL*, anterior olfactory nucleus, lateral part; *AOV*, anterior olfactory nucleus, ventral part; *Cg*, cingulate cortex; *E/OV*, ependyma and subependymal layer/olfactory ventricle; *Fr*, frontal cortex; *Gl*, glomerular layer of the olfactory bulb; *IGr*, internal granular layer of the olfactory bulb; *LO*, lateral orbital cortex; *Mi*, mitral cell layer of the olfactory bulb; *MO/VO*, medial/ventral orbital cortex; *Pir*, piriform cortex; *TT*, tenia tecta; *VLO*, ventrolateral orbital cortex; *VN*, vomeronasal nerve layer.

reproduction of the staining, we modified the contrast and luminosity of the crude scans with Adobe Photoshop 4.0. The illustration plates were printed on a Kodak 7700 dye sublimation printer.

RESULTS

Antiserum specificity

We used two antisera, one raised against the C-terminal 17 amino acids of preprohypocretin (#2050) and a second raised against the bacterially expressed preproprotein hcrt (#2123). Competition studies showed that the immunohistochemical staining was absent when antiserum #2050 was preincubated for 24 hr at 4°C with the preproprotein or with the 17 aa portion of hcrt. In contrast, the labeling was still present after preincubation of the antibody with synthetic hcrt-2 or with rat secretin, showing that

antiserum #2050 specifically recognizes the 17 aa portion of hcrt but not hcrt-2 or rat secretin (Fig. 1). No labeling was obtained after preincubation of #2123 with the preproprotein. However, the labeling was still present after preincubation of #2123 with the 17 aa portion, orexin A, or hcrt-2, indicating that #2123 is a polyclonal antiserum against multiple epitopes of the preproprotein and not just the C-terminal 17 aa portion recognized by #2050. The cell body staining obtained with both antisera (#2050 and #2123) and by autoradiographic (refer to de Lecea et al., 1998) and colorimetric *in situ* hybridization is indistinguishable (Figs. 1, 2). The combination of *in situ* hybridization and immunohistochemistry for hcrt showed that neurons that expressed the mRNA coding for hcrt also made the protein (Fig. 2C,D).

Figure 6. Schematic drawings of 20 μ m rostrocaudal coronal sections illustrating the distribution and relative density of hcrt fibers in the telencephalon after immunohistochemistry for hcrt using antibody #2050. *aca*, Anterior commissure, anterior part; *AcbC*, accumbens nucleus, core; *AcbSh*, accumbens nucleus, shell; *AI*, agranular insular cortex; *Cg*, cingulate cortex; *Cl*, claustrum; *CPu*, caudate putamen; *DEn*, dorsal endopiriform nucleus; *DI*, dysgranular insular cortex; *fmi*, forceps minor of the corpus callosum; *Fr*, frontal cortex; *gcc*, genu of the corpus callosum; *GI*, granular insular cortex; *HDB*, nucleus of the horizontal limb of the diagonal band; *ICj*, islands of Calleja; *ICjM*, islands of Calleja, major island; *lo*, lateral olfactory tract; *LSI*, lateral septal nucleus, intermediate part; *LSV*, lateral septal nucleus, ventral part; *LV*, lateral ventricle; *Par*, parietal cortex; *Par1*, parietal cortex, area 1; *Pir*, piriform cortex; *SHi*, septohippocampal nucleus; *TT*, tenia tecta; *Tu*, olfactory tubercle; *VP*, ventral pallidum.

Figure 7. Schematic drawings of 20 μ m rostrocaudal coronal sections illustrating the distribution and relative density of hcrt fibers at the level of the preoptic area after immunohistochemistry for hcrt using antibody #2050. 3V, 3rd ventricle; ac, anterior commissure; acp, anterior commissure, posterior part; AHA, anterior hypothalamic area, anterior part; AI, agranular insular cortex; BSTL, bed nucleus of the stria terminalis, lateral division; BSTM, bed nucleus of the stria terminalis, medial division; BSTV, bed nucleus of the stria terminalis, ventral division; cc, corpus callosum; (Figure legend continues) Cg, cingulate cortex; Cl, claustrum; CPu, caudate putamen; DEn, dorsal endopiriform nucleus; f, fornix; FL, forelimb area of the cortex; Fr, frontal cortex; FStr, fundus striati; GI, granular insular cortex; GP, globus pallidus; HDB, nucleus of the horizontal limb of the diagonal band; HL, hindlimb area of the cortex; ic, internal capsule; LA, lateroanterior hypothalamic nucleus; lo, lateral olfactory tract; LOT, nucleus of the lateral olfactory tract; LPO, lateral preoptic area; LV, lateral ventricle; MCPO, magnocellular preoptic nucleus; MPA, medial preoptic area; MPO, medial preoptic nucleus; ox, optic chiasm; Par, parietal cortex; Pir, piriform cortex; PVA, paraventricular thalamic nucleus, anterior part; Sch, supraoptic nucleus; SFO, subformal organ; SHy, septohypothalamic nucleus; SI, substantia innominata; sm, stria medullaris of the thalamus; SO, supraoptic nucleus; st, stria terminalis; TS, triangular septal nucleus; VP, ventral pallidum.

Because MCH neurons are known to be located in the same tuberal region of the hypothalamus as hcrtr cells, we combined *in situ* hybridization for MCH with hcrtr immunohistochemistry. In this combination, we did not observe any double-labeled cells, showing that MCH and hcrtr were synthesized by distinct neuronal populations and therefore that there was no cross-reactivity between the *in situ* and immunohistochemistry procedures (Fig. 2*E,F*). Hcrtr neurons appeared smaller in size than did MCH cells in Figure 2, *E* and *F*. This is likely an artifact because of the different staining procedures used. The granulation of the precipitate obtained with the Vector red substrate is larger than that obtained with Fast red (alkaline phosphatase substrate) or DAB–nickel (substrate of the peroxidase used for immunohistochemistry). Therefore, cells stained with Vector red substrate appeared larger with poorly defined cell borders (Fig. 2*E,F*). When MCH cells are stained using the other substrates, they do not differ in size in comparison with hcrtr cells.

Distribution of hcrtr-immunoreactive cell bodies

In situ hybridization and immunohistochemistry against hcrtr (mRNA and protein, respectively) showed that hcrtr neurons were distributed exclusively in a restricted area of the tuberal region of the hypothalamus (1 mm rostrocaudal) caudal to the paraventricular nucleus of the hypothalamus. On coronal sections, labeled neurons showed a bilaterally symmetric organization. They were observed in the perifornical nucleus, the dorsomedial hypothalamic nucleus, and the dorsal and lateral hypothalamic areas. A few cells were seen in the posterior hypothalamic area and the subincertal nucleus at the junction of the thalamus and the hypothalamus (Fig. 3; also see Fig. 8). The labeled neurons were medium in size (25–30 μm in large diameter) and multipolar to fusiform in shape. They typically gave rise to two to three primary dendrites that were either smooth or only very sparsely invested with dendritic spines. Secondary branching was often observed, but third-order divisions were rarely seen.

Using the method of Abercrombie (1946), we found 682.62 ± 11.97 neurons on each side based on four brains stained with each antibody. The mean diameter of labeled cells was $\sim 25 \mu\text{m}$. Therefore, the total number of hcrtr cells in the brain is estimated to be: $682 (120/145) \times 2 = 1128$ cells. The perifornical nucleus contains $\sim 50\%$ of the hcrtr-labeled neurons. Approximately the same number of hcrtr neurons was observed by autoradiographic and colorimetric *in situ* hybridization as by immunohistochemistry.

Electron microscopy

Immunostaining was found in the cytoplasm of cell bodies and proximal dendrites (Fig. 4*A*). In strongly stained cells, peroxidase label was found distributed throughout the cytoplasm. In more lightly stained cells (Fig. 4), the staining was more punctate. With electron microscopy, the light peroxidase immunolabel was found to be restricted to regions of the Golgi apparatus and dense core granules (Fig. 4*B*, *thin arrows*). Immunoreactive cells generally had a partially invaginated nucleus and a single large nucleolus and were rich in cytoplasmic organelles (Fig. 4*B*). Two to three thick dendrites were found in immunoreactive cells, and their proximal region also contained a high density of cytoplasmic organelles.

Distribution of fibers

Hcrtr-IR fibers were distributed throughout the brain as illustrated in Figures 5–12. We obtained indistinguishable distribution of fibers with both antibodies (#2050 and #2123). The relative

density of fibers observed in different brain regions is reported in Table 1. Hcrtr-IR fibers were spread throughout the entire hypothalamus (Figs. 7–9). The density of fibers was homogeneous in the tuberal region of the hypothalamus. A high density of fibers was also seen in the other regions of the hypothalamus, although fewer fibers were seen in the medial preoptic nucleus, the anterior part of the ventromedial hypothalamic nucleus, and the paraventricular nucleus (Figs. 7, 8). Hcrtr fibers were found around the suprachiasmatic nucleus and the supraoptic nucleus in the anterior hypothalamus (Fig. 7), but few axons were found in these nuclei. Long, thick hcrtr fibers with numerous boutons innervated the arcuate nucleus (Fig. 13*C*) and followed the border of the brain to end laterally in the tuberomammillary nucleus. Hcrtr fibers avoided the mammillary bodies and went through the supramammillary nucleus and the posterior hypothalamic area (Fig. 9).

The tracts taken by fibers out of the hypothalamus are subjectively subdivided into four different pathways: dorsal and ventral ascending pathways and dorsal and ventral descending pathways.

Dorsal ascending pathway

Hcrtr neurons sent axons through the zona incerta to the paraventricular nucleus of the thalamus (anterior and posterior part) (Fig. 13*D,F*), the central medial nucleus of the thalamus, and the lateral habenula, avoiding the other thalamic nuclei (see Table 1). Hcrtr fibers were also found in the substantia innominata, the bed nucleus of the stria terminalis, the septal nuclei (medial and lateral), and the dorsal anterior nucleus of the olfactory bulb (Fig. 5). Fibers in these nuclei were long and thick with numerous boutons. Hcrtr axons going through this pathway avoided the caudate putamen and the globus pallidus and innervated the cortex where fibers were mainly long and thin with varicosities (Fig. 13*B*). Although they were widespread in the cortex, fibers were slightly denser and shorter at the border of the corpus callosum. Axons were also sent more laterally through the zona incerta, the subincertal nucleus of the thalamus, and the subthalamic nucleus following the optic tract to the central, anterior, and medial amygdaloid nuclei (Fig. 8).

Ventral ascending pathway

Hcrtr fibers were found in the ventral pallidum, the vertical and horizontal limb of the diagonal band of Broca, the medial part of the accumbens nucleus, and the olfactory bulb. In the olfactory system, fibers were mainly seen in the anterior olfactory nuclei (Fig. 5). A few fibers were observed in the glomerular layer and the internal granular layer, but no fibers were seen in the mitral cell layer (Fig. 5).

Dorsal descending pathway

Hcrtr fibers were directed up through the mesencephalic central gray to innervate the colliculi and the pontine central gray, particularly the locus coeruleus, the dorsal raphe nucleus, and the laterodorsal tegmental nucleus (Figs. 10, 11). In these nuclei, hcrtr fibers were long and thick with numerous boutons. Hcrtr fibers also went through the dorsal tegmental area to the pedunculopontine nucleus, the parabrachial nucleus, and the dorsal and α subcoeruleus area (Fig. 11). Then, avoiding the vestibular nuclei, they descended to the dorsolateral part of the nucleus of the solitary tract and the parvocellular reticular area and more caudally to the dorsal medullary area and the gelatinous layer of the caudal spinal trigeminal nucleus (Fig. 12). In these last structures, fibers were long and thick with numerous varicosities. Along this path-

Figure 8. Schematic drawings of 20 μ m rostrocaudal coronal sections illustrating the distribution and relative density of hcr fibers at the level of the tuberal region of the hypothalamus after immunohistochemistry for hcr using antibody #2050. The position of hcr cell bodies is indicated as dots in the left hemisphere. 3V, 3rd ventricle; AHP, anterior hypothalamic area, posterior part; Arc, arcuate nucleus; AV, anteroventral thalamic nucleus; BLA, basolateral amygdaloid nucleus, anterior part; BMA, basomedial amygdaloid nucleus, anterior part; CA1–CA3, fields CA1–CA3 of Ammon's horn; cc, corpus callosum; Ce, central amygdaloid nucleus; CM, central medial thalamic nucleus; CPu, caudate putamen; DEn, dorsal endopiriform nucleus; DG, dentate gyrus; f, fornix; fi, fimbria of the hippocampus; G, gelatinous thalamic nucleus; GP, globus pallidus; I, intercalated nuclei of the amygdala; ic, internal capsule; LDVL, laterodorsal thalamic nucleus, ventrolateral part; LH, lateral hypothalamic area; LHb, lateral habenular nucleus; LV, lateral ventricle; Me, medial amygdaloid nucleus; mt, mammillothalamic tract; opt, optic tract; PaMP, paraventricular hypothalamic nucleus, medial parvocellular part; Pir, piriform cortex; PoDG, polymorph layer of the dentate gyrus; PV, paraventricular thalamic nucleus; PVA, paraventricular thalamic nucleus, anterior part; Re, reuniens thalamic nucleus; Rh, rhomboid thalamic nucleus; Rt, reticular thalamic nucleus; sm, stria medullaris of the thalamus; SOR, supraoptic nucleus, retrochiasmatic part; st, stria terminalis; SubI, subincertal nucleus; TC, tuber cinereum area; VL, ventrolateral thalamic nucleus; VMH, ventromedial hypothalamic nucleus; VPL, ventral posterolateral thalamic nucleus; VPM, ventral posteromedial thalamic nucleus; ZI, zona incerta.

Figure 9. Schematic drawings of 20 μ m rostrocaudal coronal sections illustrating the distribution and relative density of hcr fibers in the caudal part of the hypothalamus after immunohistochemistry for hcr using antibody #2050. 3V, 3rd ventricle; AHi, amygdalohippocampal area; APir, amygdalopiriform transition area; APT, anterior pretecal nucleus; ar, acoustic stria; Arc, arcuate nucleus; BLP, basolateral amygdaloid nucleus, posterior part; CA1–CA3, fields CA1–CA3 of Ammon's horn; cp, cerebral peduncle; ctg, central tegmental tract; DEn, dorsal endopiriform nucleus; DG, dentate gyrus; Dk, nucleus Darkschewitsch; dlf, dorsal longitudinal fasciculus; DLG, dorsal lateral geniculate nucleus; f, fornix; F, nucleus of the fields of Forel; fi, fimbria of the hippocampus; fr, fasciculus retroflexus; Gem, gemini hypothalamic nucleus; ic, internal capsule; LH, lateral hypothalamic area; LHB, lateral habenular nucleus; LM, lateral mammillary nucleus; LPLR, lateral posterior thalamic nucleus, laterorostral part; LV, lateral ventricle; MG, medial geniculate nucleus; ml, medial lemniscus; MM, medial mammillary nucleus, medial part; mt, mammillothalamic tract; pc, posterior commissure; PF, parafascicular thalamic nucleus; PH, posterior hypothalamic area; Pir, piriform cortex; PLCo, posterolateral cortical amygdaloid nucleus; PMCo, posteromedial cortical amygdaloid nucleus; PVP, paraventricular thalamic nucleus, posterior part; SNR, substantia nigra, reticular part; SPf, subparafascicular thalamic nucleus; STh, subthalamic nucleus; SuM, supramammillary nucleus; sumx, supramammillary decussation; TM, tuberomammillary nucleus; VLG, ventral lateral geniculate nucleus; VPL, ventral posterolateral thalamic nucleus; VPM, ventral posteromedial thalamic nucleus; VTA, ventral tegmental area; ZI, zona incerta.

Figure 10. Schematic drawings of 20 μ m rostrocaudal coronal sections illustrating the distribution and relative density of hcrt fibers in the midbrain after immunohistochemistry for hcrt using antibody #2050. *APir*, Amygdalopiriform transition area; *APT*, anterior pretectal nucleus; *Aq*, aqueduct; *ATg*, anterior tegmental nucleus; *CA1–CA3*, fields CA1–CA3 of Ammon's horn; *CG*, central gray; *cp*, cerebral peduncle; *ctg*, central tegmental tract; *Dk*, nucleus Darkschewitsch; *DpMe*, deep mesencephalic nucleus; *DR*, dorsal raphe nucleus; *Ent*, entorhinal cortex; *fr*, fasciculus retroflexus; *HiF*, hippocampal fissure; *IC*, inferior colliculus; *InCo*, intercollicular nucleus; *InG*, intermediate gray layer of the superior colliculus; *lfp*, longitudinal fasciculus of the pons; *LL*, lateral lemniscus; *LPMC*, lateral posterior thalamic nucleus, mediocaudal part; *Me5*, mesencephalic trigeminal nucleus; *MGV*, medial geniculate nucleus, ventral part; *MiTg*, microcellular tegmental nucleus; *ml*, medial lemniscus; *mlf*, medial longitudinal fasciculus; *MnR*, median raphe nucleus; *OT*, nucleus of the optic tract; *PBG*, parabigeminal nucleus; *pc*, posterior commissure; *PMCo*, posteromedial cortical amygdaloid nucleus; *Pn*, pontine nuclei; *PoDG*, polymorph layer of the dentate gyrus; *PPT*, posterior pretectal nucleus; *PPTg*, pedunculopontine tegmental nucleus; *PRh*, perirhinal cortex; *R*, red nucleus; *SC*, superior colliculus; *SNC*, substantia nigra, compact part; *SNL*, substantia nigra, lateral part; *SNR*, substantia nigra, reticular part; *SuG*, superficial gray layer of the superior colliculus; *Te*, temporal cortex; *VTA*, ventral tegmental area; *xscp*, decussation of the superior cerebellar peduncle.

Figure 11. Schematic drawings of 20 μ m rostrocaudal coronal sections illustrating the distribution and relative density of hcr fibers in the pons after immunohistochemistry for hcr using antibody #2050. 2,3, Cerebellar lobules; 4V, 4th ventricle; 6, abducens nucleus; 7n, facial nerve or its root; 8vn, vestibular root, vestibulocochlear nerve; Acs6/7, accessory abducens and facial nuclei; CnF, cuneiform nucleus; g7, genu of the facial nerve; IC, inferior colliculus; KF, Kölliker–Fuse nucleus; LC, locus coeruleus; LDTg, laterodorsal tegmental nucleus; LPB, lateral (Figure legend continues) parabrachial nucleus; LSO, lateral superior olive; mcp, middle cerebellar peduncle; Me5, mesencephalic trigeminal nucleus; mlf, medial longitudinal fasciculus; MSO, medial superior olive; PCRtA, parvocellular reticular nucleus, α part; PDTg, posterodorsal tegmental nucleus; PnC, pontine reticular nucleus, caudal part; PnO, pontine reticular nucleus, oral part; Pr5, principal sensory trigeminal nucleus; py, pyramidal tract; RMg, raphe magnus nucleus; RPa, raphe pallidus nucleus; RPh, raphe pontis nucleus; rs, rubrospinal tract; RtTg, reticulotegmental nucleus of the pons; scp, superior cerebellar peduncle; sp5, spinal trigeminal tract; Sp5O, spinal trigeminal nucleus, oral part; SubCA, subcoeruleus nucleus, α part; SubCV, subcoeruleus nucleus, ventral part; SuVe, superior vestibular nucleus; Tz, nucleus of the trapezoid body.

Figure 12. Schematic drawings of 20 μ m rostrocaudal coronal sections illustrating the distribution and relative density of hcr fibers in the medulla after immunohistochemistry for hcr using antibody #2050. 4V, 4th ventricle; 7, facial nucleus; 10, dorsal motor nucleus of vagus; 12, hypoglossal nucleus; Amb, ambiguus nucleus; AP, area postrema; cu, cuneate fasciculus; Cu, cuneate nucleus; DPGi, dorsal paragigantocellular nucleus; ECu, external cuneate nucleus; Gi, gigantocellular reticular nucleus; GiA, gigantocellular reticular nucleus, α part; GiV, gigantocellular reticular nucleus, ventral part; Gr, gracile nucleus; icp, inferior cerebellar peduncle; IntA, interposed cerebellar nucleus, anterior part; IOC, inferior olive, subnucleus C of medial nucleus; IRt, intermediate reticular nucleus; Lat, lateral cerebellar nucleus; LPGi, lateral paragigantocellular nucleus; LRt, lateral reticular nucleus; LVe, lateral vestibular nucleus; MdD, medullary reticular nucleus, dorsal part; MdV, medullary reticular nucleus, ventral part; Med, medial cerebellar nucleus; mlf, medial longitudinal fasciculus; MVe, medial vestibular nucleus; MVeV, medial vestibular nucleus, ventral part; PCRtA, parvocellular reticular nucleus, α part; PrH, prepositus hypoglossal nucleus; py, pyramidal tract; RMg, raphe magnus nucleus; Ro, nucleus of Roller; ROb, raphe obscurus nucleus; RPa, raphe pallidus nucleus; RVL, rostroventrolateral reticular nucleus; Sol, nucleus of the solitary tract; sp5, spinal trigeminal tract; sp5, spinal trigeminal nucleus; Sp5I, spinal trigeminal nucleus, interpolar part; Sp5O, spinal trigeminal nucleus, oral part; SpVe, spinal vestibular nucleus; Y, nucleus Y.

Table 1. Relative density of hypocretin-immunoreactive fibers in various regions of the rat brain

Brain region	Fiber density ^a
Cortex	
Layers 1–3	++
Layer 4	+(+)
Layers 5–6	++(+)
Olfactory bulb	
Main bulb	–
Anterior olfactory nuclei	+
Endopiriform nucleus	++
Claustrum	++
Tenia tecta	+
Hippocampus	+
Amygdala	
Amygdalohippocampal area	+
Anterior amygdaloid area	+++
Basolateral nucleus	+
Basomedial nucleus	+
Cortical nucleus	+
Central nucleus	+++
Intercalated nuclei	+
Lateral nucleus	+
Medial nucleus	++
Septum	
Lateral nucleus	+++
Medial nucleus	+++
Septofimbrial nucleus	++
Subfornical organ	–
Diagonal band of Broca	
Horizontal limb	++
Vertical limb	+++
Bed nucleus of stria terminalis	
Lateral nucleus	+++
Medial nucleus	+++
Ventral nucleus	++++
Posteromedial nucleus	+++
Posterolateral nucleus	++++
Basal ganglia	
Accumbens nucleus, core	+
Accumbens nucleus, shell	++
Caudate putamen	–
Globus pallidus	–
Fundus of the striatum	+++
Thalamus	
Anteroventral thalamic nucleus	–
Anterior pretectal nucleus	–
Central medial nucleus	++++
Medial habenular nucleus	–
Mediodorsal thalamic nucleus	–
Lateral habenular nucleus	+++
Laterodorsal nucleus	+
Paraventricular nucleus	++++
Reuniens nucleus	++
Rhomboid nucleus	++
Ventrolateral nucleus	–
Ventroposteriomedial nucleus	–
Ventroposteriolateral nucleus	–
Lateral geniculate nucleus	–
Medial geniculate nucleus	–
Zona incerta, rostral	++++

Table 1. Continued

Brain region	Fiber density ^a
Zona incerta, caudal	++
Subincertal nucleus	++++
Subthalamic nucleus	++++
Subparafascicular thalamic nucleus	++++
Parafascicular nucleus	++
Central gray of the thalamus	++++
Anterior hypothalamus	
Medial preoptic area	+++
Medial preoptic nucleus	++
Lateral preoptic nucleus	+++
Magnocellular preoptic nucleus	+++
Supraoptic nucleus	–
Suprachiasmatic nucleus	–
Anterior hypothalamic area, anterior part	++
Lateroanterior nucleus	+++
Substantia innominata	++++
Ventral pallidum	+++
Posterior hypothalamus	
Paraventricular nucleus	+++
Ventromedial nucleus, rostral	++
Ventromedial nucleus, caudal	++++
Dorsomedial nucleus	++++
Dorsal hypothalamic area	++++
Lateral hypothalamic area	++++
Tuberum cinereum	++++
Tuberomammillary nucleus	++++
Perifornical nucleus	+++++
Posterior hypothalamic area	++++
Arcuate nucleus	++++
Submammillary nucleus	++++
Mammillary bodies	–
Pineal gland	–
Midbrain	
Substantia nigra, compact part	++++
Substantia nigra, reticular part	–
Substantia nigra, lateral part	+++
Ventral tegmental area	+++
Central gray	++++
Red nucleus	–
Mesencephalic reticular formation	++
Interpeduncular nucleus	+++
Brainstem	
Oculomotor nuclei	
Abducens nucleus (VI)	–
Oculomotor nucleus (III)	–
Trochlear motor nucleus (IV)	–
Trigeminal motor nucleus (V)	+
Facial motor nucleus (VII)	+
Motor nucleus of the vagus (X)	+
Hypoglossal nucleus (XII)	+
Somatosensory nuclei	
Superior colliculus	++
Inferior colliculus	++
Mesencephalic nucleus	–
Parabigeminal nucleus	+
Olivary nuclei	–
Cochlear nucleus, dorsal	++
Cochlear nucleus, ventral	–
Lateral lemniscus	+++

Table 1. *Continued*

Brain region	Fiber density ^a
Trapezoid body	–
Vestibular nuclei	++
Nucleus of the solitary tract	++++
Area postrema	++
Parabrachial nucleus	+++
Kölliker-Fuse nucleus	+++
Spinal trigeminal nucleus	–
Spinal trigeminal nucleus, gelatinous layer of the caudal part	+++
Central gray nuclei	
Nucleus of Darkschewitsch	–
Anterior tegmental nucleus	+
Ventral tegmental nucleus	+
Dorsal tegmental nucleus	–
Laterodorsal tegmental nucleus	+++
Barrington's nucleus	+++
Locus coeruleus	+++++
Periaqueductal gray	++++
Raphe nuclei	
Raphe dorsalis	++++
Raphe median	+++
Raphe magnus	++++
Raphe pallidus	++
Raphe obscurus	++
Raphe linearis	++
Raphe pontis	++
B9	+++
Reticular formation	
Central tegmental field	++
Pedunculopontine nucleus	+++
Cuneiform nucleus	++
Parvocellular reticular area (and its α part)	+++
Gigantocellular reticular area	++
Gigantocellular reticular area, α part	+++
Gigantocellular reticular area, ventral part	++++
Dorsal paragigantocellular reticular area	++
Lateral paragigantocellular reticular area	+++
Intermediate reticular field	+++
Lateral reticular nucleus	+
Medullary reticular nuclei, dorsal part	++++
Medullary reticular nuclei, ventral part	++
Pontine nuclei (oral, ventral, and caudal)	+
Other brainstem nuclei	
Nucleus prepositus hypoglossi	++
Rostroventrolateral medulla	+++
Caudoventrrolateral medulla	++
Nucleus subcoeruleus (α , ventral, and dorsal)	++
Cerebellum (cortex and nuclei)	–
Ependyma	++

^aRating scale: +++++, greatest fiber density; +, lowest fiber density; and –, insignificant number of fibers.

way, hcrt fibers avoided the geniculate nuclei, the trigeminal motor nucleus, and the spinal trigeminal nuclei.

Ventral descending pathway

Hcrt fibers went through the interpeduncular nucleus, the ventral tegmental area, and the substantia nigra pars compacta (Fig. 10).

Hcrt fibers in these nuclei were long with boutons. In the pons and medulla, hcrt fibers were distributed through the raphe nuclei and the reticular formation in the pontis oralis, caudal and ventral, the ventral and α gigantocellular reticular nuclei, and the ventral medullary area (Figs. 10–12). Hcrt fibers were particularly dense in the raphe magnus, the lateral paragigantocellular nucleus, and the ventral subcoeruleus area where the A5 noradrenergic cell group is located (Figs. 11, 12). However, fibers avoided several nuclei implicated in motor functions such as the red nucleus, the pontine nucleus, and the facial motor nucleus, as well as auditory structures such as the trapezoid body and the superior and inferior olive nuclei (Figs. 10–12).

DISCUSSION

In the present work, we used two antibodies, one against the 17 aa C-terminal part of preprohypocretin and one against the entire preprohypocretin molecule. With each antiserum, we obtained a strong signal with minimal background. The preabsorption experiments, the consistency of staining with both antibodies, and the agreement with *in situ* hybridization, as well as the double staining of hcrt-positive cells by both *in situ* hybridization and immunohistochemistry, showed that both antibodies are highly specific to preprohypocretin. The immunostaining pattern of these preprohypocretin antisera was similar to that of an antibody raised against the active peptide, hypocretin-2 (van den Pol et al., 1998), suggesting that the immunoreactive cells and fibers described in the present study represent the distribution pattern of the neuroactive peptide.

We found that hcrt-containing neurons are restricted to a subregion of the hypothalamus. They were located in the perifornical nucleus, the dorsomedial hypothalamic nucleus, and the dorsal and lateral hypothalamic areas. A few cells were seen in the posterior hypothalamic area and the subincertal nucleus. Sakurai et al. (1998) described orexin (hcrt) neurons as a discrete set of cells in the hypothalamic and subthalamic areas such as the zona incerta and the subincertal and the subthalamic nuclei. In our study, we conducted immunohistochemistry with a color-precipitation reaction, and we counterstained the labeled sections with neutral red. Therefore, we were able to identify directly structures containing the labeled neurons and found that hcrt neurons were just ventral to the zona incerta and rostral to the subthalamic nucleus.

We found that ~50% of the hcrt cells are located in the perifornical nucleus at the tuberal level of the hypothalamus. Some of the hcrt projections observed are similar to those described previously as projections of the perifornical nucleus such as those to the dorsal, dorsomedial, lateral, and posterior hypothalamic areas, the tuberomammillary nuclei, the basal forebrain bundle, the bed nucleus of the stria terminalis, the substantia innominata, the paraventricular nuclei of the thalamus and hypothalamus, the central nucleus of the amygdala, the arcuate nucleus, the central gray, the dorsal and median raphe nuclei, the laterodorsal tegmental nucleus, the locus coeruleus, Barrington's nucleus, the reticular formation, and the nucleus of the solitary tract (Saper et al., 1976; Allen and Cechetto, 1992, 1993; Valentino et al., 1994; Luppi et al., 1995; Touzani et al., 1996; Peyron et al., 1998). However, we also observed hcrt efferent projections that were not described as projections from the perifornical nucleus such as those to the olfactory bulb, the ventral pallidum, the compact part of the substantia nigra, the ventral tegmental area, and the nucleus raphe magnus. Furthermore, Allen and Cechetto (1992, 1993) report projections from the perifornical nucleus to

Figure 13. Photomicrographs of hcrt immunoreactive axons in the rat brain using the antiserum #2123. *A*, Dark-field illustration of thick hcrt fibers (in white) located in the locus coeruleus, lateral to the 4th ventricle. Notice that fibers are restricted to the locus and contain numerous boutons. *B*, Photomicrograph showing that hcrt-IR fibers were mainly long with varicosities. The density of fibers was relatively low in all cortical areas as shown in this picture of the frontal cortex. *C*, Illustration of the numerous long and thick fibers seen in the caudal part of the arcuate nucleus. Hcrt fibers contain numerous boutons. *D*, *F*, Photomicrographs illustrating one of the main projections for hcrt neurons, the paraventricular nucleus of the thalamus. Fibers were long with numerous varicosities as illustrated in *F* on a dark-field enlargement of *D*. *E*, Photomicrograph showing the high density of varicose fibers located in the lateral periaqueductal gray at the level of the dorsal raphe nucleus. Scale bars: *A*, 36 μ m; *B*–*D*, 70 μ m; *E*, 50 μ m; *F*, 25 μ m. *3V*, 3rd ventricle; *Aq*, Aqueduct; *LHb*, lateral habenular nucleus.

Figure 14. Schematic summary drawing of pathways taken by hcr1 processes that widely innervate rat brain. The sagittal section used is taken from the atlas of Paxinos and Watson (1986). *Purple dots:* Hypocretin-labeled neurons; *red:* dorsal ascending pathway; *light blue:* ventral ascending pathway; *green:* dorsal descending pathway; *dark blue:* ventral descending pathway.

the mediodorsal thalamic nucleus, the ventral premammillary nucleus, and the dorsal tegmental nucleus that we did not observe; these projections are therefore unlikely to use hcr1 as a neurotransmitter and/or neuromodulator. Consequently, the hcr1 cell group is a unique system and not simply a subset of the perifornical nucleus.

Although hcr1-containing neurons represent a relatively small number of cells, their projections are widely distributed in the CNS (Fig. 14), suggesting that hcr1 might be involved in multiple functions as we discuss in the following paragraphs.

Feeding

The perifornical nucleus contains ~50% of the hcr1-labeled neurons. This nucleus has been shown to be intimately involved in the neural control of food intake (Winn et al., 1984; Stanley et al., 1996) and is the most sensitive brain region for both the orexigenic effect of neuropeptide Y (Stanley et al., 1993) and the suppressive effects of catecholamines on feeding (Leibowitz and Stanley, 1986). Neurons in the perifornical nucleus and the lateral hypothalamic area also respond to internal signals related to the nutritional state of the animal (Himmi et al., 1988). We observed hcr1 fibers in nuclei that are known to be involved in the regulation of food intake such as the ventromedial hypothalamic nucleus, the arcuate nucleus, the paraventricular nucleus of the hypothalamus, the parabrachial area, the nucleus of the solitary tract, and the area postrema (Leibowitz and Brown, 1980; Roberts, 1980; Stanley and Leibowitz, 1984; Luiten et al., 1987; Ritter and Stone, 1987; Akabayashi et al., 1994; Ritter et al., 1994; Nishijo and Norgren, 1997; Shimura et al., 1997). Taken together, these data suggest that hcr1 might be involved in the regulation of feeding. Indeed, Sakurai et al. (1998) recently showed that orexin A (hcr1-1) and B (hcr1-2) stimulate food intake when injected intracerebroventricularly and that the mRNA accumulates during fasting.

MCH, whose cell bodies are also found in the tuberal region of the hypothalamus (Skofitsch et al., 1985; Nahon et al., 1989; Bittencourt et al., 1992), has also been reported to have potent

orexigenic activity (Presse et al., 1996; Qu et al., 1996; Rossi et al., 1997). Based on the absence of colocalization (Fig. 2*E,F*), MCH and hcr1 cells are distinct neuronal populations although they are partly intermingled anatomically. MCH and hcr1 neurons innervate some of the same brain regions such as the medial septum/diagonal band, the bed nucleus of the stria terminalis, the zona incerta, the entire hypothalamus, the arcuate nucleus, the ventral tegmental area, the periaqueductal gray, the locus coeruleus, and the nucleus of the solitary tract (Bittencourt et al., 1992), suggesting that MCH and hcr1 might be involved in the same physiological functions. Håkansson et al. (1998) showed that leptin receptors are located in the perifornical nucleus and the lateral hypothalamic area and are found on MCH neurons as well as other cell types. Whether leptin receptors are also present on hcr1 cells remains to be determined.

Blood pressure regulation

Electrical or chemical stimulation of the perifornical nucleus increases blood pressure and heart rate and activates neurons of the lateral paraventricular area (Sun and Guyenet, 1986; Allen and Cechetto, 1992). The perifornical nucleus and adjacent lateral hypothalamic area have been identified as the source of neurons responsible for producing cardiovascular responses associated with emotion (Smith et al., 1990). In our study, we found hcr1 fibers located in nuclei that are well known to be involved in blood pressure regulation such as the rostral ventrolateral medulla, the lateral paraventricular nucleus, the locus coeruleus, the nucleus of the solitary tract, the midbrain periaqueductal gray, the A5 noradrenergic cell group, the parabrachial region, and the area postrema (for review, see Dampney, 1994). Furthermore, the perifornical nucleus receives ascending afferent inputs mainly from the ventromedial central gray, the dorsal raphe nucleus, the laterodorsal tegmental region, and the nucleus of the solitary tract (Allen and Cechetto, 1992). These brainstem structures are known to be closely associated with cardiovascular function (Lindgren, 1961; Reis and Cuénod, 1965; Calaresu and Ciriello, 1980; for review, see Dampney, 1994). Consequently, our results

combined with the literature suggest that hcrt could be involved in the regulation of blood pressure.

Neuroendocrine regulation

The arcuate nucleus, highly involved in neuroendocrine regulation, is among the most densely hcrt-innervated areas of the hypothalamus, suggesting that hcrt might also be involved in hormonal regulation. This hypothesis has recently been tested electrophysiologically in hypothalamic slices containing the arcuate nucleus and the median eminence (van del Pol et al., 1998). Application of hcrt increased presynaptic activity of GABAergic cells terminating on electrophysiologically identified neuroendocrine neurons. These data indicate that hcrt modulates the afferent input controlling the neuroendocrine neurons in the arcuate nucleus. Furthermore, the paraventricular nucleus of the hypothalamus (PVN) is innervated by the perifornical nucleus (Allen and Cechetto, 1993; Larsen et al., 1994). The perifornical nucleus, along with the dorsomedial hypothalamic nucleus, provides most of the local excitatory synaptic input to PVN neurons (Boudaba et al., 1997). We have shown previously that hcrt has an excitatory effect on hypothalamic cells *in vitro* (de Lecea et al., 1998) and, in the present study, that the perifornical input to the PVN includes hcrt axons. These data suggest that hcrt might also modulate hormonal release controlled by PVN neurons.

Thermoregulation

The lateral hypothalamic area, in which we observed hcrt-labeled cells, has been implicated in the behavioral regulation of temperature (Corbett et al., 1988). The lateral hypothalamic area is also involved in heat loss responses, and the neural pathway involved in shivering passes through this region (Hemingway, 1963). The presence of hcrt fibers in the raphe magnus and the subcoeruleus areas, brain regions that also have been implicated in thermoregulation (Werner and Bienck, 1990), suggests that hcrt could modulate the regulation of body temperature.

Sleep–waking cycle

Neurons in the lateral hypothalamic area have been involved in the maintenance of waking state (Vanni-Mercier et al., 1984). Our results showed that hcrt fibers were seen in numerous structures involved in the sleep–waking cycle such as the locus coeruleus, the tuberomammillary nucleus, the pontine reticular formation, the raphe nuclei, the preoptic area, and the laterodorsal tegmental nucleus (for review, see Vertes, 1990; Jones, 1994). The high density of fibers in the locus coeruleus and the tuberomammillary nucleus of the hypothalamus, nuclei containing neurons responsible for the maintenance of the waking state, suggests that hcrt might have an effect on arousal.

Conclusion

The hypothalamus has a major role in regulating various behaviors that contribute to homeostasis such as arousal, feeding, and thermoregulation by integrating external and internal stimuli (Simerly, 1995). As discussed above, hcrt could be involved in the regulation of feeding, blood pressure, hormonal release, temperature, and arousal. Animals encounter situations that require or favor coordinated responses of several autonomic systems. We suggest that the hypocretins could provide such a coordinating signal.

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