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Opioid Precursor Gene Expression in the Human Hypothalamus

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Abstract

Using in situ hybridization histochemistry, we studied the distribution of neurons that express preproopiomelanocortin (pre-POMC), preprodynorphin (pre-PDYN), and preproenkephalin (pre-PENK) gene transcripts within the human hypothalamus and surrounding structures. Of the three opioid systems, pre-POMC neurons have the most restricted distribution. Pre-POMC cells are most numerous in the infundibular nucleus and retrochiasmatic area of the mediobasal hypothalamus; a few labeled cells are present within the boundaries of the ventromedial nucleus and infundibular stalk. Pre-POMC message was not found in the limited samples of structures adjacent to the hypothalamus.

In contrast to neurons that express pre-POMC, neurons expressing pre-PDYN and pre-PENK are more widely represented throughout the hypothalamus and extrahypothalamic structures. However, pre-PDYN and pre-PENK cells differ from one another in distribution. Pre-PDYN

message is especially abundant in neurons of the tuberal and mammillary regions, with a distinct population of labeled cells in the premammillary nucleus and dorsal posterior hypothalamus. Pre-PDYN gene expression also is found in neurons of the dorsomedial nucleus, ventromedial nucleus, caudal magnocellular portion of the paraventricular nucleus, dorsolateral supraoptic nucleus, tuberomammillary nucleus, caudal lateral hypothalamus, and retrochiasmatic area. In structures immediately adjacent to the hypothalamus, pre-PDYN neurons were observed in the caudate nucleus, putamen, cortical nucleus of the amygdala, and bed nucleus of the stria terminalis.

Pre-PENK neurons occur in varying numbers in all hypothalamic nuclei except the mammillary bodies. The chiasmatic region is particularly rich in pre-PENK neurons, with the highest packing density in the intermediate nucleus {the intermediate nucleus (Braak and Braak [1987] Anat. Embryol. 176:315-330) has also been termed the sexually dimorphic nucleus of the preoptic area (SDA-POA, Swaab and Fliers [1985] Science 228:1112-1115) or the interstitial nucleus of the anterior hypothalamus 1 (Allen et al. [1989] J. Neurosci. 9:497-506)}, dorsal suprachiasmatic nucleus, medial preoptic area, and rostral lateral hypothalamic area. Pre-PENK neurons are numerous in the infundibular nucleus, ventromedial nucleus, dorsomedial nucleus, caudal parvicellular portion of the paraventricular nucleus, tuberomammillary nucleus, lateral hypothalamus, and retrochiasmatic area. Only a few lightly labeled cells were found in the periphery of the supraoptic nucleus and lateral tuberal nucleus. In areas adjacent to the hypothalamus, cells that contain pre-PENK message occur in the nucleus basalis of Meynert, central nucleus of amygdala, bed nucleus of the stria terminalis, caudate nucleus, and putamen. The differential distribution of pre-POMC, pre-PDYN, and pre-PENK neurons in the human hypothalamus suggests that these three opioid systems influence hypothalamic functions in quite different ways.

Keywords

basal forebrain; dynorphin; enkephalin; intermediate nucleus; proopiomelanocortin

INTRODUCTION

In mammals, cerebral opioid systems are anatomically heterogeneous and consist of three different classes of peptides, β-endorphins, enkephalins, and dynorphins (Dores et al., 1990; Brownstein, 1993). Each of these putative neurotransmitters is synthesized by a different gene with its own precursor molecule (Höllt, 1993). Proopiomelanocortin (POMC) is a 267-amino-acid precursor peptide that is coded for by the pre-POMC gene and yields a group of opioid peptides, the β-endorphins, as well as the nonopioid hormones adrenocorticotrophic hormone, and three melanocyte- stimulating hormone-like molecules (Mains et al., 1977; Young et al., 1993). The maturation and cleavage of the precursor molecule into its various products appear to be tissue-specific phenomena, and posttranslational processing plays a crucial role in determining the biological activities of POMC derivatives in the brain (Smith and Funder, 1988; Benjannet et al., 1991; Roberts et al., 1993). For example, different regions of the brain preferentially produce different POMC cleavage products that are processed into smaller forms and exhibit neurotransmitter/neuromodulatory functions (Akil et al., 1984; Smith and Funder, 1988; Young et al., 1993).

The most recently discovered class of endogenous opioid peptides consists of derivatives of the 256-amino-acid precursor proenkephalin (PENK B), or prodynorphin (PDYN), coded for by the pre-PDYN gene (Kakidani et al., 1982). The cleavage of PDYN yields three main opioid peptides (i.e., neoendorphin, dynorphin A, and dynorphin B), all of which contain the sequence of leu-enkephalin (Day and Akil, 1989). Neoendorphin can exhibit two different forms, α-neoendorphin and β-neoendorphin, that differ by one amino acid (Minamino et al., 1980, 1981; Watson et al., 1982; Nakao et al., 1983; Akil et al., 1984). Currently, the best-known cleavage products of dynorphin A are two smaller fragments, DYN A1-8 and DYN A1-17 (Minamino et al., 1980; Seizinger et al., 1981). Processing of the dynorphin B domain can produce the 29-amino-acid peptide leumorphin or dynorphin B1-13 (Day and Akil, 1989). Mechanisms of complex PDYN tissue-specific processing are being investigated in a number of systems at the cellular, integrative, and behavioral levels (Day et al., 1993).

Enkephalins are produced by the 267-amino-acid peptide PENK, coded for by the pre-PENK gene (Gubler et al., 1982; Noda et al., 1982). All cleavage products of PENK, including four met-enkephalins, two carboxyl-extended met-enkephalins, and one leu-enkephalin, exhibit opioid-like activity. Larger fragments isolated from the adrenals, peptides E and F, contain met- and leu-enkephalin sequences (Schultzberg et al., 1978; Costa et al., 1979; Viveros et al., 1979; Lewis et al., 1980), but it is undetermined whether these adrenally derived peptides are processed into active fragments in the brain (Rossier, 1993). It is also possible that complex PENK tissue-specific processing may generate several nonopioid peptides with physiological activity (Rossier, 1993).

The opioid peptides are involved in a variety of behavioral and physiological processes such as eating, drinking, reproduction, stress, pain, emotions, learning, and homeostasis (Morley et al., 1983; Ferin and Vande Wiele, 1984; Olson et al., 1990; Dondi et al., 1991; Laatikainen, 1991; Neumann et al., 1992; Russell et al., 1992), and opioid dysfunction has been implicated in several neurological/psychiatric disorders (Berger et al., 1981; Franceschi et al., 1986; Seizinger et al., 1986; Berger and Nemeroff, 1987; Cavagnini et al., 1987; Terenius et al., 1987; Franceschi et al., 1988; Chamberlain and Herman, 1990; Zis and Garland, 1991). A number of immunocytochemical and biochemical studies have shown that the peptides are concentrated heavily in the hypothalamus of various species, including humans (Bloch et al., 1978; Gramsch et al., 1979, 1982; Kubek and Wilber, 1980; Maysinger et al., 1982; Pittius et al., 1983, 1984; Suda et al., 1985). Because the hypothalamus is a key modulator and integrator of numerous behavioral and physiological functions, it is important to determine the distribution of opioid peptides within hypothalamic nuclei.

Previous studies have used immunocytochemistry to study human cerebral opioid systems (Haber and Watson, 1985; Abe et al., 1988; Haber et al., 1990). However, the interpretation of immunocytochemical results can be compromised by the potential cross-reactivity of antibodies with certain members of the three opioid peptide families (Simerly et al., 1988; Haber et al., 1990) and by the loss of epitopes resulting from extended postmortem intervals common in obtaining human material. In situ hybridization histochemistry is particularly useful for localizing transmitter-specific substances in human postmortem tissues (Mengod

et al., 1992). In this study, we exploited the postmortem stability of mRNA (Johnson et al., 1986) and the high specificity and sensitivity of oligonucleotide probes to map opioid peptide precursor gene expression in neurons of the human hypothalamus.

MATERIALS AND METHODS

Tissue blocks were taken at autopsy from four 16–61-year-old human males with a mean postmortem interval of 9 hours (Walker et al., 1991; Sukhov et al., 1993). All subjects died acutely of non-neurological conditions, and complete autopsies did not disclose disorders that would be expected to compromise our analysis (Table 1). Autopsy specimens were collected in accordance with guidelines set forth in Federal Register 46 and with the institutional guidelines of The Johns Hopkins University School of Medicine and The University of Arizona College of Medicine. Each tissue sample included the hypothalamus and all or part of the parolfactory/paraterminal cortex, septum, substantia innominata, dorsal amygdala, caudate nucleus, putamen, globus pallidus, and thalamus. Blocks of tissue were placed on foil-coated glass slides, frozen at −30°C in isopentane, and stored at −80°C.

Hybridization histochemistry

Sections (20 microns thick) were cut coronally (cases 814, 426) or sagittally (cases 814, 871, 810) on a cryostat-microtome at −20°C and thaw-mounted onto gelatin-coated slides. Every twentieth section was processed for hybridization histochemistry using a specific probe as described previously (Young, 1989; Walker et al., 1991; Sukhov et al., 1993; Rance et al., 1994). Briefly, sections were brought to room temperature, postfixed in 4% formaldehyde in phosphate-buffered saline for 5 minutes, treated with 0.25% acetic anhydride for 10 minutes, and delipidated in a graded series of ethanols and chloroform. After drying, sections were incubated for 20 hours at 37°C in a buffer consisting of 600 mM NaCl, 80 mM Tris-HC1, pH 7.5, 4 mM ethylenediaminetetraacetic acid, 0.1% sodium pyrophosphate, 0.2% sodium dodecyl sulfate, 10% dextran sulfate, 0.2 mg/ml heparin sulfate, 100 mM dithiothreitol, and ~10^6 dpm of ^{35}S-labeled probe per 50 microliters (see below). Sections were then washed in a solution of 0.3 M NaCl/30 mM sodium citrate buffer (2x SSC) and 50% formamide at 45°C. After drying, sections were dipped in Kodak NTB-3 nuclear emulsion and exposed at 4°C for pre-POMC (7–14 days), pre-PDYN (60–120 days), and pre-PENK (30–60 days). Oligonucleotide probes were targeted toward bases of the human pre-POMC (7,106–7,153; Takahashi et al., 1983), rat pre-PDYN [862–909; Civelli et al., 1985; 94% homology with human (Horikawa et al., 1983)], and human pre-PENK (963–1,010; Comb et al., 1982) mRNA sequences. Oligonucleotide probes were labeled on the 3’ end using terminal deoxynucleotidyl transferase (Boehringer-Mannheim, Indianapolis, IN) and [^{35}S]deoxyadenosine triphosphate (> 1,000 Ci/mmol; New England Nuclear, Boston, MA). Probes used in these experiments were designed to preclude cross-hybridization with other transcripts. The specificity of probes was determined through a series of controls. First, probes were used for Northern analysis under similar conditions, which identified the appropriate size of transcripts (Rance and Young, 1991). Second, probes in this study gave distributions different from each other, as did a number of other 48mers in our previous studies (Rance and Young, 1991, 1994; Sukhov et al., 1993). Third, a 48-base vasopressin

sense probe gave no signal above background (Young, unpublished observations). After hybridization histochemistry, sections were stained with toluidine blue.

Analysis of tissue

Sections were mapped with a computerized microscopic mapping system (Sukhov et al., 1993). Computer-assisted mapping was carried out using a combination of brightfield and darkfield illumination. The boundaries of various hypothalamic nuclei and extrahypothalamic structures were first drawn under brightfield illumination using a 2.5x Plan-Neofluar (Zeiss Axiophot) objective. Sections were then systematically scanned under darkfield illumination for labeled pre-POMC, pre-PDYN, and pre-PENK neurons using a 20x Plan-Neofluar objective. Neuronal perikarya were considered “labeled” (i.e., containing pre-POMC, pre-PDYN, or pre-PENK mRNA) only if silver grains were localized over the cell soma (verified at 40x under brightfield illumination) and if the density of superimposed silver grains exceeded three times that of the surrounding neuropil. (Hereafter, we will refer to pre-POMC, pre-PDYN, and pre-PENK mRNA-containing cells as POMC, PDYN, and PENK neurons, respectively.) Based on labeling intensity, positive neurons were subdivided into lightly, moderately, and heavily labeled groups (for illustration, see Fig. 1B in Sukhov et al., 1993). Lightly labeled neurons were mapped only if grains within the neuronal somata exceeded three times background and the nucleus of each cell was present. In some cases, heavily labeled neurons were completely covered with superimposed silver grains, obscuring cellular details. Several sources were consulted to assist in the identification of basal forebrain structures (Le Gros Clark, 1936, 1938; Nauta and Haymaker, 1969; Veazey et al., 1982; Bleier, 1984; Braak and Braak, 1987, 1992; Gai et al., 1990; Saper, 1990).

RESULTS

POMC

The distribution of POMC mRNA in human hypothalamus is restricted to neurons of the mediobasal hypothalamus (Table 2). Most POMC neurons are confined to two major neuronal groups, the infundibular nucleus and the retrochiasmatic area. A small number of POMC neurons also are found in the ventromedial nucleus and infundibular stalk (Figs. 1,2).

POMC in the infundibular nucleus.—The infundibular nucleus, also known as the arcuate nucleus (Nauta and Haymaker, 1969; Saper, 1990), occupies a considerable portion of the mediobasal hypothalamus, extending rostrocaudally from the infundibular stalk to the origin of the mammillothalamic tract (Bleier, 1984; Saper, 1990; Swaab et al., 1993). Significant populations of neurons express POMC throughout the entire length of the infundibular nucleus (Figs. 1A–C, 2A–D). Most neurons that contain POMC transcripts are small and round, with moderate-to-heavy labeling over the cytoplasm (Fig. 3A). At the most rostral part of the infundibular nucleus that borders the retrochiasmatic area, infundibular POMC neurons overlap with oblong, medium-sized, labeled neurons of the retrochiasmatic area.

POMC in the retrochiasmatic area.—Numerous POMC neurons were identified in the retrochiasmatic area. The human retrochiasmatic area is a poorly delineated area that lies

in the anterior hypothalamus, caudal to the optic chiasm (Figs. 1, 2). The caudoventral retrochiasmatic region merges with the infundibular nucleus. Most POMC neurons in the retrochiasmatic area are oriented with their long axes toward the infundibular stalk (Fig. 3B).

POMC in the ventromedial nucleus.—We detected a few labeled POMC neurons in the ventrolateral part of the ventromedial nucleus (Fig. 3C). Some moderately and heavily labeled POMC cells appear to have strayed rostrocaudally from the infundibular nucleus into the ventromedial nucleus in the tuberal hypothalamus (Fig. 2C). Other cells seem to extend from the ventromedial nucleus into the lateral hypothalamic area and dorsomedial nucleus (Fig. 3C).

POMC in the infundibular stalk.—Labeled POMC cells were found in the infundibular stalk adjacent to the optic chiasm (Figs. 1A, 2A–C).

PDYN

PDYN neurons are more widely distributed in hypothalamic structures than are POMC cells (Table 2). We detected labeled PDYN cells in the supraoptic, paraventricular, dorsomedial, ventromedial, premammillary, and tuberomammillary nuclei, in the retrochiasmatic area, and in lateral and posterior hypothalamic areas. However, the packing density of PDYN neurons varied among hypothalamic cell groups; in the caudal aspect of the paraventricular nucleus, the premammillary nucleus, and the posterior hypothalamic area, a significant number of neurons express pre-PDYN. The packing density of PDYN cells was lower in other PDYN-containing hypothalamic nuclei (Figs. 4A–F, 5A–D). In general, PDYN neurons predominate in the tuberal and dorsal posterior regions, with only rare labeled neurons in more rostral parts of the hypothalamus.

PDYN in the premammillary nucleus.—The premammillary nucleus is located in the tuberal region just rostral to the medial mammillary nucleus (Figs. 4E, 5B,C). Many neurons of the premammillary nucleus express pre-PDYN. In coronal sections, these cells are round, with moderately labeled cytoplasm (Fig. 6F); in sagittal sections, neurons of the premammillary nucleus appear more elongated. Because of the high packing density of PDYN neurons, the premammillary nucleus is highlighted in pre-PDYN hybridization histochemical preparations (Figs. 4E, 5B,C). Some PDYN neurons in the periphery of the nucleus are medium-sized to small, round or pear-shaped neurons that resemble cells of the ventrolateral division of the ventromedial nucleus.

PDYN in the paraventricular nucleus.—Viewed in sagittal sections, the human paraventricular nucleus extends along the medial surface of the hypothalamus as a rostroventral-to-caudodorsal continuum (Sukhov et al., 1993). PDYN cells are scattered throughout this nucleus (Figs. 4D,E, 5A,B) but are most abundant caudally. Only a few labeled neurons were detected in the rostroventral paraventricular nucleus. The majority of PDYN neurons are magnocellular with lightly to moderately labeled perikarya (Fig. 6A), but some medium-sized neurons also express pre-PDYN.

PDYN in the supraoptic nucleus.—We detected only a few PDYN neurons in the dorsolateral part of the human supraoptic nucleus. We did not observe magnocellular neurons that express the pre-PDYN gene in the nucleus. Usually, cells that contain pre-PDYN mRNA are medium-sized or small, with moderate labeling intensity (Fig. 6B).

PDYN in the dorsal posterior hypothalamus.—Two major groups of PDYN neurons were found in the posterior hypothalamus. First, there is a prominent subpopulation of posterior hypothalamic neurons, sometimes called the posterior hypothalamic nucleus in primates (Veazey et al., 1982; Bleier, 1984). Large, elongated neurons of the posterior hypothalamic nucleus span the posterior hypothalamic area rostrocaudally at the level of the medial mammillary nucleus (Figs. 4F, 5C,D; Braak and Braak, 1992). Numerous PDYN neurons in this group are heavily labeled (Fig. 6D). Second, posterior hypothalamic PDYN neurons are widely dispersed throughout the region and intermingle laterally with unlabeled neurons of the zona incerta and fields of Forel. Labeled neurons in this diffuse collection of cells are morphologically heterogeneous, reflecting, in general, the complex cytology of the dorsal posterior hypothalamic area (Veazey et al., 1982; Bleier, 1984).

PDYN in the dorsomedial nucleus.—A large subpopulation of PDYN neurons occupies a relatively cell-sparse region within the conventional boundaries of the dorsomedial nucleus. Although the columns of the fornix serve as landmarks that divide the human hypothalamus into medial and lateral zones, PDYN neurons of the more medial dorsomedial nucleus extend into the lateral hypothalamic area at the rostral perifornical area (Figs. 4C–E, 5A–C). Predominantly medium-sized, round and pyramidal PDYN neurons of the dorsomedial nucleus (Fig. 6H) intermingle with the more oblong and pear-shaped PDYN neurons of the lateral hypothalamic area near the descending columns of the fornix.

PDYN in the ventromedial nucleus.—The human ventromedial nucleus occupies a relatively large area in the tuberal region (Braak and Braak, 1992). PDYN neurons are dispersed sporadically throughout the ventromedial nucleus, and most are lightly labeled (Fig. 6G).

PDYN in the tuberomammillary nucleus.—The tuberomammillary nucleus consists of three subdivisions that extend from the basolateral surface of the hypothalamus in three directions, ventromedial, dorsal, and dorsolateral. The tuberomammillary nucleus contains only a few large, lightly labeled PDYN neurons, mostly in the dorsolateral subdivision (Figs. 4C–E, 6C).

PDYN in the retrochiasmatic area.—In the rostral hypothalamus, PDYN neurons can be found in the retrochiasmatic area, where oblong neurons with moderately labeled cytoplasm occupy the territory caudoventral to the optic chiasm (Figs. 4B, 6E).

PENK

Neurons that express pre-PENK are widely distributed in the human hypothalamus and basal forebrain (Table 2). We observed PENK neurons in virtually all hypothalamic nuclei except the mammillary complex. The packing density of PENK neurons is greatest in

anterior hypothalamic structures, especially in the chiasmatic region. Numerous heavily labeled PENK neurons are located in the medial preoptic area, intermediate nucleus, dorsal suprachiasmatic nucleus, and rostral part of the lateral hypothalamus (Figs. 7A–D, 8B–D). Other subsets of PENK neurons were dispersed throughout the retrochiasmatic area, the dorsolateral supraoptic nucleus, and the area called the hypothalamic gray by Braak and Braak (1992; Figs. 7, 8). In the tuberal region, PENK neurons are present in the infundibular nucleus, ventromedial nucleus, dorsomedial nucleus, paraventricular nucleus, tuberomammillary nucleus, lateral tuberal nucleus, and lateral hypothalamic area. The posterior hypothalamic area dorsal to the mammillary complex contains a sparse population of PENK neurons (Fig. 8C,D).

PENK in the chiasmatic region.—The medial preoptic area contains many PENK neurons (Figs. 7A–D, 8A–D). Labeling in the intermediate nucleus is particularly prominent; almost all cells in this nucleus, which lies midway between the rostroventral tip of the paraventricular nucleus and the dorsomedial supraoptic nucleus, express pre-PENK (Figs. 7C, 8C, 9). Neurons of the intermediate nucleus are medium-sized and slightly elongated (Fig. 10D). Another nucleus in the chiasmatic area containing densely packed PENK neurons is the suprachiasmatic nucleus (Figs. 7A,B, 8B,C). Recently, five major subdivisions of the suprachiasmatic nucleus were described in humans: dorsal, central, ventral, medial, and external (Mai et al., 1991). Numerous small, round or oval neurons in the dorsal subdivision of the suprachiasmatic nucleus are lightly labeled (Fig. 10C). In contrast, only sparse cells are labeled in other parts of the suprachiasmatic nucleus (data not shown). We detected dispersed PENK neurons in the rostroventral portion of the paraventricular nucleus just medial and dorsal to the suprachiasmatic nucleus (Fig. 7A,B): Most paraventricular PENK neurons are parvicellular and resemble the more superiorly located PENK cells of the periventricular region. Only a few small, lightly labeled neurons expressing pre- PENK were detected in the periphery of the dorsolateral and ventromedial parts of the supraoptic nucleus (Fig. 10B); the core of the supraoptic nucleus is devoid of PENK cells. In the rest of the chiasmatic region, PENK neurons are dispersed throughout the anterior hypothalamus in the hypothalamic gray.

In the mediobasal anterior hypothalamus, neurons of the retrochiasmatic area express pre-PENK, and the pattern of labeling is similar to that described above for POMC and PDYN cells. More caudally, these cells intermingle with round, medium-sized, and small PENK cells of the infundibular nucleus, which are less abundant than POMC neurons but show similar robust labeling.

PENK in the tuberal and mammillary regions.—As was mentioned above, PENK neurons are present in all nuclei of the tuberal and mammillary regions except for the mammillary complex itself. The greatest packing density of PENK neurons is in the ventromedial nucleus and paraventricular nucleus (Figs. 7E,F, 8B,C). Significant numbers of labeled neurons also are present in the infundibular nucleus, lateral hypothalamic area, and dorsomedial nucleus (Figs. 7, 8). A few labeled neurons are also present in the tuberomammillary nucleus, premammillary nucleus, and posterior hypothalamic area (Fig. 7, 8). Most PENK neurons of the tuberomammillary nucleus are found in the

dorsolateral subdivision (Fig. 10E). Most neurons of the lateral tuberal nucleus and posterior hypothalamic nucleus are devoid of pre-PENK; only a few neurons of the lateral tuberal nucleus express pre-PENK (very weakly; Fig. 10F).

Extrahypothalamic opioid precursor gene expression

Our tissue samples also included all or part of the amygdala, caudate nucleus, putamen, parolfactory gyrus, globus pallidus, nucleus basalis of Meynert, bed nucleus of the stria terminalis, septum, and infundibular stalk. In several of these regions, we detected PDYN and PENK neurons; extrahypothalamic POMC cells were noted only in the infundibular stalk. Although a detailed description of extrahypothalamic opioids is beyond the scope of this study, we will briefly summarize some of our observations. The caudate nucleus and putamen contain robustly labeled cells expressing both pre-PDYN and pre-PENK. In the caudate nucleus, PDYN cells form islands, whereas PENK cells are distributed more uniformly. We detected numerous robustly labeled PENK neurons but relatively few PDYN neurons in the putamen. PDYN and PENK cells have similar distributions in the anteriormost part of the lateral hypothalamus, where peripheral islands of small neurons of the bed nucleus of the stria terminalis accompany the main core of the nucleus as it traverses the rostral lateral hypothalamic area toward the amygdala (Martin et al., 1991). We observed, at the level of the suprachiasmatic nucleus, only a few PDYN cells in the bed nucleus of the stria terminalis, whereas PENK neurons were more abundant (Figs. 4A,B, 7A,B). We observed, in parts of the amygdala, a differential distribution of PDYN and PENK neurons. The central nucleus of the amygdala contains PENK cells, whereas PDYN neurons are present in the cortical nucleus (Figs. 4C–F, 7E,F). We observed morphologically distinct types of PENK neurons in the nucleus basalis of Meynert (including the nucleus of the diagonal band of Broca; Fig. 11) but did not identify PDYN neurons in this part of the basal forebrain. Clusters of densely packed, very small PDYN and PENK neurons in the nucleus accumbens appeared to invade the territory of the septum and parolfactory gyrus (Figs. 5D; 8C,D).

DISCUSSION

Our knowledge of opioid systems in the brain is based mainly on immunocytochemical and hybridization histochemical studies in rodents and in nonhuman primates (for review, see Khachaturian et al., 1993). Only a few immunocytochemical reports have been devoted to human cerebral opioid systems (Bloch et al., 1978; Gramsch et al., 1979; Maysinger et al., 1982; Haber and Watson, 1985; Abe et al., 1988; Haber et al., 1990; Martin et al., 1991). The present study provides the first detailed description of opioid peptide precursor gene expression in the human hypothalamus and basal forebrain.

The differential distribution of separate classes of opioid peptide-containing neurons has been described previously in rodents and in nonhuman primates (Khachaturian et al., 1985b; Haber et al., 1990). We observed a distinct anatomical segregation of the three different opioid peptide precursor mRNA in the human hypothalamus and adjacent structures. Specifically, pre-POMC gene expression is largely restricted to neurons of the mediobasal hypothalamus. PDYN and PENK neurons are more widely represented in the human

basal forebrain, although they differ in distribution. PDYN neurons are located primarily in the tuberal and dorsal posterior hypothalamus, whereas PENK neurons predominate in the chiasmatic region. The expression of PDYN and PENK genes in several different extrahypothalamic cell populations contrasts sharply with the absence, except in the infundibular stalk, of POMC neurons outside the hypothalamus.

POMC:

Our study shows that the distribution of POMC neurons in humans is similar to that observed in rodents and in nonhuman primates (Khachaturian et al., 1984; Lewis et al., 1984; Haber et al., 1990). In particular, the human retrochiasmatic area and infundibular nucleus contain significant populations of POMC cells. In rodents and monkeys, these POMC-enriched areas have extensive projections throughout the brain and innervate several diencephalic, limbic, mesencephalic, and lower brainstem structures (Khachaturian et al., 1985a; Haber et al., 1990). Because POMC efferents are comparable in rodents and monkeys, it is conceivable that similar connections are present in humans.

Numerous lines of evidence indicate that opioid peptides have an inhibitory influence in the regulation of gonadotropin secretion (Ferin et al., 1984; Howlett and Rees, 1986; Kalra and Kalra, 1986; Gindoff and Ferin, 1987). This role is best documented for the POMC system. POMC neurons are located within the medial basal hypothalamus, the primary control center for reproduction (Krey et al., 1975; Knobil, 1980) and the location of the gonadotropin-releasing hormone pulse generator (Wilson et al., 1984). POMC efferents terminate on capillaries in the median eminence, and β-endorphin is secreted into the hypophyseal portal circulation of rats (Wehrenberg et al., 1982) and primates (Wardlaw et al., 1980; Gindoff and Ferin, 1987). In addition, levels of β-endorphin in portal blood are modified by ovarian steroids and vary with the menstrual cycle (Wardlaw et al., 1982). Numerous studies have shown that POMC gene expression in the medial basal hypothalamus is modified by gonadal steroids (Wilcox and Roberts, 1985; Chowen-Breed et al., 1989; Tong et al., 1990; Wise et al., 1990; Adams et al., 1991; Rasmussen et al., 1992; Treiser and Wardlaw, 1992; Pelletier, 1993). Only a small population of β-endorphin-immunoreactive neurons in the medial basal hypothalamus of rats and mice concentrates estrogen (Morrell et al., 1985; Jirikowski et al., 1986). However, there are increased numbers of arcuate β-endorphin-immunoreactive neurons after estrogen treatment in the guinea pig (Thornton et al., 1994), and estrogen receptors are colocalized in 15–20% of β-endorphin cells in the medial basal hypothalamus of sheep (Lehman and Karsch, 1993).

PDYN:

Information on the distribution of PDYN is limited to immunocytochemical studies in rodents, rhesus monkeys (Khachaturian et al., 1982, 1985a), and humans (Abe et al., 1988). We confirmed a previous description of PDYN immunoreactivity in magnocellular neurons of the human paraventricular nucleus (Abe et al., 1988), especially its caudal part. Whether these neurons coexpress PDYN and vasopressin (Watson et al., 1981) remains to be assessed in double-labeling studies. We found in the SON only a few PDYN neurons, in contrast to immunocytochemical data that indicate many dynorphin-like immunoreactive neurons in this area (Abe et al., 1988). In agreement with the demonstration of dynorphin-like

immunoreactive perikarya in the perifornical area of rats (Khachaturian et al., 1985a; Fallon and Leslie, 1986) and humans (Abe et al., 1988), we observed relatively large numbers of PDYN neurons in the dorsomedial nucleus and lateral hypothalamic area surrounding the fornix from the medial and lateral sides, respectively. In rats, the perifornical area is implicated in the control of cardiovascular function (Allen and Cechetto, 1992, 1993; Oppenheimer et al., 1992) and eating (Stanley et al., 1993a,b).

The most striking finding in the human hypothalamic PDYN system is the presence of densely packed PDYN neurons in the premammillary nucleus. Knowledge of premammillary nuclear function and connectivity is extremely limited. Unlike the case in rodents, where the premammillary nucleus has been subdivided into dorsal and ventral components (Krieg, 1932; Saper et al., 1979), in nonhuman primates (Veazey et al., 1982) and humans this nucleus is relatively meager and is composed of small and medium-sized neurons.

Recently, anterograde and retrograde tracing studies have described connections of the dorsal and ventral parts of the premammillary nucleus in rodents (Canteras and Swanson, 1992; Canteras et al., 1992). Current evidence suggests that the premammillary nucleus may play an important role in goal-oriented behavior associated with hunger, thirst, and reproduction (Canteras and Swanson, 1992). The connectivity of the ventral premammillary nucleus implicates this structure in neuroendocrine and sexually dimorphic circuitry (Canteras et al., 1992). Our hybridization histochemical preparations show that the majority of neurons in the human premammillary nucleus express the pre-PDYN gene, readily distinguishing this nucleus from adjacent hypothalamic nuclei. Thus, PDYN may be a key peptide in the function of the premammillary nucleus.

Another significant group of PDYN neurons occurs in the dorsal posterior hypothalamic area. The neuronal composition of the posterior hypothalamic area is heterogeneous (Veazey et al., 1982). The posterior hypothalamic nucleus consists of large neurons, some of which stray into the posterior hypothalamic area in ventrodorsal and mediolateral directions. The majority of posterior hypothalamic nucleus neurons express pre-PDYN. The remainder of the posterior hypothalamic area consists of small and medium-sized neurons that make up the posterior hypothalamic gray; some of these neurons contain pre-PDYN. There is no information on PDYN in posterior hypothalamic structures of rodents or monkeys.

PENK:

Of the three classes of opioid cells, PENK neurons are the most abundant in the human hypothalamus. All structures except the mammillary bodies contain PENK neurons. The massive numbers of PENK neurons throughout hypothalamic structures suggest that PENK neurons participate in numerous homeostatic functions. However, the chiasmatic region has the greatest number of neurons, especially in the intermediate nucleus, but also in the suprachiasmatic nucleus and scattered throughout the medial and lateral preoptic regions. There was also robust labeling of neurons by the pre-PENK probe in the bed nucleus of the stria terminalis and in the central nucleus of the amygdala. Several of these structures have been reported to be sexually dimorphic in humans (Swaab and Fliers, 1985; Allen et al., 1989; Hofman and Swaab, 1989; Allen and Gorski, 1990; LeVay, 1991) and rats (Gorski,

1968; Simerly et al., 1984, 1988; Turkenburg et al., 1988; De Jonge et al., 1989; Herbison and Dye, 1993).

The present study provides the first identification of PENK mRNA in the intermediate nucleus of the hypothalamus. Nearly all neurons in the intermediate nucleus were labeled with the pre-PENK probe. The intermediate nucleus is a well-defined collection of darkly staining neurons that lies within the preoptic area between the supraoptic nucleus and paraventricular nuclei and rostral to the accessory magnocellular nuclei (Brockhaus, 1942; Braak and Braak, 1987; Saper, 1990). In 1985, Swaab and Fliers reported that the volume of the intermediate nucleus is more than twice as large in men as in women. These authors referred to the intermediate nucleus as the "sexually dimorphic nucleus of the preoptic (SDN-POA)," implying that this nucleus is homologous to the SDN-POA described in rat brain (Gorski et al., 1978, 1980; Simerly et al., 1984); however, this issue has become controversial (Allen et al., 1989; LeVay, 1991).

Additional studies will be necessary to determine whether and where PENK neurons are sexually dimorphic in the human hypothalamus. Sexual dimorphism has been found in the number of enkephalin-immunoreactive cells within an anteroventral periventricular nucleus of the rat (Simerly et al., 1988). However, enkephalin-immunoreactive neurons were not identified in SDN-POA of the rat preoptic area (Simerly et al., 1988).

For the hypothalamic magnocellular complex, we observed PENK cells mainly in the parvicellular caudal paraventricular nucleus and in the periphery of dorsolateral and ventromedial portions of the supraoptic nucleus. A hybridization histochemical study in rats showed labeling in both parvicellular and magnocellular neurons (Harlan et al., 1987). Immunocytochemical studies have been inconsistent, showing labeling in parvicellular neurons only (Wamsley et al., 1980; Hokfelt et al., 1983; Khachaturian et al., 1983), in both parvicellular and magnocellular neurons (Sar et al., 1978; Sawchenko et al., 1982) of rodents or, in bovine brain, in magnocellular neurons only (Vanderhaeghen et al., 1983). The location of PENK neurons in the periphery of the supraoptic nucleus and the small size of PENK neurons in both the paraventricular nucleus and the supraoptic nucleus indicate that PENK could colocalize with oxytocin in some neurons (Meister et al., 1990; Sukhov et al., 1993). Recently, it has been suggested that the expression of enkephalin in both vasopressin and oxytocin neurons in rats may increase in response to chronic stress, thus supplementing parvicellular neurons as a source of enkephalin (Young and Lightman, 1992). It remains to be determined whether similar processes occur in nonhuman primates and humans.

The presence of a significant population of PENK neurons in the ventromedial nucleus corresponds well with results obtained in rats (Shivers et al., 1986; Harlan et al., 1987). One suggested function of the ventromedial nucleus in rats is the regulation of steroid-dependent, motivated behavior through progesterone, estrogen, and androgen receptors (Pfaff, 1980; Simerly et al., 1990). In addition, the ventromedial nucleus exhibits sexually dimorphic features (Matsumoto and Arai, 1983), and pre-PENK gene expression is regulated by estrogens (Romano et al., 1990). Because we found the greatest expression of pre-PENK in structures such as the medial preoptic area, ventromedial nucleus, amygdala, and bed nucleus of the stria terminalis, which are thought to mediate the hormonal control of

copulatory behavior, it may be informative to relate the expression of androgen and estrogen receptors to that of pre-PENK in these areas.

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Abbreviations

3v	third ventricle
A	amygdala, central nucleus
AC	anterior commissure
Ac	amygdala, cortical nucleus
BNST	bed nucleus of the stria terminalis
DBB	diagonal band of Broca
DMN	dorsomedial nucleus
F	fornix
FF	fields of Fore1
GPe	globus pallidus pars externa
GPi	globus pallidus pars interna
HG	hypothalamic gray
IC	internal capsule
IN	intermediate nucleus
INAH-1	intermediate nucleus of anterior hypothalamus-1
INF	infundibular nucleus
IS	infundibular stalk
LH	lateral hypothalamus
LT	lateral tuberal nucleus
LV	lateral ventricle
MB	mammillary body
MPA	medial preoptic area

MT	mammillothalamic tract
NA	nucleus accumbens
NBM oc	nucleus basalis of Meynert
OT	optic tract
P	putamen
PDYN	prodynorphin
PENK	proenkephalin
PH	posterior hypothalamus
PN	premammillary nucleus
POA	preoptic area
POG	parolfactory gyrus
POMC	proopiomelanocortin
por	preoptic recess
pre-PDYN	preprodynorphin
pre-PENK	preproenkephalin
pre-POMC	preproopiomelanocortin
PS	precommissural septum
PVN	paraventricular nucleus
RA	retrochiasmatic area
S	septum
SCN	suprachiasmatic nucleus
SDN-POA	sexually dimorphic nucleus of preoptic area
SI	substantia innominata
SONd	supraoptic nucleus, dorsolateral
SONv	supraoptic nucleus, ventromedial
TM	tuberomammillary nucleus
VMN	ventromedial nucleus
VP	ventral pallidum
ZI	zona incerta

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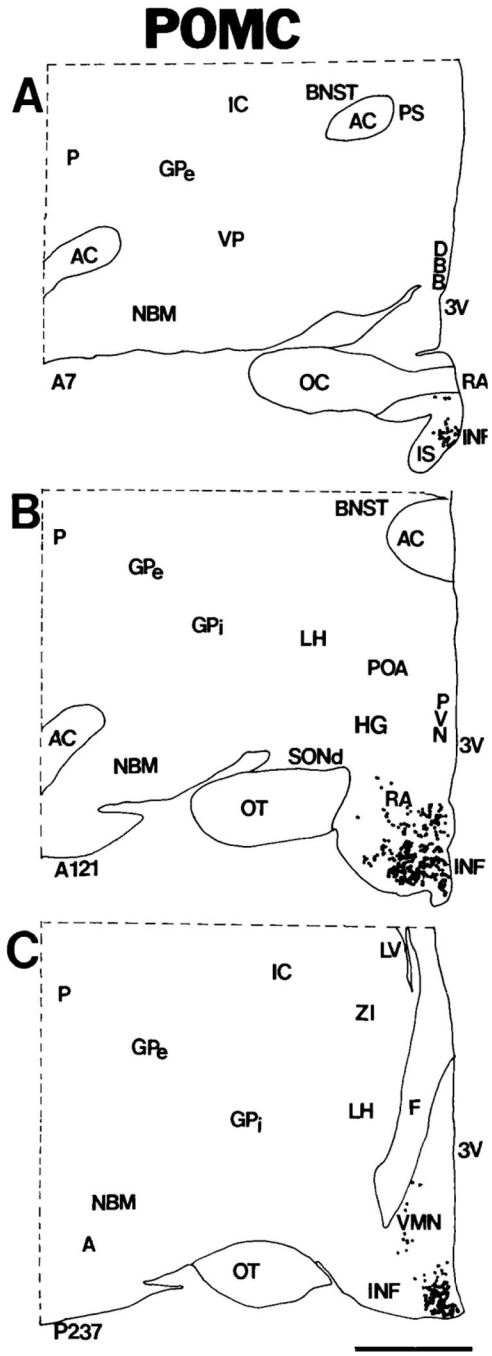

Figure 1.

A-C: Computer-assisted maps of the distribution of POMC cells in coronal sections of human hypothalamus arranged rostrocaudally from A to C. Each dot represents a single neuron. Numbers at the lower left correspond to sequential locations of sections from anterior to posterior. Each section is 20 microns thick; hence, the distance between A and C is ~4.6 mm. Scale bar = 5 mm.

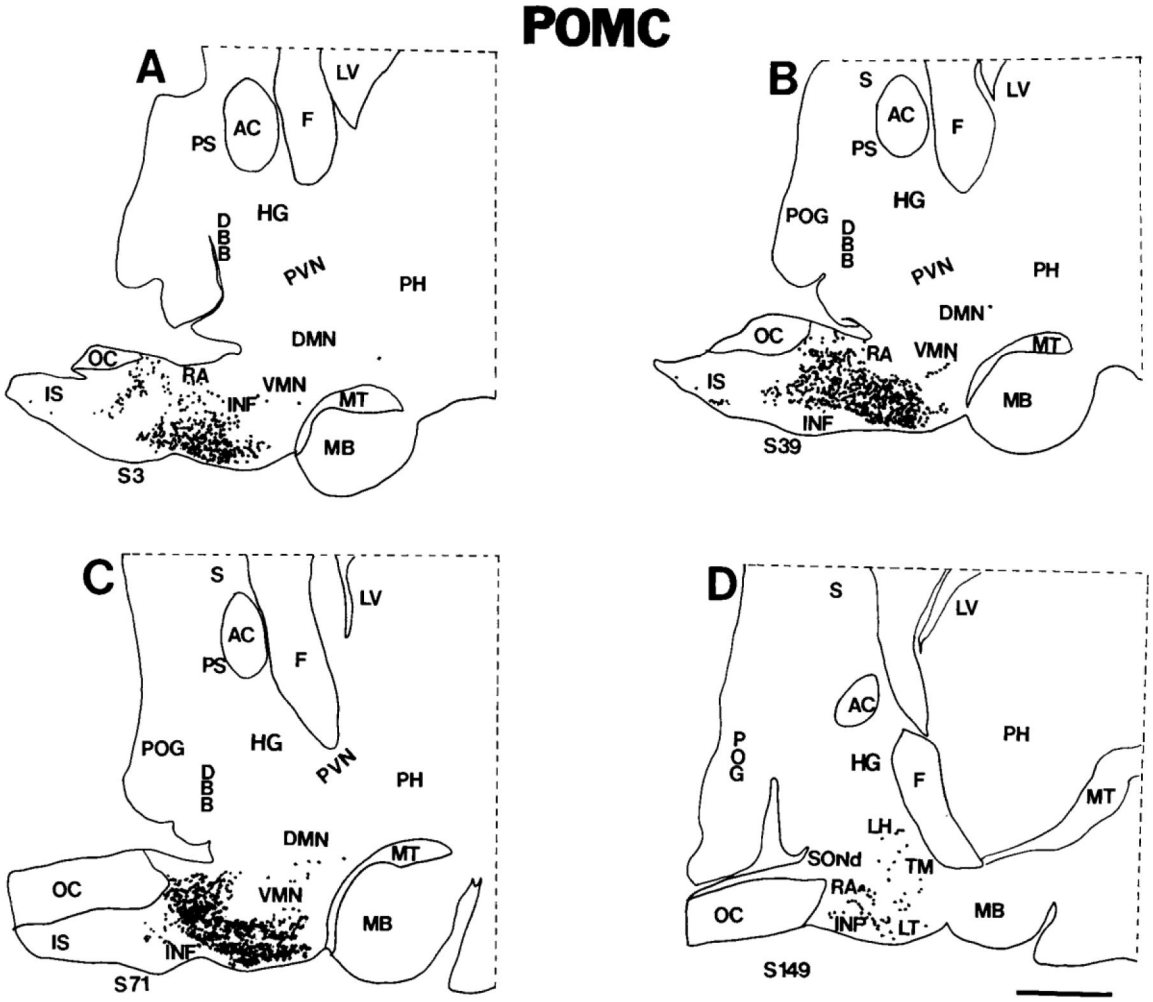

Figure 2.

A-D: Computer-assisted maps of the distribution of POMC cells in sagittal sections of the human hypothalamus. The most medial section is A, and the most lateral section is D. Sagittal sections are numbered serially from medial to lateral; each section is 20 microns thick; hence, the distance between A and D is ~1.9 mm. Each dot represents a single neuron. Scale bar = 5 mm.

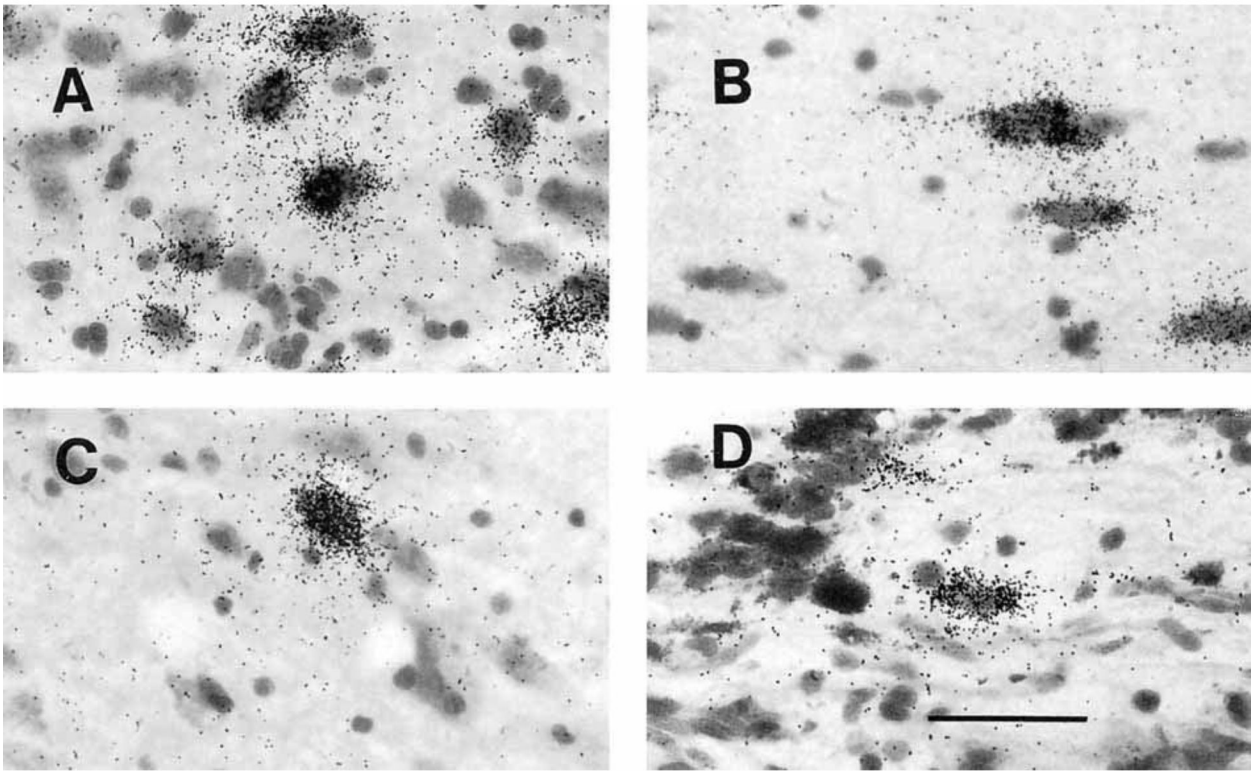

Figure 3.

Photomicrographs of POMC cells in the human hypothalamus. A: Robustly labeled neurons of the infundibular nucleus. B: Typical oblong neurons of the retrochiasmatic area. The long axis is oriented toward infundibular stalk. C: One of the rare POMC neurons in the territory of the ventromedial nucleus. D: Cell that contains POMC mRNA in the infundibular stalk. Scale bar = 50 microns.

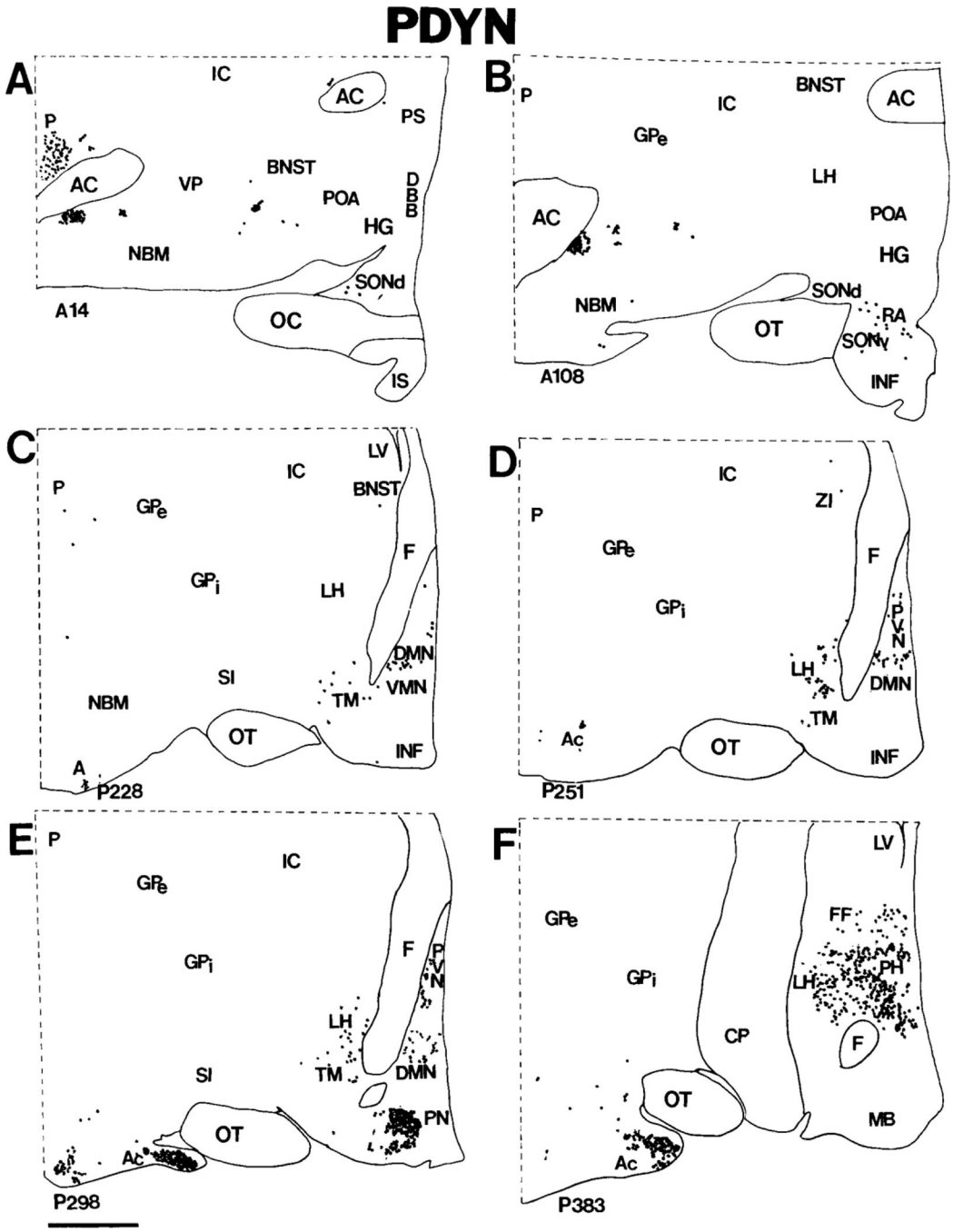

Figure 4.

A-F: Computer-assisted maps of the distribution of PDYN cells in coronal sections of the human hypothalamus arranged rostrocaudally from A to F. The most anterior section is A, and the most posterior section is F. Numbers at the lower left correspond to sequential locations of sections from anterior to posterior. Each section is 20 microns thick. Each dot represents a single neuron. Scale bar = 5 mm.

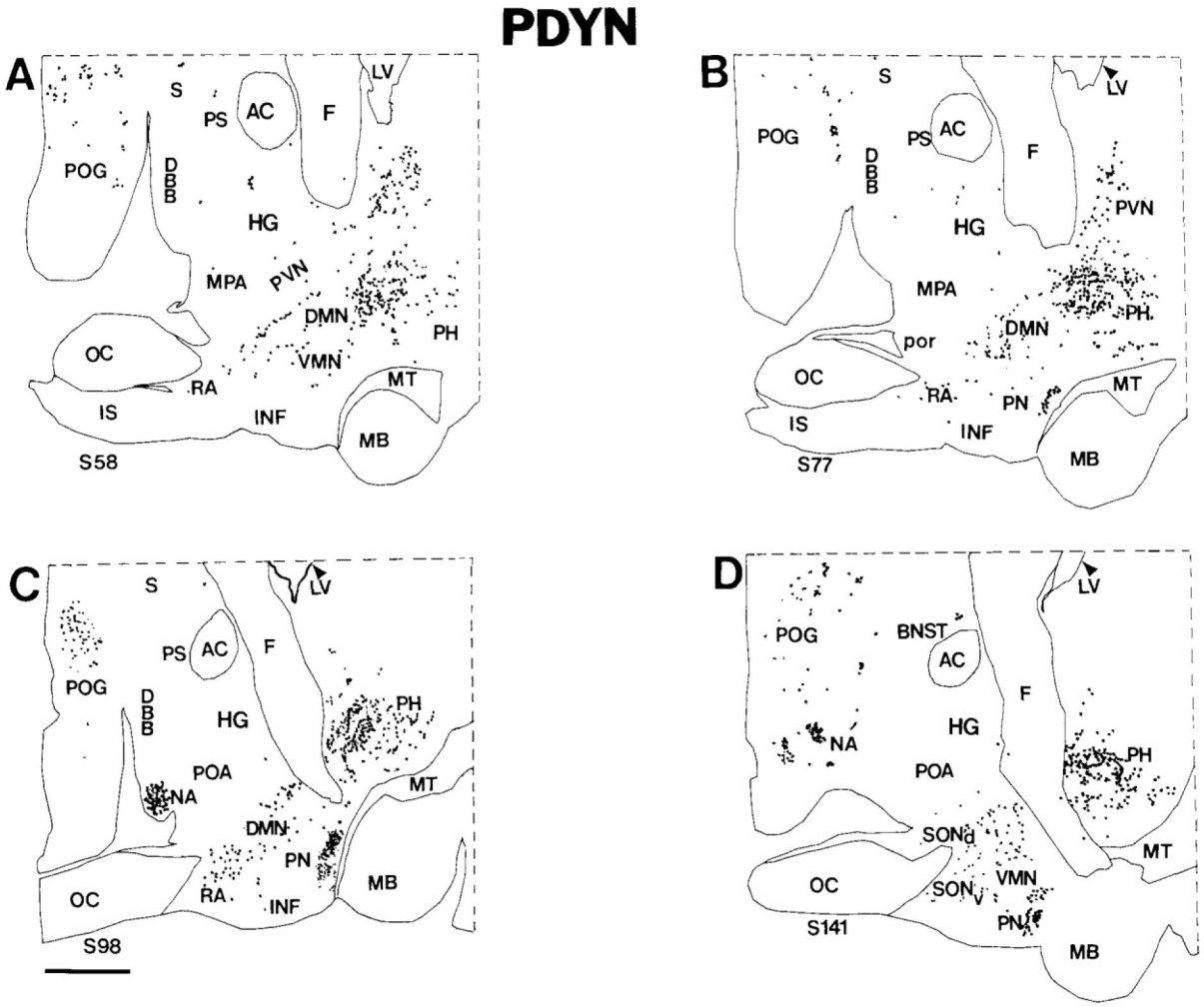

Figure 5.

A-D: Computer-assisted maps of the distribution of PDYN cells in sagittal sections of the human hypothalamus. The most medial section is A, and the most lateral section is D. Each dot represents a single neuron. Scale bar = 5 mm.

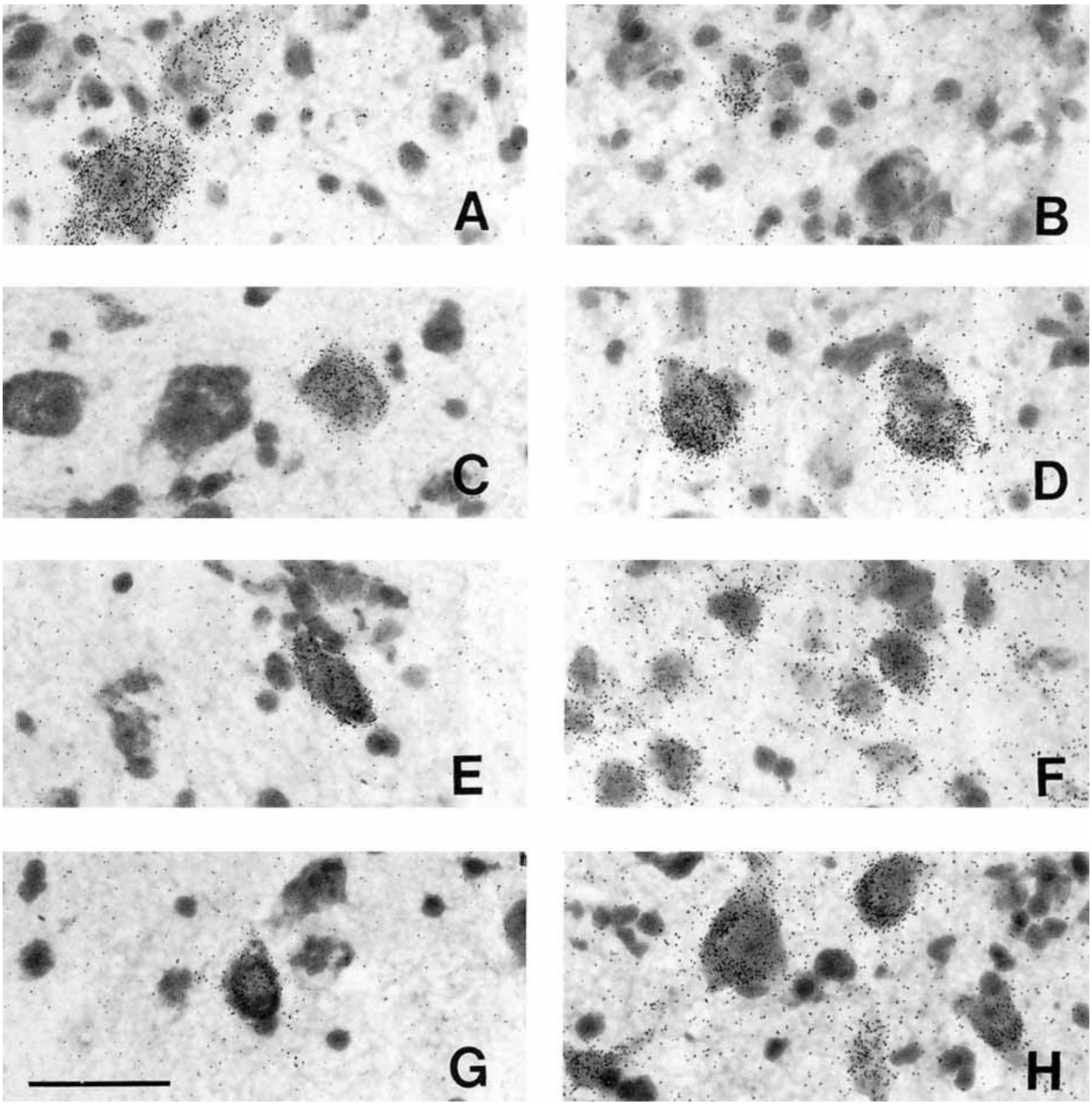

Figure 6.

Photomicrographs of PDYN neurons in the human hypothalamus. A: Moderately and lightly labeled magnocellular neurons of the caudal paraventricular nucleus. B: Lightly labeled, small, peripherally located neuron in the dorsolateral supraoptic nucleus. C: Typical PDYN neuron of the tuberomammillary nucleus. D: Large, heavily labeled neurons of the posterior hypothalamic nucleus (coronal view). E: One of the few PDYN neurons of the retrochiasmatic area. F: PDYN neurons of the premammillary nucleus. G: Lightly labeled neuron of the ventromedial nucleus. H: PDYN neurons of the dorsomedial nucleus. Scale bar = 50 microns.

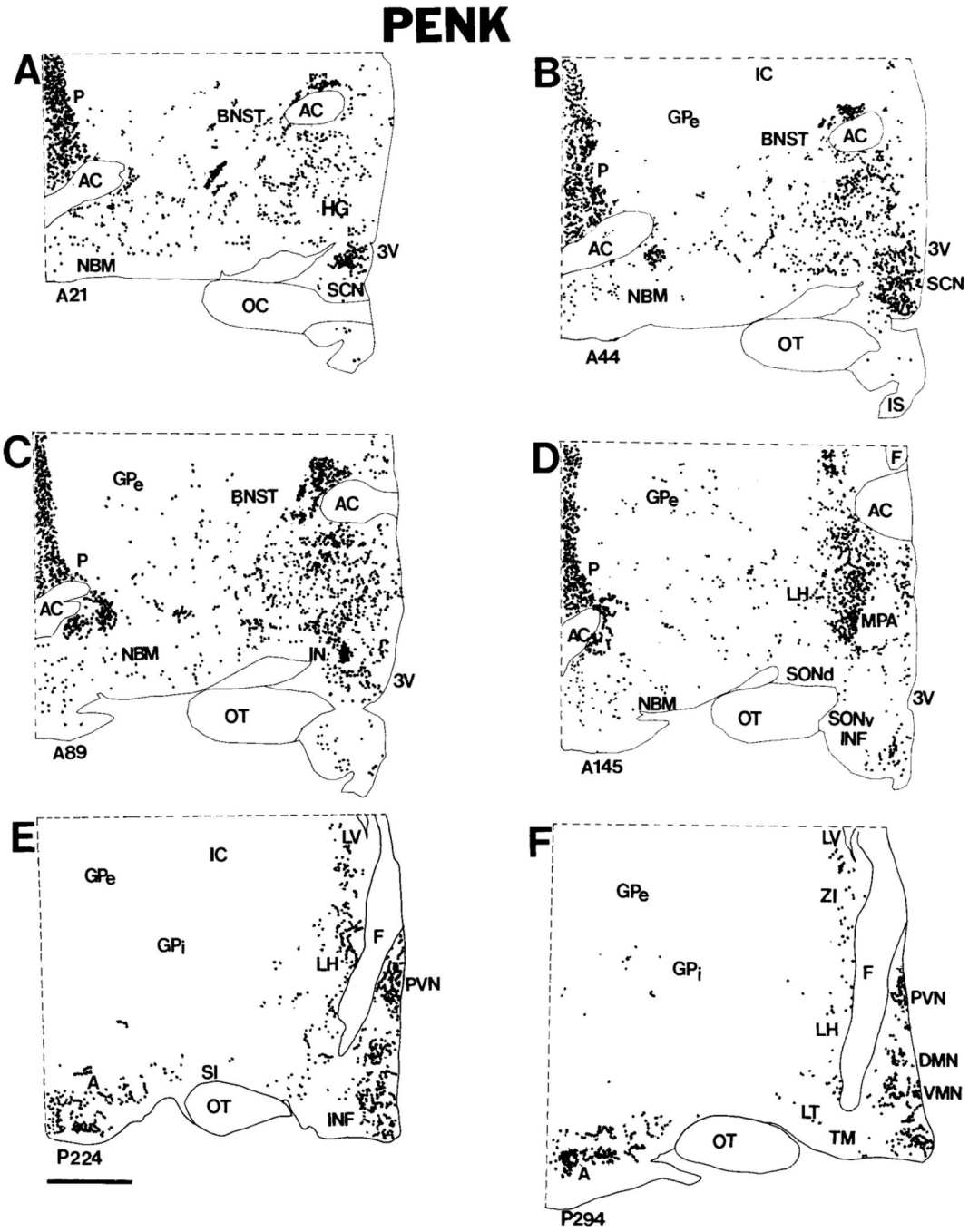

Figure 7.

A-F: Computer-assisted maps of the distribution of PENK cells in coronal sections of the human hypothalamus. The most anterior section is A, and the most posterior section is F. Each dot represents a single neuron. Numbers at the lower left correspond to sequential locations of sections from anterior to posterior. Each section is 20 microns thick. Scale bar = 5 mm.

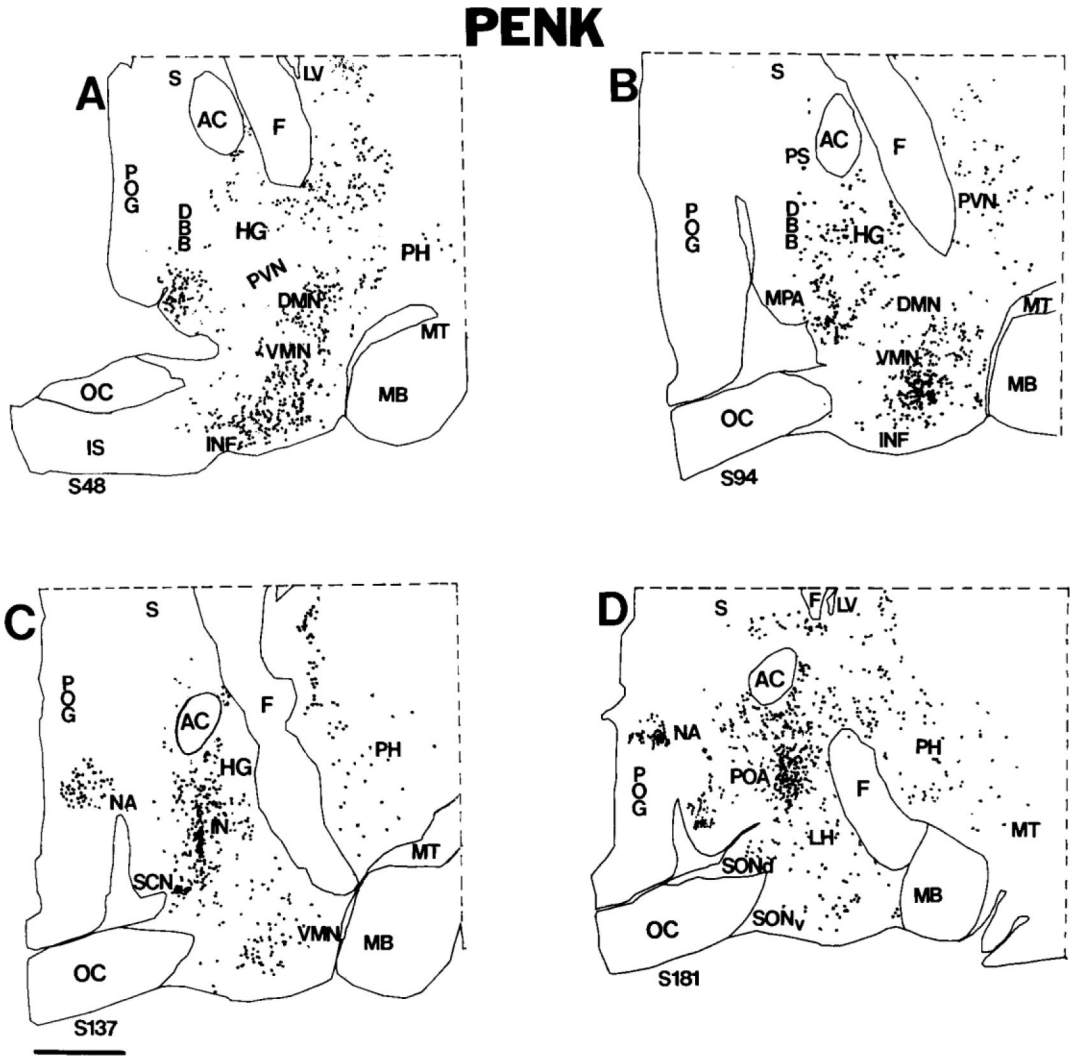

Figure 8.

A-D: Computer-assisted maps of the distribution of PENK cells in 20-micron-thick sagittal sections of the human hypothalamus. The most medial section is A, and the most lateral section is D. Each dot represents a single neuron. Scale bar = 5 mm.

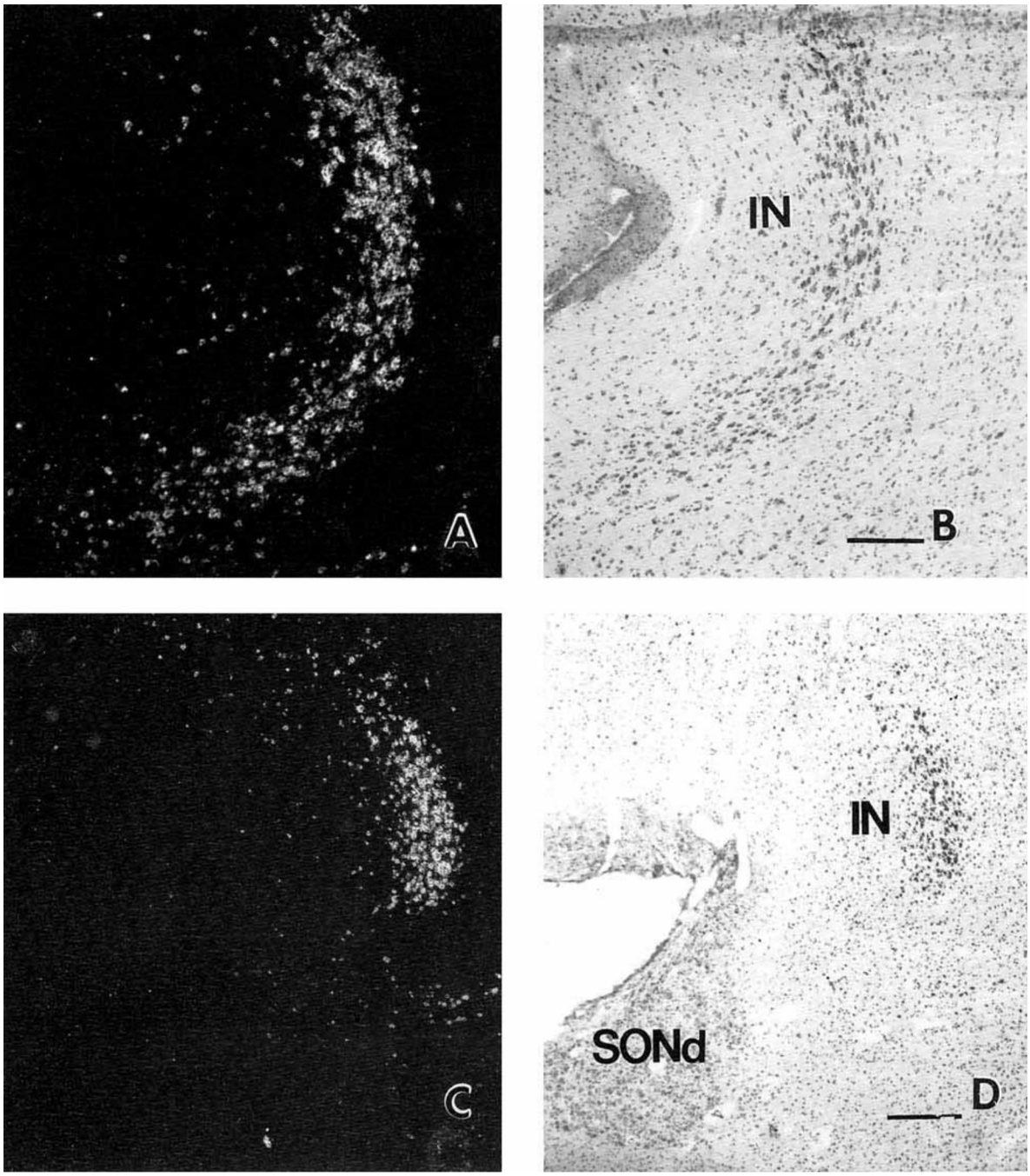

Figure 9.

Pre-PENK gene expression in neurons of the intermediate nucleus of the human hypothalamus. Darkfield (A) and brightfield (B) photomicrographs of the intermediate nucleus in a sagittal section; rostral is to the left, and ventral is toward the bottom. Lower magnification darkfield (C) and brightfield (D) photomicrographs of the human intermediate nucleus in a coronal section. Scale bars = 740 microns in A,B, and 1,480 microns in C,D.

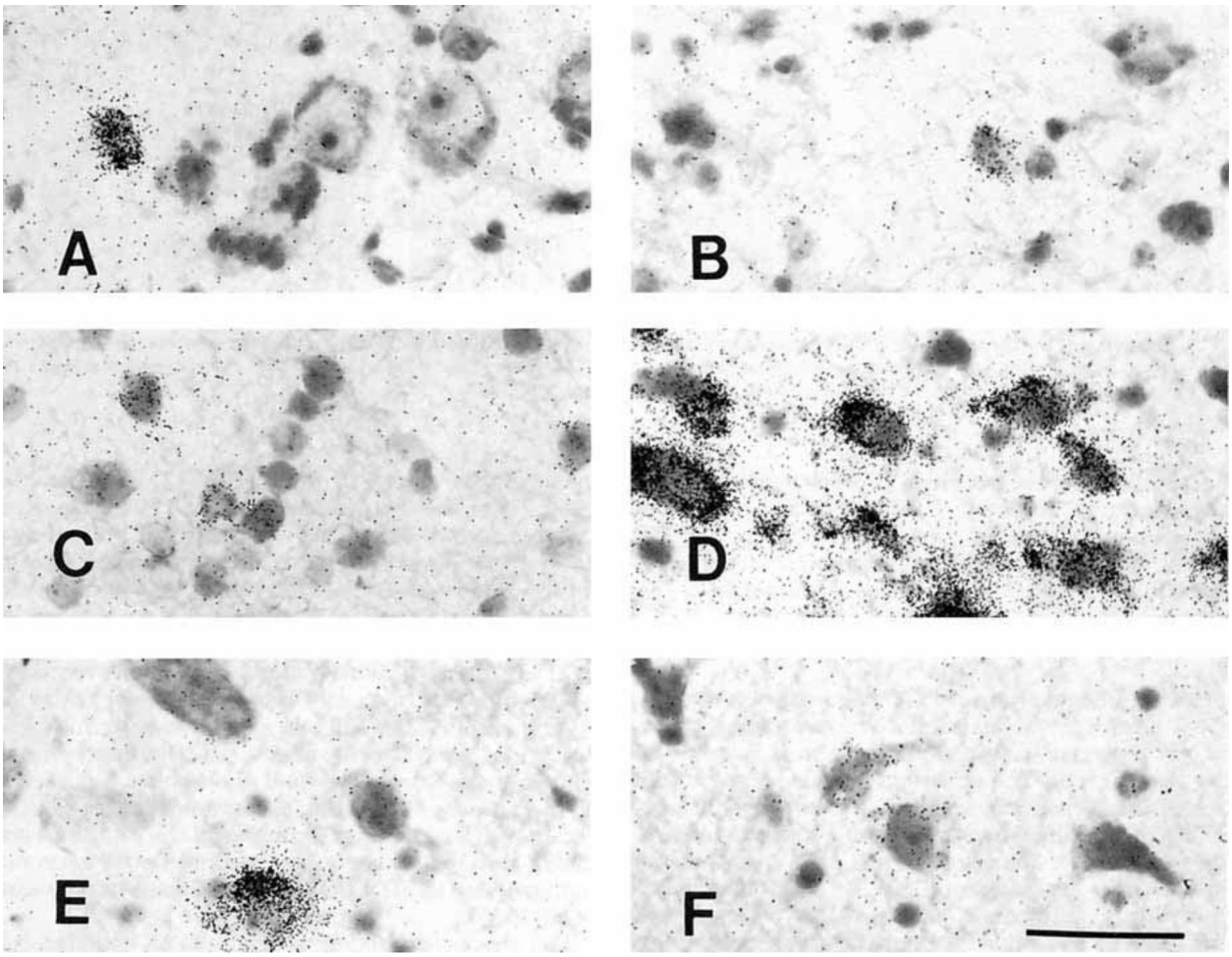

Figure 10.

Photomicrographs of PENK cells in the human hypothalamus. A: Typical parvicellular PENK neuron in the caudal paraventricular nucleus. B: Small, lightly labeled neuron of the dorsolateral supraoptic nucleus. C: PENK neurons of the dorsal suprachiasmatic nucleus; cells are typically small and lightly labeled. D: Robustly labeled, medium-sized, elongated neurons of the intermediate nucleus. E: PENK neurons of the tuberomammillary nucleus. F: Lightly labeled neurons of the lateral tuberal nucleus. Scale bar = 50 microns.

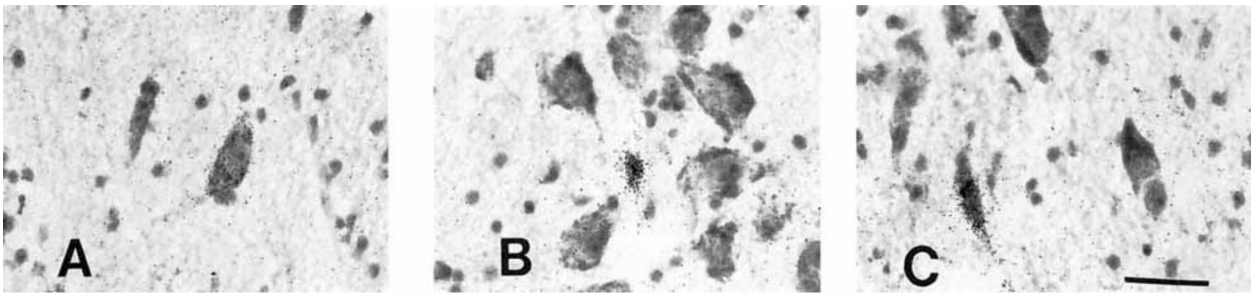

Figure 11.

Examples of the three types of PENK neurons in the basal forebrain magnocellular complex. A: Magnocellular neuron of the nucleus of the diagonal band of Broca. B: Small neuron of the nucleus basalis of Meynert. C: Typical elongated neurons of the nucleus basalis of Meynert; oblong cells usually abut the anterior commissure. Scale bar = 40 microns.

TABLE 1.

Perimortem Histories of Human Subjects

Case no.	Sex	Age (years)	Cause of death	Postmortem interval (hours)
426	Male	61	Acute myocardial infarction	13
810	Male	16	Lacerated lung	12
814	Male	52	Asphyxiation	4
871	Male	23	Gunshot wound	7

TABLE 2.

Opioid Peptide Precursor Gene Expression in Neurons of the Hypothalamic Nuclei and Some Extrahypothalamic Structures[1]

Region	Structure	Pre-POMC	Pre-PDYN	Pre-PENK
Chiasmatic	Supraoptic nucleus, dorsolateral	−	+	+
	Supraoptic nucleus, ventromedial	−	+	+
	Medial preoptic area	−	−	+++
	Intermediate nucleus	−	−	+++
	Suprachiasmatic nucleus	−	−	+++
	Paraventricular nucleus	−	+	+++
	Retrochiasmatic area	+++	++	++
Tuberal	Paraventricular nucleus	−	++	++
	Dorsomedial nucleus	−	+++	+
	Ventromedial nucleus	+	++	+++
	Premammillary nucleus	−	+++	+
	Lateral tuberal nucleus	−	−	+
	Tuberomammillary nucleus	−	+	+
	Infundibular nucleus	+++	−	+++
Mammillary	Mammillary body	−	−	−
	Posterior hypothalamus	−	+++	++
	Paraventricular nucleus	−	++	++
Extrahypothalamic	Amygdala, central nucleus	−	−	+++
	Amygdala, cortical nucleus	−	+++	−
	Bed nucleus of the stria terminalis	−	++	+++
	Nucleus basalis of Meynert	−	−	+
	Nucleus accumbens	−	+++	+
	Putamen	−	+	+++
	Caudate nucleus	−	++	+
	Globus pallidus	−	−	++
	Parolfactory gyrus	−	+	+
	Zona incerta	−	++	++

[1]−, Opioid peptide precursor mRNA-containing cells were absent (0% of the whole neuronal population); +, only a few cells that express the opioid peptide precursor gene were present in the structure (1–10%); ++, cells that express the opioid peptide precursor gene were present in moderate numbers in the nucleus (10–60%); +++, cells that express the opioid peptide precursor gene were very abundant in the nucleus (60–100%).