

Parathyroid hormone-related peptide gene is expressed in the mammalian central nervous system

(neuropeptide/*in situ* hybridization)

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ABSTRACT A parathyroid hormone-related peptide (PTHrP) has been identified in human tumors associated with the syndrome of humoral hypercalcemia of malignancy. While parathyroid hormone (PTH) gene expression appears to be limited to the parathyroid glands, PTHrP mRNA has been identified in a variety of normal tissues. To investigate the apparent expression of the PTHrP in the central nervous system, we examined extracts of whole rat brain for PTHrP bioactivity by measuring adenylate cyclase-stimulating activity (ACSA) in a PTH-sensitive assay. Extracts consistently contained ACSA and this activity was completely inhibited by a PTHrP antiserum but was unaffected by a PTH antiserum. ACSA was found in a number of anatomic subregions of rat brain, being greatest in the cortex and telencephalon. RNase protection analysis revealed PTHrP transcripts in total RNA prepared from whole rat brain and from the same anatomic subregions. By *in situ* hybridization histochemistry, we found that the highest levels of PTHrP gene expression occurred in neurons of the cerebral cortex, hippocampus, and cerebellar cortex. These studies demonstrate that both PTHrP mRNA and biological activity are present in a number of regions of rat brain. The widespread expression of this peptide by multiple types of neurons suggests that the PTHrP may play a general role in neuronal physiology.

A parathyroid hormone-related peptide (PTHrP) has been identified and its cDNA was cloned (1–3) from human tumors associated with the syndrome of humoral hypercalcemia of malignancy (HHM). The deduced PTHrP product is almost twice the size of parathyroid hormone (PTH) and has sequence similarity to human PTH confined to its N terminus (1–3). N-terminal PTHrP fragments have been synthesized and shown to stimulate PTH-sensitive systems *in vivo* and *in vitro* and to bind to PTH receptors in bone and kidney *in vitro* (4). However, the final secretory form of the PTHrP has yet to be established for any cell type, and unique PTHrP receptors have not yet been demonstrated.

The PTH and PTHrP genes appear to have arisen by duplication and to represent members of a gene family (2, 5). Whereas the PTH gene seems to be expressed exclusively in the parathyroid glands, PTHrP transcripts have been identified in poly(A)⁺ RNA prepared from a variety of normal tissues, including skin, the parathyroids, lactating mammary tissue, and whole rat brain (2, 6, 7). The precise role of the PTHrP in these tissues is unknown.

To investigate further expression of the PTHrP in the central nervous system, we used *in situ* hybridization histochemistry in conjunction with RNase protection studies to show that neurons of diverse regions of the mammalian

nervous system express PTHrP mRNA. A bioassay, based on the ability of the PTHrP to stimulate adenylate cyclase, confirmed that the high levels of PTHrP mRNA found are translated. The widespread expression of this peptide by multiple neuronal types, as compared to the restricted pattern of expression observed for many neuropeptides, suggests that the PTHrP may play a general role in normal neuronal physiology.

METHODS

Tissue Extraction and Characterization of Adenylate Cyclase-Stimulating Activity (ACSA). Rats underwent euthanasia by carbon dioxide asphyxiation; whole brains were removed and, in some cases, were dissected into five anatomic subregions. All tissues were stored at -70°C . Acid-urea extracts were prepared as described (8). We used the rat osteosarcoma cell (ROS) assay (9) to measure ACSA in brain tissue extracts. This method uses a PTH-sensitive ROS cell line (17/2.8) to measure the conversion of [³H]ATP to [³H]cAMP in response to binding to PTH receptors (9). The detection limit of the assay is 5×10^{-11} M bovine PTH (bPTH)-(1–34). The dose-response curve of ACSA in brain extract in the ROS assay was compared with that of synthetic [bPTH-(1–34)] (Bachem) and human [Tyr³⁶]PTHrP-(1–36) amide (4). Whole brain extract was also assayed in the presence of the synthetic competitive PTH antagonist [Nle^{8,18},Tyr³⁴]bPTH-(7–34) amide (Bachem).

Antibody neutralization studies were performed with a rabbit polyclonal anti-bPTH-(1–34) antiserum, JH-42 (Meloy Laboratories) (10), and a rabbit polyclonal antiserum (R13) prepared to human PTHrP [hPTHrP-(1–74)]. hPTHrP-(1–74), synthesized as described (11), was conjugated to bovine serum albumin (Sigma), emulsified in Freund's adjuvant (Difco), and injected monthly into New Zealand White rabbits. Antiserum R13 was harvested after the sixth injection. This antiserum inhibited hPTHrP-(1–36) activity in the ROS assay but had no effect on bPTH-(1–34) activity (data not shown). For immunoneutralization experiments, brain extract was incubated for 24 hr at 4°C with phosphate-buffered saline (PBS) or with an equivalent volume of antiserum diluted in PBS to give a final dilution of 1:50. This first-generation antiserum has not proven useful for immunohistochemistry and has thus far not been used to determine PTHrP immunoreactivity in tissue extracts by radioimmunoassay.

RNase Protection Assay. Whole brain or anatomic subregions were used to prepare total RNA by the guanidinium

Abbreviations: PTH, parathyroid hormone; bPTH, bovine PTH; PTHrP, PTH-related peptide; hPTHrP, human PTHrP; HHM, humoral hypercalcemia of malignancy; ACSA, adenylate cyclase-stimulating activity; ROS, rat osteosarcoma.

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thiocyanate/cesium chloride technique (12). Total RNA was analyzed by RNase protection assay (13) using 2×10^5 cpm of antisense RNA corresponding to the coding region of the rat PTHRP cDNA (6). A 343-base-pair *Pvu* II/*Bgl* II fragment of the rat cDNA was subcloned into a BlueScript vector (Stratagene) and 32 P-labeled antisense RNA prepared with T7 RNA polymerase (7).

In Situ Hybridization Histochemistry. Adult female Sprague-Dawley rats were decapitated under pentobarbital anesthesia and their brains were frozen on dry ice in cryostat embedding medium (Histo-prep, Fisher). Tissues were stored at -70°C until use within 1 month. Ten-micrometer cryostat sections were thaw-mounted onto slides coated with gelatin/chrome alum, dried 5 min at room temperature, and stored at -70°C . Three pairs of sense and antisense oligonucleotide probes were used in these experiments. We initially used a pair of 45-mers corresponding to amino acids -2 to $+13$ of the hPTHRP (12). These oligonucleotides had a G+C content of 58% and had eight mismatches with the rat PTHRP nucleotide sequence. We subsequently synthesized and used a pair of 40-mers (55% G+C) corresponding to amino acids 31–43 and a pair of 26-mers (50% G+C) corresponding to amino acids 62–70; these regions are PTHRP specific, and the human and rat nucleotide sequences are identical (1–3, 6).

Hybridization was accomplished by standard techniques (14) using tissue fixed by 3% paraformaldehyde and probes 3' end-labeled with terminal transferase and either ^{35}S (15, 16) or digoxigenin-UTP by the protocol provided by Boehringer Mannheim. For hybridization, 5×10^5 cpm of radioactive or 60 ng of digoxigenin probe was added per slide in a total vol of 300 μl of hybridization buffer. Sections were incubated overnight at 37°C (5°C below the calculated hybrid melting point of 42°C for $4\times$ SSC/47% formamide) ($1\times$ SSC = 0.15 M NaCl/0.015 M sodium citrate), followed by washing at high stringency (46°C in $0.5\times$ SSC/24% formamide) for a total of 3.5 hr. Radioactively labeled sections were exposed to x-ray film (Hyperfilm Bmax; Amersham) for 2–6 wk. Hybridization to digoxigenin-labeled tissue was visualized by incubation with an anti-digoxigenin antibody complexed to alkaline phosphatase and developed with a nitroblue tetrazolium color solution for 6–24 hr. Control incubations performed with colorization solution and/or antibody but without probes were negative. Since the neurons found to express high levels of PTHRP mRNA possess abundant total RNA, as evidenced by their intense Nissl staining, a variety of controls were prepared to ensure that hybridization was specific for PTHRP. First, sense probes failed to hybridize or hybridized only weakly (e.g., over cerebellar granule cells) under high-stringency conditions. Second, identical findings were obtained with three different oligonucleotide pairs. Third, a pronounced loss of labeling was observed when hybridizations were carried out 10°C above the melting temperature (data not shown). Fourth, hybridization of antisense probe was eliminated or greatly reduced by pretreatment of tissue sections with RNase or a 100-fold excess of unlabeled probe, respectively (data not shown). Finally, we have used these same hybridization techniques with probes for prosomatostatin and prodynorphin and have found completely different, much more restricted, labeling as compared to PTHRP (data not shown).

RESULTS

Bioactivity in Whole Brain Extracts. Extracts of whole brain harvested from 11 groups of adult rats consistently contained ACSA, ranging from 3.3- to 13.9-fold stimulation over basal (mean \pm SEM, 6.5 ± 0.9). These values corresponded to a mean \pm SEM specific activity of 4.0 ± 0.6 ng equivalents of PTH-(1–34) per mg of protein (range, 2.2–8.6). Extracts of

adult rat spleen and liver, included as negative controls (7), contained no ACSA. There was no difference in activity between brain extracts from male or female rats. Extracts of 18-day-old fetal rat, adult mouse, and adult rabbit brain also contained ACSA (8.0-, 22.6-, and 24.0-fold stimulation, respectively). Thus, ACSA was present in developing brain, in several mammalian species, and in both sexes.

The dose-response curve for adult rat brain extract in the ROS assay paralleled that of synthetic bPTH-(1–34) and $[\text{Tyr}^{36}]\text{hPTHRP}$ -(1–36) amide (Fig. 1). To further demonstrate specificity, adult rat brain extract was assayed in the presence of a competitive inhibitor of PTH binding to its receptor $[\text{Nle}^{8,18}, \text{Tyr}^{34}]\text{bPTH}$ -(7–34) amide. This resulted in 75% inhibition of activity at an antagonist concentration of 1×10^{-5} M (Fig. 2A). Specificity was also examined by preincubating brain extract with PTHRP and PTH antisera. Preincubation of adult rat brain extract with a PTHRP antiserum inhibited ACSA almost to basal levels, whereas preincubation with a PTH antiserum resulted in no loss of activity (Fig. 2B). ACSA in extracts of rabbit brain was similarly inhibited with the PTHRP antiserum (without antibody, 27.0-fold stimulation; with antibody, 3.4-fold stimulation).

Anatomic Localization of PTHRP Bioactivity and Transcripts. The regional distribution of ACSA in the brain was further defined by preparation of extracts from the cerebral cortex, the telencephalon (excluding the cortex), the cerebellum, the diencephalon, and the brainstem. ACSA ranging from 2.1- to 5.7-fold stimulation over basal was found in all regions, being greatest in the cortex and the telencephalon. The activity in all regions examined was completely abolished by the PTHRP antiserum, confirming that the ACSA was due to PTHRP (Fig. 3).

PTHRP transcripts were demonstrated in total RNA prepared from whole rat brain and the five brain regions noted above by RNase protection analysis using an antisense probe corresponding to the coding region of the rat PTHRP cDNA (6). As shown in Fig. 4, RNA prepared from each brain region protected an identical 343-base fragment. Similar results were obtained with total RNA prepared from human prefrontal, lateral temporal, and motor cortex (data not shown).

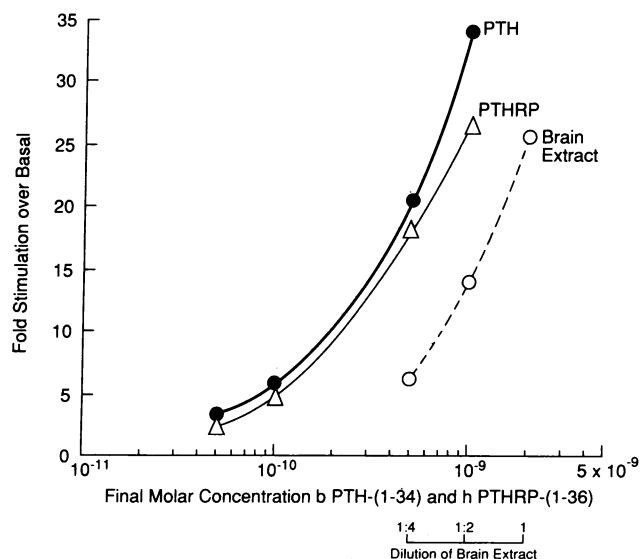


FIG. 1. Dose-response curves of adult rat brain extract (○), synthetic bPTH-(1–34) (●), and synthetic $[\text{Tyr}^{36}]\text{hPTHRP}$ -(1–36) amide (Δ) in the ROS assay. At the maximum concentration of brain extract assayed, protein added per well was 58 μg . ACSA was determined in duplicate and expressed as \pm fold stimulation over basal. bPTH-(1–34) and hPTHRP-(1–36) dilutions are shown as molar concentrations in the final reaction mixture. Basal activity in this assay was 245 cpm.

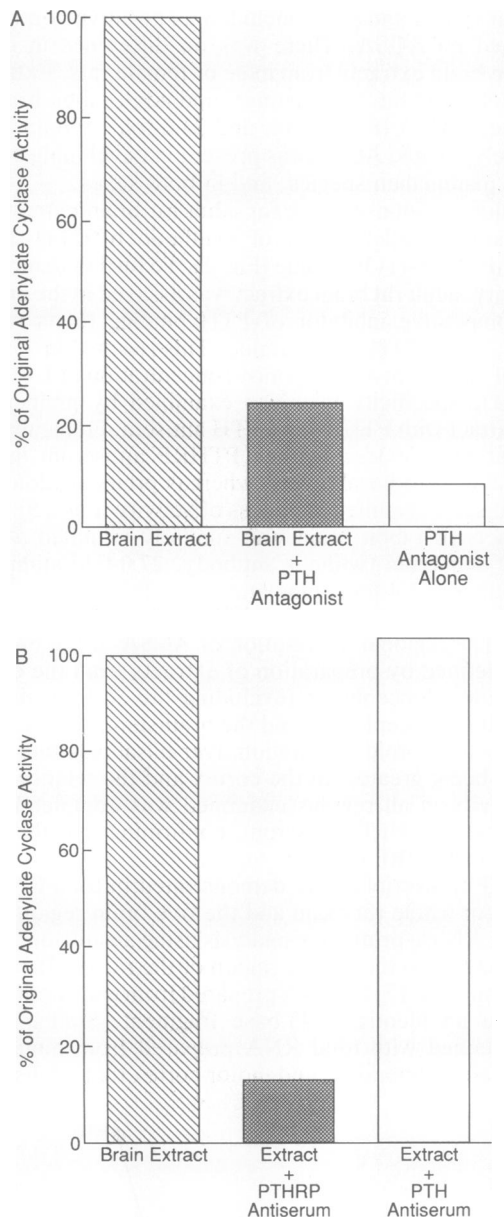


FIG. 2. (A) Effect of coincubation of brain extract with [Nle^{8,18}, Tyr³⁴]bPTH-(7-34) amide at a concentration of 1×10^{-5} M. The antagonist was added to unconcentrated extract immediately prior to assay. The quantity of protein added to each well was 58 μ g. The mean -fold stimulation without inhibitor was 10.7 and with inhibitor was 2.6. Antagonist alone had no activity in the assay (0.9-fold). Results are expressed as percentage of ACSA without inhibition. Basal activity in this assay was 231 cpm. (B) Effect of preincubating brain extract with antisera to bPTH-(1-34) or hPTH(1-74) at a dilution of 1:50. The effects of the PTHRP and PTH antisera on ACSA were determined in two separate experiments, in which the values for -fold stimulation in the absence of antiserum were 16.6- and 4.9-fold, respectively. The variability in -fold stimulation in this assay results primarily from the variation in basal activity. The results are expressed as a percentage of ACSA in the absence of antiserum. The human and rat PTHRPs differ by only one residue through the first 74 amino acids (10).

In Situ Hybridization. The localization of PTHRP transcripts by *in situ* hybridization agreed well with the anatomical distribution of PTHRP bioactivity. Strong hybridization signals were observed in three distinct regions: the cerebral cortex, the hippocampal formation, and the cerebellar cortex (Fig. 5). The marked variation of hybridization intensity between and within brain regions suggested a neuronal localization.

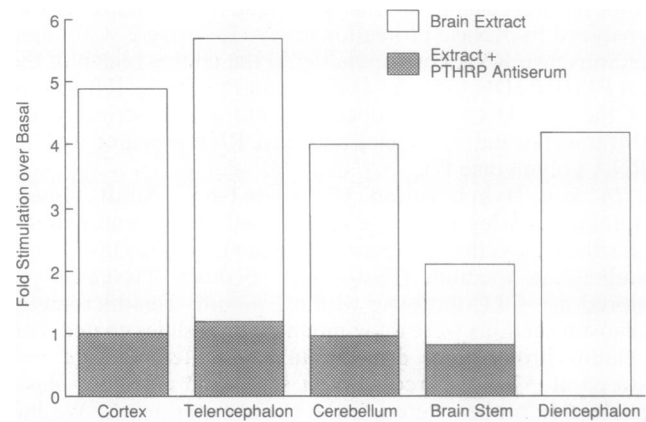


FIG. 3. Adenylylate cyclase stimulating activity in extracts of five regions of rat brain and the effect of preincubation with PTHRP antiserum R13 at a dilution of 1:50. Specific activities of extracts (ng equivalents of PTH per mg of protein) were as follows: cerebral cortex, 3.4; telencephalon excluding cerebral cortex, 2.7; cerebellum, 1.1; brain stem, 1.5; diencephalon, 1.2. Extracts of these regions have been prepared on two occasions with similar results. The diencephalon extract was not included in the immunoneutralization experiment because of insufficient quantity. Results are expressed as -fold stimulation over basal and are the means of duplicate determinations. Basal activity in this assay was 760 cpm.

High-resolution studies carried out with digoxigenin-labeled 40- and 26-mers (Fig. 6) confirmed that hybridization occurred over neuronal perikarya and was in a speckled pattern, sparing the nucleus, and thus consistent with cytoplasmic RNA. The most intense specific hybridization signals were observed over dentate granule cells, cornu ammonis (CA) field pyramidal neurons, and large dentate hilar interneurons of the hippocampal formation (Fig. 6 Top). In the cerebral cortex, many large pyramidal neurons in deeper layers as well as granule cells in layer II expressed PTHRP mRNA, as shown here for the cingulate cortex (Fig. 6 Middle). This was in marked contrast to the horizontal cells in the molecular layer and other small neurons throughout the cortex, which hybridized at a weak but detectable level. In the cerebellum (Fig. 6 Bottom), the probes hybridized strongly in granule cell perikarya but only faintly to Purkinje

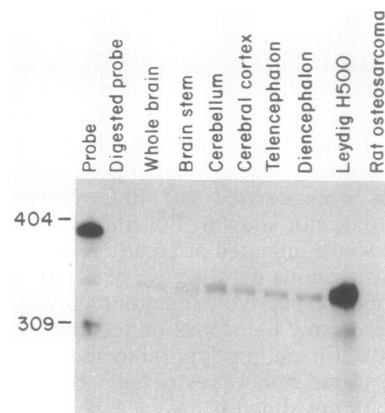


FIG. 4. Expression of PTHRP transcripts in subregions of rat brain as determined by RNase protection assay. Sixty micrograms of total RNA was analyzed, except for the H500 Leydig cell specimen [3 μ g of poly(A)⁺ RNA] and the ROS cell specimen (150 μ g of total RNA). The rat H500 Leydig cell tumor is a well-characterized animal model of HHM (12) and was included as a positive control. The ROS cell line (17/2/8) was included as a negative control (12). The probe contains polylinker sequences from the vector and is \approx 390 bases long.

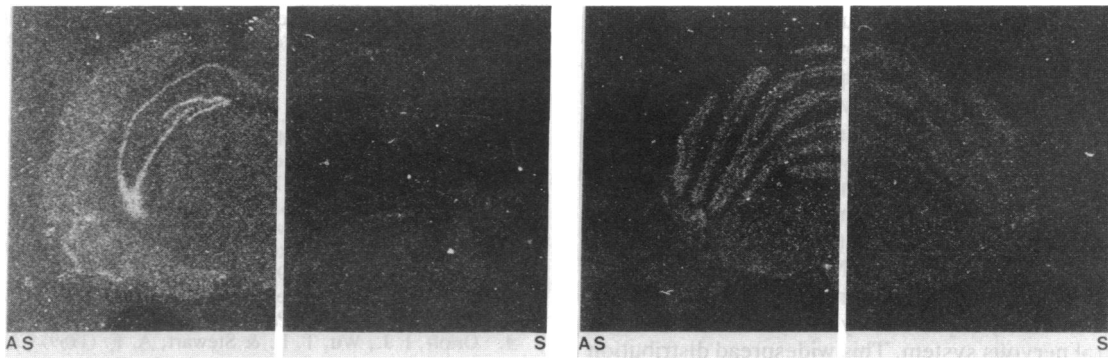


FIG. 5. *In situ* hybridization histochemistry of PTHRP mRNA in rat brain using oligonucleotides. The dark-field images were obtained with ^{35}S -labeled 45-base sense (S) and antisense (AS) oligonucleotides. The AS probes hybridized most intensely to regions of the hippocampal formation and cerebral cortex (Left) as well as to cerebellum (Right). Only a low level of binding of the S probe was observed, which was highest in the cerebellum. White matter appeared unlabeled. Exposure time was 18 days.

cells and the smaller basket and stellate neurons in the molecular layer. In addition, much lower but significant levels of hybridization were observed over many neurons throughout all regions of the central nervous system examined, including the brainstem. No signal was observed over Nissl-negative cells under the high-stringency conditions used. Thus, glia appeared unlabeled. White matter was also unstained throughout the brain.

DISCUSSION

The PTHRP was initially discovered by virtue of its expression, eutopically or ectopically, by malignant tumors associated with the syndrome of HHM. The PTH-like actions of the peptide

appear to result from N-terminal sequence similarity to PTH, and initial studies of PTHRP biological activity have focused largely on synthetic N-terminal fragments containing this sequence (4). However, this homologous region is confined to the first 13 amino acids of the deduced PTHRP product, following which the sequence of the peptide, comprising some 90% of its structure, is unique (1–3). The region of residues 88–108 of this sequence is highly enriched in basic amino acids and contains two putative amidation signals (1–3), suggesting that the PTHRP may be subject to one or several forms of posttranslational processing. The processed and/or secretory form of the peptide has not been defined, and specific PTHRP receptors have yet to be identified.

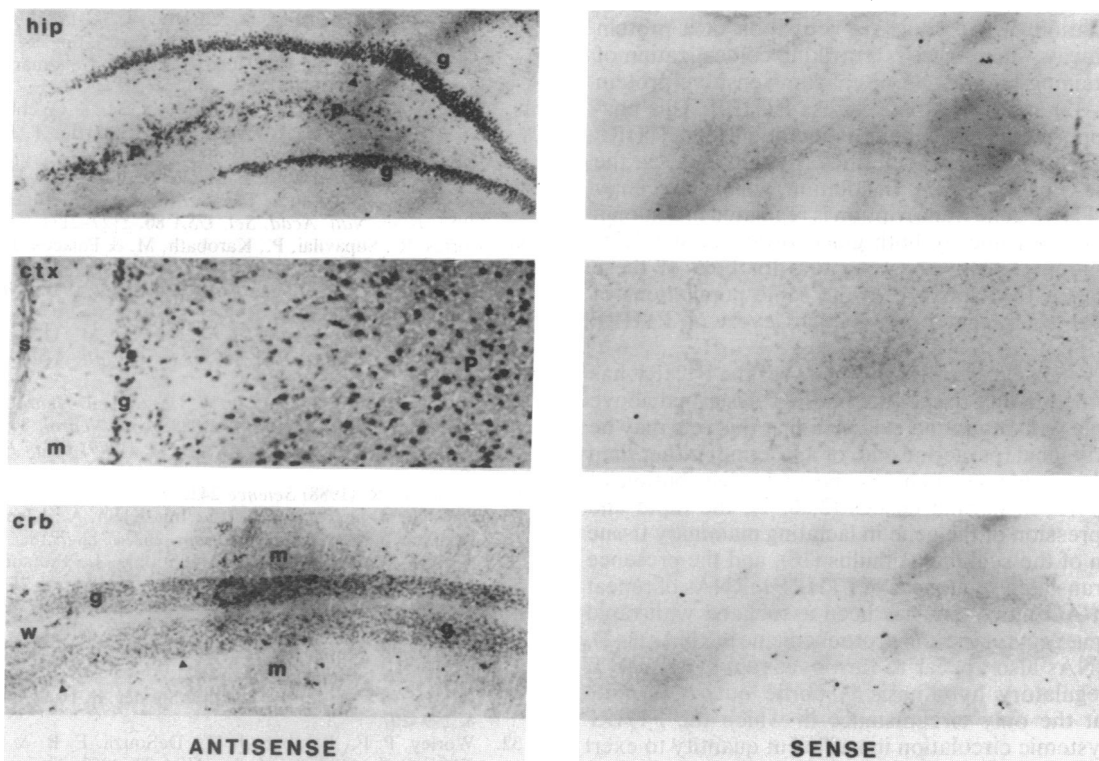


FIG. 6. Nonradioactive *in situ* hybridization using 26-mer oligonucleotide pairs. The immunohistochemical localization of digoxigenin end-labeled oligonucleotides revealed dense hybridization only over neuronal perikarya. A wide range of hybridization intensities was observed, with many positive neurons throughout all regions of the nervous system studied. In the hippocampus (hip; $\times 35$), dentate granule (g) cells, pyramidal (p) neurons, and large dentate interneurons (arrowhead) were densely labeled. In the cerebral cortex (ctx; cingulate cortex; $\times 90$) intercerebral surface (s) is to the left. Many large pyramidal (p) neurons in the deep layers and granule (g) cells in layer II exhibited strong hybridization, in contrast to small neurons like horizontal cells in the molecular (m) layer. In the cerebellum (crb; $\times 35$), granule (g) cell perikarya hybridized intensely, in contrast to weak signals observed for Purkinje cells (arrowheads) and basket and stellate neurons in the molecular (m) layer. Glia did not appear to stain positively.

Chromosomal localization and structural data indicate that the PTH and PTHRP genes arose by an ancient duplication event (2, 5, 17–19). Following this duplication event, the PTH and PTHRP genes have clearly evolved separately. The PTH gene is a comparatively simple structure (19), and its expression appears to be confined to the parathyroid glands. In contrast, the PTHRP gene has developed a complex organization at both its 5' and 3' ends (5, 17, 18), and it seems to be widely expressed in both endocrine and nonendocrine tissues (2, 6, 7).

Taken together, the bioassay, RNase protection, and *in situ* hybridization findings we report here indicate that the PTHRP gene is widely expressed by many neurons throughout the central nervous system. This widespread distribution of expression suggests that the PTHRP may play a fundamental role in normal neuronal function. One common feature of the diverse neurons that express PTHRP mRNA in large abundance is a high level of electrical activity, as characterized by large transmembrane ionic fluxes, including calcium. These neurons are enriched in at least one calcium channel (L type) (20), excitatory glutamate receptors (21), and Na,K-ATPase activity (22), and they display marked sensitivity to excitotoxins (23, 24). Furthermore, a number of these cells are known to exhibit prominent calcium currents, including dendritic calcium action potentials (25, 26). Thus, one possible role of the PTHRP could be related to regulation of intracellular calcium. Because PTHRP is a member of the PTH gene family and both PTH and the PTHRP appear to regulate calcium fluxes in other tissues (27–29), it seems reasonable to suppose that PTHRP may have a similar function in the nervous system.

We have not yet studied the regulation of PTHRP expression and/or secretion within the central nervous system, but there are a number of interesting possibilities in this regard. One is stimulation of secretion via activation of a protein kinase C pathway, since there is a striking colocalization of neurons containing high levels of calcium-sensitive protein kinase C (30–32) and those that express PTHRP. This possibility is supported by the recent observation that PTHRP secretion by neuroendocrine cell lines is stimulated via the protein kinase C pathway (33). In addition, we have recently shown that PTHRP gene transcription is regulated in a human neuroendocrine cell line by both glucocorticoids and 1,25-dihydroxyvitamin D (34). The receptors for both of these hormones appear to localize to the same populations of neurons in rat brain that express high levels of PTHRP mRNA (35, 36).

Although the exact physiological role(s) of the PTHRP has yet to be defined in any tissue, the findings described above are compatible with mounting evidence that this role may be predominantly local (paracrine and/or autocrine) rather than systemic. This evidence includes the wide distribution of PTHRP mRNAs in normal tissues (2, 6, 7), the rapid and transient expression of the gene in lactating mammary tissue as a function of the suckling stimulus (16), and the presence in the 3' untranslated sequences of PTHRP mRNAs of repeat copies of an AU motif that has been associated with rapid turnover of many cytokine and protooncogene mRNAs (1–3). PTHRP mRNAs also appear to turn over rapidly (6, 34). If this local regulatory hypothesis is borne out, it is quite possible that the only circumstance in which the PTHRP enters the systemic circulation in sufficient quantity to exert a conventional endocrine effect is in the specific pathological setting of the syndrome of HHM.

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