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UNIVERSITY OF ALBERTA

SEQUENCE ANALYSIS OF HYPOTHALAMIC PARATHYROID HORMONE (PTH) mRNA

by

MARK TERRENCE NUTLEY 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science.

DEPARTMENT OF PHYSIOLOGY

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The undersigned certify that they have read and recommended to the Faculty of Graduate Studies and Research for acceptance a thesis entitled Sequence Analysis of Hypothalamic Parathyroid Hormone (PTH) mRNA submitted by Mark Terrence Nutley in partial fulfilment of the requirements for the degree of Masters of Science.

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Date: 09/25/95

To my Grandfather, Mom, Dad, Shiela, Shawn and Kim with love.

ABSTRACT

Parathyroid hormone (PTH)-like peptides and mRNA have recently been detected in neural tissues but it is uncertain if this reflects the transcription of the PTH gene or a closely related gene. This possibility has therefore been investigated.

PTH-like cDNA moieties of predicted size were readily generated from reverse transcribed (RT) brain (hypothalamic and extra-hypothalamic tissue) and pituitary RNA, using PCR with 3 sets of overlapping oligonucleotide primers designed to amplify PTH cDNA fragments of 285 bp, 372 bp and 459 bp. PCR re-amplification of the largest hypothalamic moiety with an internal set of primers also generated a cDNA fragment of predicted size (372 bp). Restriction endonuclease digestion with *BstN1* cleaved the largest hypothalamic cDNA moieties into smaller fragments of 217 bp and 242 bp, identical to the cleavage of parathyroidal PTH cDNA. RACE amplification of the 3' flanking cDNA sequences also produced hypothalamic and extra-hypothalamic cDNA moieties identical in size (499 bps) to parathyroidal PTH cDNA. Southern analysis of these PCR and RACE cDNA fragments further indicated homology with PTH cDNA. This homology was subsequently confirmed by nucleotide sequencing, which demonstrated complete homology between the neural and parathyroidal cDNA fragments. This homology extended over 673 bp (spanning nucleotides 31 to 709 of PTH cDNA), encompassing 95% of the entire parathyroidal gene. The mRNA for this gene, determined by Northern blotting with a riboprobe for PTH mRNA, was of identicle size to the parathyroidal PTH but its abundance in brain was < 0.01% of that expressed in the parathyroid glands. This transcript was not however detected in liver. The translation of this moiety in hypothalamic tissues was

indicated by the presence of a protein in the rat hypothalamus that was immunoreactive with PTH_{1-84} antisera and of comparable size to that in parathyroidal tissue. The abundance of this protein in hypothalamic tissue was approximately 0.25% of that in the parathyroid glands, suggesting tissue-specific differences in its rate of synthesis, processing or degradation.

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LIST OF ABBREVIATIONS

AC	adenylate cyclase
Ach	acetylcholine
ACTH	adrenocorticotropic hormone
bPTH	bovine parathyroid hormone
Ca^{++}	free calcium
Ca_i	intracellular calcium
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CRE	cAMP response element
CSF	cerebral spinal fluid
DOP	dopamine
DOPAC	dihydroxyphyenyl acetic acid
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalograph
Gp	guanine nucleotide regulatory protein
h	hour
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
i.c.v.	intracerebroventricular

i.v.	intravenous
IGF	insulin-like growth factors
IR	immunoreactive
Kb	kilobase
kDa	kilodalton
min	minutes
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
PKC	protein kinase C
PLC	phospholipase C
PRL	prolactin
PTH	parathyroid hormone
PTHrp	parathyroid hormone related peptide
RACE	random amplification of cDNA ends
RER	rough endoplasmic recticulum
RNA	ribonucleic acid
rPTH	rat parathyroid hormone
RT	reverse transcribed
Taq	Thermus aquaticus
VDR	vitamin D receptor
w/v	weight per volume ratio

1.1. GENERAL OVERVIEW

Parathyroid hormone (PTH) is a basic single-chain peptide of 84 amino acids synthesized and secreted by the parathyroid glands. PTH has a critical role in the normal physiologic processes of calcium homeostasis. It protects the organism from hypocalcemia and is responsible for the minute-to-minute fine regulation of calcium levels in extracellular fluid within the narrow limits required for the normal functioning of the heart and other muscles, nerves, clotting factors of blood coagulation, and numerous enzymes. PTH-regulated Ca^{++} is responsible for the secretion and expression of biologic activity of numerous other hormones that use calcium as a "second messenger" (Broadus 1981). Removal of the parathyroid glands in humans has been shown to lead to tetany, seizure, and even death within hours. The effects of PTH on calcium metabolism result from its direct action on bone in stimulating bone resorption and the release of calcium salts into the blood and extracelluar fluid. It has a direct action on the kidney promoting calcium reabsorption and phosphate excretion, and an indirect action on the gut is in promoting calcium absorbtion from dietary intake (Goltzman 1979; Broadus 1981; Potts *et al.* 1982). Most classical PTH effects seem to be linked to the stimulation of adenylate cyclase by parathyroid hormone and the generation of cyclic AMP (Goltzman 1979; Broadus 1981; Potts *et al.* 1982). There exists an ever growing number of "non-classical" actions of PTH which have also been described. For example, PTH is known to be important in specific vasodilatory effects in smooth muscle (Pang *et al.* 1980). More recently however, the possibility of neural PTH having a role in the brain (Harvey *et al.* 1993a) has been examined.

1.2. EMBRYOLOGY AND ANATOMY

1.2.1. Development

Once the embryo has attained 8-10 mm in size, the parathyroids will begin to develope from the third and fourth branchial pouches. The third branchial pouch gives rise to the thymus and the parathyroid complex. The parathyroids then migrate to and remain at the lower poles of the thyroid. The inferior parathyroids migrate with the thymus and come to rest below the parathyroids derived from branchial pouch four.The fourth branchia! pouch, or the fourth-fifth pharyngeal complex, gives rise to the superior parathyroid glands (Gilmour *et al.* 1937).

1.2.2. Gross Anatomy

The parathyroid gland is a redish brown, gelatinous, encapsulated structure usually found on the surface of each of the two lobes of the thyroid gland. In humans, the parathyroid glands measure between 2 and 7 mm in length, between 2 and 4 mm in width, and between 0.5 and 2 mm in thickness (Akerstrom *et al.* 1984). In mammals, one to twelve parathyroid glands can be found. In rats the inferior glands may be absent or too small to be seen even with the aid of a dissecting microscope.

1.2.3. Chief Cells

Chief cells are epithelially-derived polyhedrally shaped cells of the parathyroid gland that secrete PTH. Chief cells, oxyphil cells and clear cells reflect different morphologic/functional expressions of the same parenchymal cell. Clear cells represent

chief cells in which there is an excessive amount of glycogen in the cytoplasm (Akerstrom *et al.* 1984). Oxyphils initially are found in the glands at puberty, apparently increase with age, and may form small microscopic nodules. These cells can be observed in any one of four different phases. The chief cells respond to low serum calcium levels by recruiting neighbouring cells into active synthesis and secretion phases, as can be suggested by a reduction in the size of their intracellular lipid vacuoles.

1.3. CLASSICAL PTH ACTIONS

1.3.1. Calcium and Phosphate Metabolism

Most calcium and phosphate in the body occurs in the form of hydroxyapatite ($Ca_{10}(PO_4)_6OH_2$), the main component of bone. It is also crucial that calcium and phosphate concentrations remain relatively constant in the circulation at all times. Even slight deviations from the plasma concentrations of these ions can have drastic effects on regulatory and metabolic functions at both the cellular and the organ level.

Both extracellular calcium and phosphate concentrations are maintained under homeostatic control by means of regulating molecular mechanisms within the cell. These mechanisms maintain an almost 10,000 fold Ca^{++} concentration gradient between the intra- and extracellular region of the cell. This concentration gradient is maintained by a Na^{+}/Ca^{++} exchanger which is driven by the sodium gradient as well as Ca^{++}/H^{+} ATPase (Rasmussen and Barnett, 1984). The intracellular fluid concentration is actually relatively low as most of the calcium is stored within the confines of the mitochondria, endoplasmic reticulum (RER), and the cell nucleolus. The bulk of calcium entry into most cells is the result of agonist/receptor mediated diffusion; facilitated diffusion and voltage-dependent

channels, although Na^+/Ca^{++} exchangers also play an important role in the transport of calcium into the intracellular space (Bringhurst, 1989).

Despite the seeming invariance of the extracellular Ca^+ concentration, it too, like intracellular Ca^{++} (Ca_i), is intimately involved in its own regulation through its role as an extracellular messenger, directly regulating a variety of cell types via cell-surface Ca^+ receptors (Brown *et al.* 1991). These include cells involved in the control of extracellular calcium homeostasis, such as parathyroid, bone, and kidney cells, but calcium also has effects on other cell types such as platlets (Brown *et al.* 1991).

The regulation of phosphates is more simple as the intra- and extracellular concentrations vary only minutely. Due to the lack of a concentration gradient, inward movement of phosphate is thought to occur by means of enzymes and/or a Na^+/phosphate antiporter (Quamme et al. 1989).

1.3.2. PTH Effects on the Kidney

Even slight reductions in the extracellular ionized calcium concentration (of approximately 1-2% or less) elicit prompt increases in the rate of PTH secretion (Brent *et al.* 1988). The most rapid changes in calcium handling by the target tissues for PTH take place in the kidneys and skeleton. Alterations in renal tubular reabsorption of Ca^+ occur within minutes in vitro (Agus *et al.* 1971). In the kidney, PTH is known to modulate the reabsorption of calcium and phosphates within the renal tubules of the nephron (Rosenblatt *et al.* 1989). PTH acts on receptor mediated calcium transporters to increase the amount of calcium uptake from the lumen (Rosenblatt *et al.* 1989). Conversly, PTH

inhibits the Na^+/phosphate antiporter resulting in a reduction of plasma phosphate levels. PTH also stimulates 25(OH)-vitamin D-1-hydroxylase in the proximal tubule, which in turn catalyses formation of more active forms of vitamin D. The net effect in the kidney is the reabsorption of calcium and the excretion of phosphate. In the collecting tubule of the rabbit nephron, as in the distal tubule of other species, PTH produces a prominent increase in the reabsorption of calcium ions. This effect persists when the electrochemical forces premoting passive reabsorption of Ca^{2+} are removed, suggesting an active, transcellular mechanism for Ca^{2+} reabsorption.

1.3.3. PTH Effects on the Intestine

PTH acts indirectly on the intestine via the synthesis of renal 1,25 $(OH)_2$ vitamin D. Renal 1,25 $(OH)_2$ vitamin D is synthesised in the proximal tubules of the kidney as a result of PTH binding to specific receptors on these cells. The effect of vitamin D is the enhancement of calcium binding within the intestine which is thought to increase the absorbtion of calcium from the intestinal lumen (Bronner *et al.* 1986).

1.3.4. PTH Effects on Bone

Short-term maintenance of calcium and phosphate homeostasis is achieved through the inhibition of hydroxyapatite production as well as the resorption of insoluble calcium and phosphate into circulation. Three different levels and/or cell types corresponding to distinct time periods in which PTH has its actions have been demonstrated (Rosenblatt et al. 1983).

1.4. NON-CLASSICAL ACTIONS OF PTH

As well as the above described "classical" actions for PTH there are also many novel PTH actions unrelated to calcium and phosphate homeostasis. Some of these could be indicative of autocrine or paracrine roles of locally produced PTH, by analogy to the local production of PTH-related peptide and its widespread roles (Fraser *et al.* 1993).

1.4.1. Bone

PTH stimulates bone remodelling via specific growth factors which act on surface osteoclasts to promote bone resorption and remodelling. The phenomenon of bone remodelling is described by the fact that bone is constantly being resorbed and reformed (Linkhart and Mohan 1989). This is achieved by surface osteoblasts which respond to specific growth factors such as bone derived growth factors, insulin-like growth factor-I (IGF-I) and IGF-II (Linkhart and Mohan 1989). These growth factors are stimulated to release in response to PTH.

1.4.2. Adipose Tissue

Werrner and Low (1973) were the first to recognize that a highly active parathyroid gland such as is found in the disease state hyperparathyroidism, results in a significant increase in the amount of serum triglycerides. Since this discovery researchers have demonstrated in controlled in vitro conditions that exogenously applied PTH caused a marked increase in glycerol and free fatty acids (Druek *et al.* 1987).

1.4.3. Adrenal Gland

The ability of PTH to stimulate cortisone synthesis and release has been extensively documented (Marotta 1971). Parsons (1975) explained this by the close homology between a region of the PTH peptide and that of ACTH.

1.4.4. Vascular Smooth Muscle

The first evidence that PTH exhibited hypotensive properties in mammals (dogs) was provided by Collipp and Clark (1925). Similar studies in other vertebrates (Pang 1980) suggested that the hypotensive effects were the result of PTH having specific vasodilatory effects (Pang *et al.* 1980; Crass and Pang 1980). These vascular actions of PTH seemed to be associated with a local increase in adenylate cyclase activity (Nickols *et al.* 1986). In more recent studies, specific PTH receptors have been located on vascular smooth muscle cell membranes (Nickols *et al.* 1990) where by these receptors have been demonstrated to promote adenylate cyclase activity (Nikols *et al.* 1986; 1990).

1.4.5. Non-Vascular Smooth Muscle

PTH-induced smooth muscle relaxation has also been found in many non-vascular tissues. Although the effects of PTH in these tissues is significant, the physiological significance of these actions is still controversial.

1.4.6. Striated Muscle

The contractile effects of PTH on striated muscle has been demonstrated through both direct and indirect means. For example, elevated PTH in hyperparathyroidism is associated with a weakening in skeletal muscle contractility, as PTH increases serum calcium levels, and induces atrophy of the innervating afferent nerves (Betroni 1987). A direct positive chronotrophic and inotropic effect of PTH is found in cardiac muscle in both dogs (Crass *et al.* 1982) and rats (Tenner *et al.* 1983), which is thought to be mediated through endogenous myocardial noradrenaline (Katoh *et al.* 1981).

1.4.7. Miscellaneous Effects

A number of PTH-related effects are listed in Table 1.1. Although the structural requirements of the PTH molecule for these biological activities have been partially established, a better understanding of the second messenger systems involved may lead to a better understanding of the importance of these effects.

Additional PTH-related effects include an influence on the proliferation and activity of immuncompetent cells (Perris *et al.* 1970). Specific PTH receptors have also been described on circulating lymphocytes (Yamamoto *et al.* 1983), which when bound to PTH cause a release of calcium stores (Atkinson *et al.* 1987).

1.5. BIOSYNTHESIS OF PARATHYROID HORMONE

1.5.1. PTH TRANSCRIPTION

The gene for human PTH is located on the short arm of chromosome 11 near the end of the chromosome (Zabel *et al.* 1985). This gene contains two introns: one intervening sequence of 3400 base pairs (bp) following a noncoding region at the 5' end and a second smaller intron of 103 bps bisecting the two coding regions (Heinrich *et al.* 1984). The cell-specific parathyroid hormone gene expression is thought to result from specific DNA sequences associated with the PTH gene which respond to the enviroment of the chief cell to activate gene expression. Once expressed, there exist a number of important factors which act to regulate PTH gene transcription.

1.5.2. Factors Regulating PTH Gene Expression

PTH gene regulation occurs in a matter of hours to days post stimulation, constituting the long-term response to parathyroid regulatory factors. There are both positive and negative regulatory elements in the 5' flanking region (Okazaki *et al.* 1991; Reis *et al.* 1990).

1.5.2.1. Calcium

At the transcriptional level, calcium seems to be important in the regulation of PTH biosynthesis (Russel *et al.* 1983; Silver *et al.* 1985). Plasma PTH concentration was initially suggested to be regulated by the negative effect of calcium on PTH secretion and degradation (Habner *et al.* 1975). It was later demonstrated that pre-pro-PTH mRNA

(resulting in the precursor peptide) was directly regulated by calcium although the exact mechanism is not known (Russell *et al.* 1983). It has been suggested that the parathyroid gland synthesizes PTH, at normal concentrations of extracellular calcium, at almost a maximal rate (Russell *et al.* 1983; Macgregor *et al.* 1988; Yamamoto *et al.* 1989; Lui *et al.* 1994).

Okazaki *et al.* (1991) have defined two *cis*-acting negative transciptional elements between 2.4 and 3.6 kb upstream of the hPTH gene. These negative regulatory elements have been suggested to have a number of possible functions. One possibility is that they may be active in all cells; tissue specificity might be conferred only in the presence of other unidentified *cis*-acting elements in the hPTH gene. In addition, they may play important roles in some regulatory function in parathyroid cells. The negative regulatory elements in the hPTH gene may be responsible for modulation of transcriptional suppression of the hPTH gene by extracellualr calcium (Yamamoto *et al.* 1989).

1.5.2.2. Vitamin D

Vitamin D is also important in the regulation of PTH gene transcription. Vitamin D modulates expression of the PTH gene (Silver *et al.* 1985) through the presence in parathyroid cells of a vitamin D receptor (VDR). Following binding of 1,25-(OH)D, the 1,25-$(OH)_2$D-VDR complex binds to specific DNA sequences usually in the upstream region of the human PTH genes. A sequence motif upstream of the human PTH gene has recently been identified that binds the VDR and likely mediates transcriptional inhibition due to 1,25-$(OH)_2$D (DeMay *et al.* 1992). Vitamin D down-regulation of PTH has been

demonstrated to regulate gene transcription independent of protein synthesis (Okazaki *et al.* 1988).

1.5.2.3. Other Factors Effecting PTH Expression

There has been limited research into determining other possible factors which may regulate the expression of the PTH gene. Recent work suggests the existence of a functional cAMP response element (CRE) in the human PTH gene which confers cAMP responsiveness to a heterologous promoter when transfected into ROS 17/2.8 cells (Rupp 1990). It is still unknown however whether or not this CRE functionally regulates the response to cAMP in the parathyroid cell. In addition, glucocorticoids have been shown to increase PTH mRNA in dispersed, hyperplastic human parathyroid cells (Peraldy *et al.* 1990) and to abolish the decrease in PTH mRNA in response to 1,25-(OH)D in dispersed bovine parathyroid cells (Karmali *et al.* 1989). Furthermore, in ovariectomized rats, estradiol administration led within 24 hr to a four-fold increase in PTH mRNA (Naveh-Many *et al.* 1992), which is consistant with the occurance of estrogen receptors within the parathyroids. Although functionally the PTH gene is similar to many others, the negative secretory response to increasing calcium concentration is thought to most likely be brought about though alterations in the signal transduction pathways.

1.6. POST-TRANSCRIPTIONAL REGULATION OF PTH

Post-transcriptional control of protein synthesis has been demonstrated for many genes including ferritin (Mattia *et al.* 1989), insulin (Itoh and Okamoto, 1980) and

growth hormone (Diamond and Goodman, 1985). Hawa *et al.* (1993) showed that low calcium increases membrane-bound polysomal pre-pro-PTH mRNA content by two fold in the absence of a change in steady-state mRNA levels. This amount is comparable with the rise in secretion induced by low extracellular calcium concentrations in bovine cells (Brookman *et al.* 1986). The increase in polysomal mRNA also persisted in the presence of actinomycin D at a concentration which inhibited pre-pro-PTH gene transcription, indicating a post-transcriptional site of regulation of PTH synthesis (Hawa *et al.* 1993). These results may be explained by the possible existence of an "untranslatable" compartment of preproPTH mRNA within the cells which may be bound to a protein inhibiting translation, similar to the mechanism which suppresses translation of ferritin (Aziz & Muro, 1986; Liebold & Munro, 1988; Walden *et al.* 1988 and Rouault *et al.* 1990). Furthermore, Lui *et al.* (1994) demonstrated that incubation of human parathyroid adenoma cells with cycloheximide resulted in the reduction of PTH mRNA suggesting that protein synthesis is needed for the the maintenance of PTH mRNA levels. Cycloheximide may inhibit the synthesis of a liable protein mediator required in PTH mRNA synthesis, or the synthesis of a PTH mRNA stabilizing protein.

1.6.1. PTH Translation

PTH mRNA translation yields a basic, single chain peptide, with no intrachain disulphide bonds (Habener *et al.*1985). There is a striking conservation of primary PTH structure across species, particularly at the amino-terminus (Fig. 1.1.). While PTH (1-84) appears to represent the major secreted and circulating form of bioactive PTH, (Segre *et*

al. 1972; Goltsman *et al.*1980) most biological activity is generally believed to be completely contained within the N-terminal 34 amino acids (Potts *et al.* 1971; Tregear *et al.* 1973). Although midregion and carboxyl fragments of the hormone circulate at higher concentrations than the parent molecule, a definite biological role has not yet been discovered (Sergre *et al.* 1972; Arnaud *et al.* 1974; Goltzman *et al.* 1984).

1.6.2. Pre-Pro-PTH

PTH is synthesised as a precursor peptide comprised of 115 amino acids (Fig. 1.2.). The hydrophobic leader sequence (residues -31 to -7) contains 25 amino acids and is thought to be essential for translocation of the maturing peptide through the endoplasmic reticulum (RER) and subsequent insertion into the RER membrane (Fig. 1.3.). The membrane-inserted leader sequence (Pre-sequence) is cleaved off and destroyed upon delivery through the membrane into the cisternal space. The PTH-specific Pro-sequence of six amino acids (residues -6 to-1) are deleted prior to packaging of the final 1-84 amino acid secretion product within the golgi apparatus (Habener *et al* 1985). The Pre-Pro-PTH or the Pro-PTH have not been detected in any significant amount in the peripheral circulation. These two PTH precursor peptides have been shown to have limited (3-5%) biological activity when administered in vivo or in vitro (Parsons *et al.* 1975).

1.7. SECRETION OF PARATHYROID HORMONE

1.7.1 Factors Regulating PTH Secretion

PTH is present in secretory vesicles within the parathyroid cell and is thought to be released by exocytosis. Exocytosis in a variety of cell types is regulated by several different types of mechanisms. Constitutive secretion (i.e., continual secretion of proteins) shows little minute to minute variation and presumably involves continuous packaging and secretion of secretory vesicles (Bennett *et al.* 1993 and Sollner *et al.* 1993). In addition, regulated secretion of PTH hormone probably involves additional regulating mechanisms (Burgoyne *et al.* 1991).These additional factors may include cAMP, diacylglycerol, and other lipid mediators, Ca^{2+} itself, as well as others; and likely act by modulating the activity of cellular kinases, with resultant changes in the phosphorylation of appropriate cellular targets (Brown 1991).

1.7.1.1. Calcium

Changing extracellular calcium concentration has little effect on PTH mRNA short term expression as the parathyroid gland is synthesising PTH at near maximal capacity (Russell *et al.* 1983; Macgregor *et al.*1988; Farrow *et al.*1988). It was therefore suggested that bioavailability control may be regulated through the effects of extracellular calcium concentration on the secretion and degradation of PTH in the parathyroid gland.

The secretory rate of PTH by the parathyroid gland is significantly decreased with an increase in extracellular calcium concentration (Sherwood *et al.* 1968; Habener and Potts, 1976; Brown *et al*, 1978; Brown and Gardner, 1979; Mayer and Hurst, 1978;

Brown *et al.* 1981; Brown 1982; Brown 1983; Tanguay *et al.* 1991 and Lui *et al.* 1994). The parathyroid gland can therefore swiftly regulate the release of PTH in response to the changing calcium concentration without the necessity of drastic changes in gene expression or protein synthesis (Russell *et al.* 1983; Habner *et al.* 1975). Recent studies confirm this close link between PTH degradation and regulation of PTH release from the cell (Tanguay *et al.* 1991).

1.7.1.2. Vitamin D

Since PTH is released from the parathyroid gland and acts on the kidney to produce 1,25-D the question arises as to the possibility of a negative feedback mechanism of 1,25-D on the release of PTH from the parathyroid gland. Brumbaugh *et al.* (1975) first discovered receptors for vitamin D on the nucleus of chick, and later porcine (Cloix *et al.* 1976) and human parathyroid cells. PTH suppression from vitamin D metabolites, has been repeatedly demonstrated in bovine and rat parathyroid cultures (Au *et al.* 1984; Cantley *et al.* 1985; Sugimoto *et al.* 1989). This seems however, to be a minor role for 1,25-D as it and its metabolites have demonstrated a greater importance at the level of PTH gene expression.

1.7.2. Role of G-Protein-Coupled Ca^{2+} Receptor

Guanine nucleotide regulatory proteins (G-proteins) link the cell surface proteins to the intracellular effector systems, transducing the signal generated from the binding of a hormone to its specific receptor (Spiegel 1987). This family of proteins are functionally

related heterotrimers comprised of , *alpha*, *beta* and *gamma* subunits. Accumulating evidence implicates a cell surface, receptor-like mechanism as a key component in the regulation of parathyroid function by extracellular Ca^{2+} (Brown *et el.* 1991). The putative Ca^{2+} receptor is linked to several intracellular second messenger systems via one or more guanine nucleotide regulator (G) proteins (Fig. 1.4.). While it is possible that a single form of Ca^{2+} receptor couples to several second messenger systems in parathyroid cells, it is also possible that there are distinct forms of the receptor that couple to changes in cAMP and phosphoinositide turnover.

1.7.3. PTH Intracellular Degradation

Another potentially important factor regulating PTH is the effect of Ca^{2+} on cellular stores of this peptide. Both *in vivo* (D'Amour *et al.* 1992) and *in vitro* (Morrissay *et al.* 1980) studies have shown that there is more intracellular degradation of PTH at high than at low extracellular Ca^{2+} concentrations, with a sigmoidal relationship between the extracellular Ca^{2+} concentration and the proportion of secreted PTH fragments.

1.8. PTH Receptor mRNA Distribution

The wide distribution of PTH receptor mRNAs suggest that the functions of the PTH receptor are not restricted to tissues involved in homeostatic calcium and phosphorous metabolism. Urena *et al.* (1993) examined the distribution of the cloned PTH receptor mRNA in several rat tissues. The results from this work are shown in Table 1.2. which lists the organs expressing the PTH receptor mRNA and its relative intensity.

1.8.1. Neural PTH Receptors

Although neural effects of PTH in the brain have been well documented, the exact mechanism for which PTH acts through the receptor remains open to some debate. Earlier data supports the fact that PTH acts through the increase in intracellular cAMP as primary cultured brain cells treated with PTH demonstrated a marked increase in cAMP (Loffler *et al.* 1982). This response was thought to be specific as it could be blocked by the application of PTH antagonists (Loffler *et al.* 1982). Conversely, Fraser and Arieff (1988) and Fraser *et al.* (1988) demonstrated that the response to PTH within the rat brain synaptosomes were not associated with an increase in cAMP activity. In addition, calcium currents in snail neurones are increased by PTH independent of augmented cAMP concentration (Kostyuk *et al.* 1992). PTH receptors have been suggested to occur in peripheral nerves (Pang *et al.* 1990) as saturable binding sites for radiolabelled PTH have been observed in neuroblastoma cells (N1E-115) of mouse. The binding affinities and capacities of these binding sites are similar to those found in target tissues such as bone, kidney and intestine.

Rat and snail (Helix pometia) brain have also been shown, although not in detail, to bind radiolabled PTH (Khudaverdyan *et al.* 1993). More specifically, radiolabled PTH has been demonstrated to bind to receptor sites on rat hypothalamic plasma membranes (Harvey & Hayer,1993), and to a lesser extent cerebellum and cerebrum, but were absent in the brain stem.

1.9. EVIDENCE FOR PTH IN THE BRAIN

In addition to its classical role, acting directly on the kidneys, bone and the intestines to increase and maintain serum calcium concentrations and rid the body of excess phosphates, PTH may also be involved in a non-classical neuroendocrine role. The suggestion that PTH may have a role in the brain is supported not only by the occurrence of its synthesis and secretion, but also by its effects on other hormones and neurotransmitters in cerebral tissue.

1.9.1. Immunocytochemical Evidence

Immunoreactive PTH is now known to be located in many neural tissues of vertebrates including the brain, hypothalami and pituitary glands of fish, amphibian, reptilian, avian and mammals (Balabanova *et al.* 1985, 1986: Harvey *et al.* 1987; Pang *et al.* 1988a,b: Fraser *et al.* 1991) and in sensory ganglia of invertebrate gastropods (Wendellar Bonga *et al.* 1989). It was demonstrated that this PTH-like immunoreactivity was the result of a heat-stable non-dialysable peptide which co-eluted with human PTH (1-84) (Pang *et al.* 1988a). It has also been demonstrated that immunoreactive cell bodies in the preoptic area of gold fish brain possess axons which terminate in the neuropars-intermedia adjacent adenohypophysial membranes (Pang *et al.* 1988b). Immunoreactive PTH cell bodies were also found in brains of hagfish, bullfrogs and mice exhibiting fibre tracts traced to the neurohypophysis in the hagfish species (Pang *et al.* 1988b). Also of interest in this study was the fact that the axon terminals of the bullfrogs and mice demonstrated a close proximity to the portal blood vessels.

These results demonstrate a clear conservation of a hypothalamic PTH peptidergic system throughout vertebrate evolution.

1.9.2. Genomic Evidence

A PTH mRNA probe has been demonstrated to hybridize to mRNA from the hypothalamus of the rat, suggesting the existence of a hypothalamic PTH peptidergic system in the vertebrate brain (Fraser *et al.* 1990). Furthermore, expression of PTH mRNA in the hypothalamus of rat by two rounds of Polymerase Chain Reaction (PCR) of cDNA reverse-transcribed from RNA proved to be the same size as that in the parathyroid (Fraser *et al.* 1990). Site specific in-situ hybridization of rPTH-like mRNA in the paraventricular and supraoptic nuclei of rat hypothalamus would suggest that these may be central localized sites for the synthesis of PTH (Fraser *et. al.* 1990).

1.9.3. Effects of Calcium on Neural PTH

Balabanova *et al.* (1986) demonstrated that immunoreactive PTH release from sheep brain is stimulated *in vitro* by depletion of extracellular calcium and suppressed by 1,25-$(OH)_2$ vitamin D_3 which is the same response observed in the regulation of parathyroidal PTH (Rosenblatt *et al.* 1989; Pocotte *et al.* 1991). *In vivo*, rat brain immunoreactive PTH release is independent of supplementation or depletion of dietary calcium (Pang *et al.* 1988a).

1.9.4. Neural PTH Degradation

Non-specific microsomal enzymes have been shown to degrade PTH in a temperature dependent manner within the gyrus, brain stem, basal ganglia and cerebellum of sheep brain (Balabanova and Helmer, 1992). This is further evidence in support of the occurrence of PTH synthesis and release within the brain.

1.9.5. Action of PTH in the Brain

1.9.5.1. Neural Actions

To date PTH is known to have a variety of central actions. For example, PTH adrenergic-like effects are blocked by PTH antagonists but not by beta-adrenergic antagonists such as propanol (Pang *et al.* 1986) suggesting possible neurocrine actions of this peptide. Chronic renal failure (CRF), which secondarily leads to hyper-parathyroidism, is also supportive evidence for PTH having neural actions. Patients with this disease are known to experience mild sensorial clouding, delirium, coma and even during post dialysis therapy, many of these patients have been known to have continued impaired mentation. These include electroencephalograph (EEG) abnormalities and peripheral neuropathy (Cooper *et al.* 1978; Akmal *et al.* 1984). Neural dysfunctions with which uraemic patients present, can be reproduced experimentally with exogenous PTH in the absence of any renal disfunction or hypercalcaemia (Guisado *et al.* 1975). Furthermore, it has been demonstrated that parathyroidectomy results in the normalization of brain EEG patterns in the uraemic subjects and in patients with both primary or secondary hyperparathyroidism, which is also associated with neuro-

psychiatric illness (Allen *et al.* 1970; Cogan *et al.* 1978; Goldstien *et al.* 1980). Hypersecretion of PTH has also been associated with other diseases such as Alzheimers and senile dementia (Morimoto *et al.* 1988).

Intracerebroventricular (i.c.v.) injections of PTH result in an enhancement of learning and memory processes suggesting the existence of central sites of PTH action (Clementi *et al.* 1984, 1985). PTH also demonstrates central action by inducing a hyperalgesic state (Gennari, 1988; Gennari *et al.* 1991) which may model the abdominal, joint or bone pain that typically occurs in 50% of patients with primary hyperparathyroidism.

1.9.5.2. Mechanisms of Central PTH Action

Central actions of PTH are thought to be mediated through the increase in calcium uptake which in turn leads to an increase of neurotransmitter release (Dubovsky and Franks, 1983). By using patch clamp techniques, on snail buccal ganglion, Wang *et al.* (1993) demonstrated that bovine PTH increased the N-like calcium channel currents in isolated B5 neurons in a concentration-dependent manner. This effect of PTH on the N-like calcium channel currents depended on the activation of a G protein insensitive to pertussis toxin, but was unlikely to be mediated by the cyclic AMP dependent protein kinase.

Noradrenaline released from rat brain synaptosomes is suppressed in uraemia by exogenous PTH. This may be due to the accompanying reduction in synaptosomal

phosphatidylserine which is important in the aggregation of neurosecretory vesicles within the membrane of the neuron.

Harvey et al. (1993a) have shown similar inhibitory effects of PTH on the uptake and release of dopamine from *in vitro* hypothalamic slices of rats. In addition to reduction in the dopamine content these researchers observed that exogeneous PTH also increased dihydroxyphenyl-acetic acid (DOPAC, a dopamine metabolite), as well as the DOPAC:dopamine ratio. Hypothalamic DOPAC and the DOPAC:dopamine ratio are increased by i.c.v. injections of PTH or PTH-rp (1-34) but not after the prior administration of agonist PTH(7-34) or monoamine oxidase (Harvey *et al.* 1993b). Concentrations of DOPAC also increase in the hypothalamic extracellular fluid after PTH perfusion (Harvey *et al.* 1993b).

1.9.6. Possible Roles for PTH in the Brain

Although PTH has only been rarely considered as a neurohumeral agent limited evidence suggests PTH may have a role in the regulation of prolactin (PRL). Some evidence also suggests that PTH may play a limited role in learning and behavior modification.

1.9.6.1. Prolactin Regulation

Evidence suggests that hyperparathyroidism may be a cause of hyperprolactinaemia (Fioretti *et al.* 1986). This is supported by the fact that parathyroidectomy restores circulating prolactin concentrations to normal in some

hyperprolactinaemic patients (Raymond *et al.* 1982). Additional evidence for the role of PTH in the regulation of prolactin stems from the fact that the disease pseudohypoparathyroidism (characterized by defective PTH receptors) is demonstrated by isolated prolactin deficiency (Kruse *et al.* 1981).

Exogenous (i.v.) PTH or parathyroid extracts increase circulating prolactin levels in normal subjects and in patients with primary hypoparathyroidism (Issac *et al.* 1978; Castro *et al.* 1980; Kruse *et al.* 1981). Although it is known that calcium entry into the cell regulates hormone release from the pituitary the prolactin response observed here is independent of plasma calcium levels (Castro *et al.* 1980) and differs from the inhibitory effect of hypercalcaemia on prolactin secretion (Rodjmark *et al.* 1984).

Additional evidence for PTH regulation of prolactin stems from the observation that hypothalamic immunoreactive PTH nerve fibres terminate in areas of the fish pituitary responsible for the secretion of prolactin (Kaneko & Pang, 1987). Harvey *et al.* (1993c) were unable to demonstrate a significant change in the circulating prolactin levels in rats in response to peripheral (i.v.) injection of bPTH. In addition, a prolactin response to this same peptide could not be detected from monolayers of rat pituitary cells or GH_3 cells *in vitro*. Plasma prolactin concentrations are elevated by i.c.v. injections of bPTH (Clementi *et al.* 1984) suggesting that PTH regulation of PRL is indirect.

The effects of PTH on the regulation of prolactin may be mediated through dopamine as its increase in metabolism along with its inhibition from release may remove its known tonic inhibitory effects on the release of prolactin (Harvey et al. 1993a,b).

1.9.6.2. Cicadian PTH Effects on Prolactin

Circadian PTH (1-84) secretion in normal humans is characterized by a late afternoon/early evening rise and a surge through the night (Logue *et al.* 1989; Calvo *et al.* 1990; Logue *et al.* 1990 and Calvo *et al.* 1991). Although the mechanism for PTH (1-84) rhythmic release has not yet been elucidated, many studies support the fact that its rhythm is not based solely on serum calcium or phosphate concentrations. This suggests that the nervous system may play a direct, or indirect, role in PTH modulation of PRL secretion (Logue *et al.* 1989; Calvo *et al.* 1990; Logue *et al.* 1990 and Calvo *et al.* 1991).

PRL secretion follows a similar pattern to PTH (1-84) measured over a 24h period in normal males with an early evening rise and a major night time elevation shortly after the beginning of sleep (Desir *et al.* 1982). Comparison of the PTH (1-84) and PRL secretion profiles over a 24h period demonstrated a strong correlation between the two where it is shown that PRL release occurs approximately 2h after PTH release (Calvo *et al.* 1990). This again suggests a neuroendocrine control common to PTH (1-84) and PRL secretion. This theory has been challenged by Logue *et al.* (1992). Male subjects were divided into two groups (Group A-sleep, 0100-0800h; Group B-sleep, 0800-1400h) where blood samples were drawn at 30 minute intervals. The results showed that the sleep shift caused a shift in the PRL secretion to the new time of sleep. The overall timing of the PTH (1-84) nocturnal peak (0200-0600h) was not altered by the sleep shift. These results therefore, contradict the suggestion of a direct neuroendocrine link between PTH (1-84) and PRL (Logue *et al.* 1992).

1.9.6.3. Effects on Aquisition of Active Avoidance Behavior

Clementi *et al.* (1984) demonstrated that i.c.v. injection of pico-molar concentrations of bPTH hormone facilitated the aquisition of active avoidance behavior in the rat. This effects was mimicked by the C-terminal fragment PTH_{65-84}, while the N-terminal fragment PTH_{1-34} and PTH_{44-68} inhibited the acquistion of active avoidance behavoir in the rat. These findings suggest that PTH molecule possesses specific sequences that oppositely affect avoidance acquisition. As the whole molecule seemed to facilitate the acquisition of active avoidance behavior it appears as though the C-terminal fragment PTH_{65-84}, is responsible for this effect. However, this study showed that PTH_{65-84} is even more potent than the whole molecule in facilitating avoidance acquisition. It has therefore, been suggested that this may be due to the inhibiting action exerted, within the PTH molecule, by the other fragments PTH_{1-34} and PTH_{44-68}. It is possible that PTH, by causing hypercalcemia, may increase the release of a neurotransmitter such as acetylcholine (Boustein *et al.* 1972) as this neuropeptide plays a role in learning an memory porcessess (Bartus *et al.* 1982).

1.9.6.4. Physiological of Pharmacological Effects

Although there exists much evidence demonstrating the effects of PTH within the brain, the possibility that these effects may be pharmacological should be considered (Pang,1988c; Orloff *et al.* 1989). During primary or secondary hyperparathyroidism the concentrations of PTH may be greater than 100-1000 times that of a normal subject (Mallette *et al.* 1982). Furthermore, nanomolar or micromolar amounts of PTH are

required to ilicit a neurological response in vitro where between pico- to nanomolar concentrations of PTH normally exist in serum although the levels may be higher in the CNS (Harvey *et al.* 1993c). However, Clementi *et al.* (1984) was able to demonstrated avoidence behavior from rats injected with PTH (i.c.v.) at concentrations well within physiological limits.

1.9.7. Hypothalamo-Pituitary Actions of PTH

Although it is certain that PTH hormones act within the brain it is uncertain as to whether these actions are caused by PTH produced locally in the brain or by endocrine actions of PTH from the parathyroid glands. Although the role of PTH within the brain has not yet been determined, the fact that parathyroidectomy fails to decrease PTH dependent (Fraser & Arieff, 1988) synaptosomal calcium uptake in uraemic rats (Fraser & Sarnaki, 1988; Massry *et al.* 1988), suggests there may exist a possible role for hypothalamic PTH within the CNS.

1.9.8. Transfer of PTH Through the Blood-Brain Barrier

Currently, there exists much conflicting data as to whether or not PTH crosses the blood-brain barrier during physiological conditions. Gennari (1988) was unable to detect a change in CSF calcium or phosphorus with either an excess or deficiency of plasma PTH concentrations. In addition, Balabanova *et al.* (1984) were unable to determine a significantly higher CSF concentration of PTH within hyperparathyroid patients as compared to a normal control population, despite a marked increase in plasma PTH

levels. Additional support for the blood-brain barriers impermeability to PTH arises from observations that (i.v.) injection or artificial parathyroid stimulation did not result in a corresponding increase of PTH in the CSF (Akmal *et al.* 1983).

Contradicting results were later published for a similar experiment in sheep which demonstrated (i.v.) injections of PTH did result in significant increases of PTH within the CSF (Care & Bell, 1986). Furthermore, human experiments showed that C-terminal PTH in the CSF of ten subjects, one of whom had primary hyperparathroidism, varied relative to the concentration of PTH in the plasma (Care & Bell, 1986). A correlation has also been shown between the levels of N-terminal and mid-molecule PTH in the serum as compared to CSF in patients suffering from bone or joint pain with unrelated concentrations of serum or CSF calcium (Jaborn *et al.* 1991). Parathyroidectomy also alleviates neurological disorders resulting primarily from the pathophysiological conditions of hyperparathyroidism and uraemia (Allen *et al.* 1970; Goldstein *et al.* 1980; Genari, 1988) suggesting that PTH may penetrate the blood-brain barrier from the peripheral side. It is important to note that the blood-brain barrier can become "leaky" to many compounds during certain disease states (Ford 1976) and that hyperparathyroidism may represent such a state.

The question as to whether PTH synthesized in the brain may pass into the peripheral circulation has also been examined. It has been suggested that hypothalamic PTH may enter the systemic circulation by way of nerve terminal release from the median eminence of neurohypophysis as immunoreactive PTH nerve terminals have been

discovered in the median eminence close to hypophysial blood vessels in rats (Pang *et al.* 1988b).

More recently it has been demonstrated that the movement of PTH from the brain to the peripheral circulation readily occurs (Yao *et al.* 1993). Allotransplantation of parathyroid tissue into the cerebroventricle of parathyroidectomized rats resulted in the maintenance of the serum concentrations of both calcium and PTH at levels comparable to those before the parathyroidectomy. These results suggest that the allografts could exert their full function of secreting hormone and as well that the hormone can easily pass through the blood brain barrier into the general circulation.

1.10. HYPOTHESIS

The specific hypothesis tested in this thesis are:

1.) the PTH gene is expressed in the rat brain, specifically in the hypothalamus

2.) the neural PTH gene is very similar to that found in the parathyroid gland and,

3.) the neural PTH gene is transcribed to an immunoreative protein in the CNS

1.11. TABLES

Table 1.1. Additional "Non-Classic" PTH-Related Effects

BIOLOGICAL RESPONSE	REFERENCE
Hypotensive Activity in Dogs and Rats	Pang *et al.* (1981) Pang *et al.* (1983)
Vasodilation in Dogs	Crass and Pang (1981)
Increased Adenylate Cyclase Activity in Renal Microvessels	Helwig *et al.* (1987)
Increased Adenylate Cyclase Activity in Renal Tubules	Helwig *et al.* (1987)
Increased Glucose-6-Phosphate Dehydrogenase Activity in Renal Tubules	Sakaguchi *et al.* (1987)
Inotropic and Chronotrophic Effects on Heart Cells	Bogin *et al.* (1981)
Binding of PTH to Lymphocytes and Adenylate Cyclase Stimulation	Yamamoto *et al.* (1983)
Increased Mitotic Activity of Thymic Lymphocytes	Atkinson *et al.* (1987)

Table 1.2. Tissue Distribution of the PTH/PTHrp Receptor mRNA

TISSUE	INTENSITY	TISSUE	INTENSITY
Aorta	+	Lung	++
Adrenal Gland	+	Skel. Muscle	+
Bladder	+	Ovary	+
Cortex	+	Placenta	+
Cerebellum	+	Skin	+
Breast	+	Spleen	+
Bone	++	Stomach	+
Heart	+	Testis	++
Uterus	+		
Ileum	+	Thyroid Gland	-
Kidney	++++	Pituitary	-
Liver	++	Prostate	-

(Urena *et al.* 1993)

1.12. FIGURES

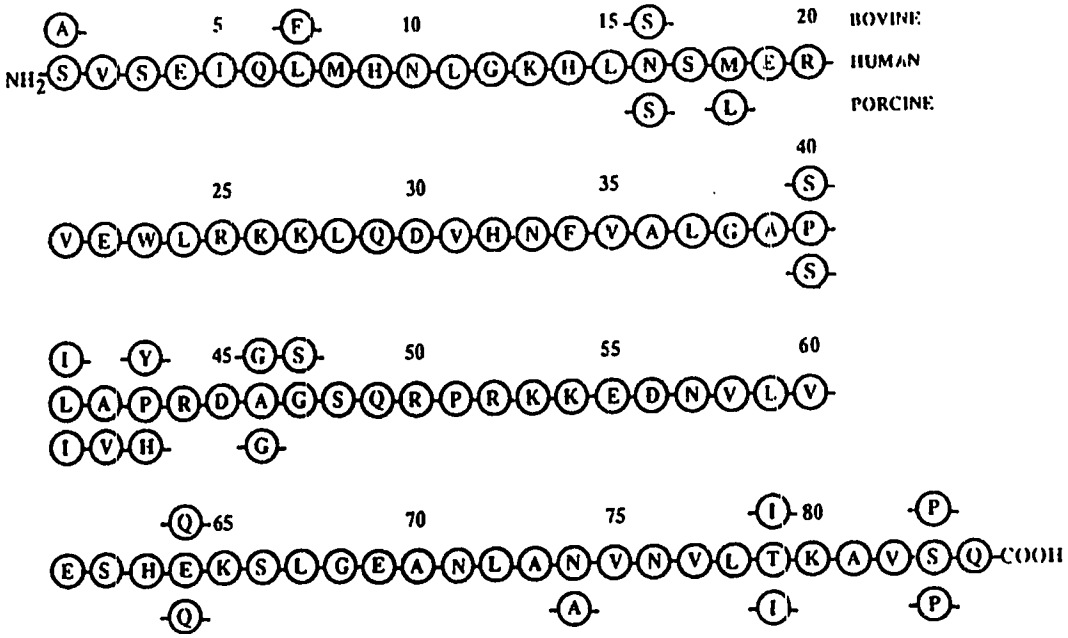

Figure 1.1. Primary structures of human, bovine and porcine PTH. Note the high degree of sequence homology between the three forms and the fact that most substitutions are conservative (e.g. isoleucine (I) for leucine (L)) (Watson and Hanley, 1993).

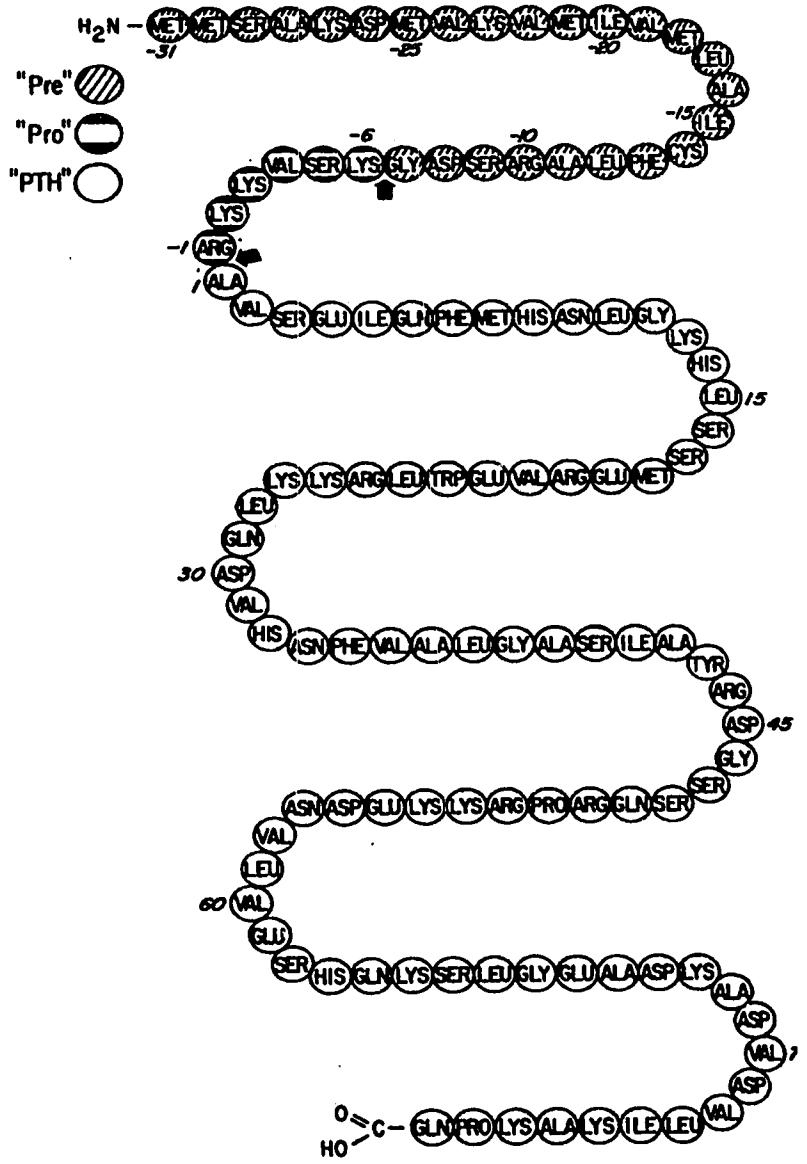

Figure 1.2. Primary structure of Pre-Pro-Parathyroid Hormone (Habener and Potts, 1978)

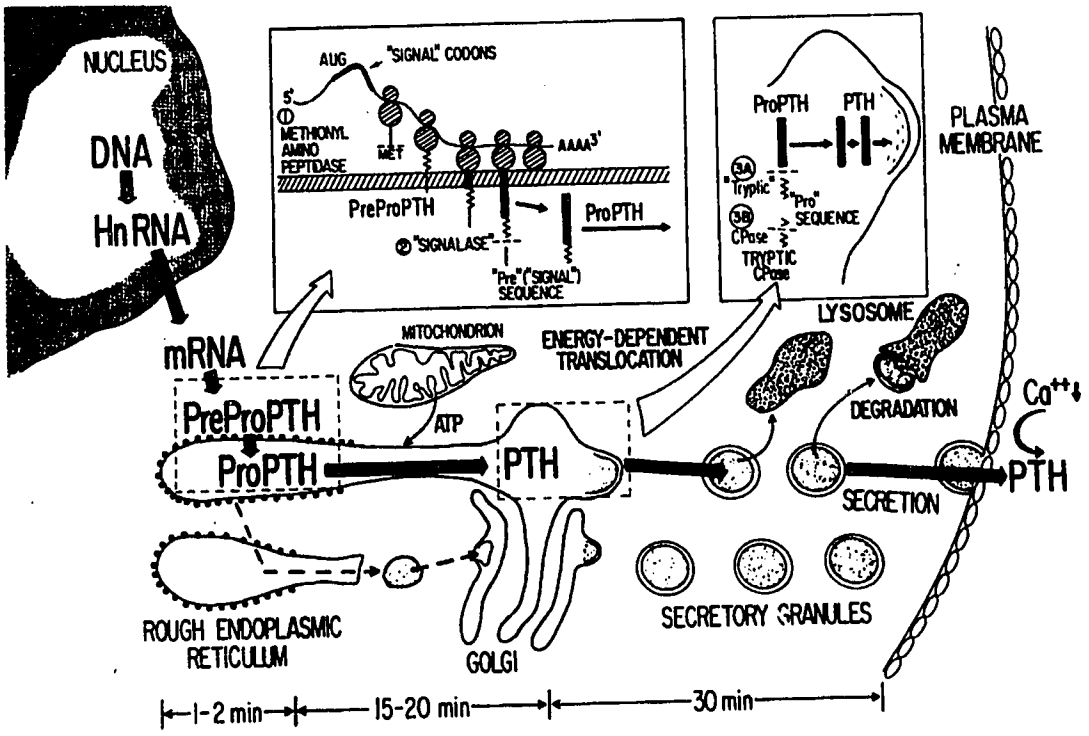

Figure 1.3. Biosynthesis of PTH (Habner and Potts1978).

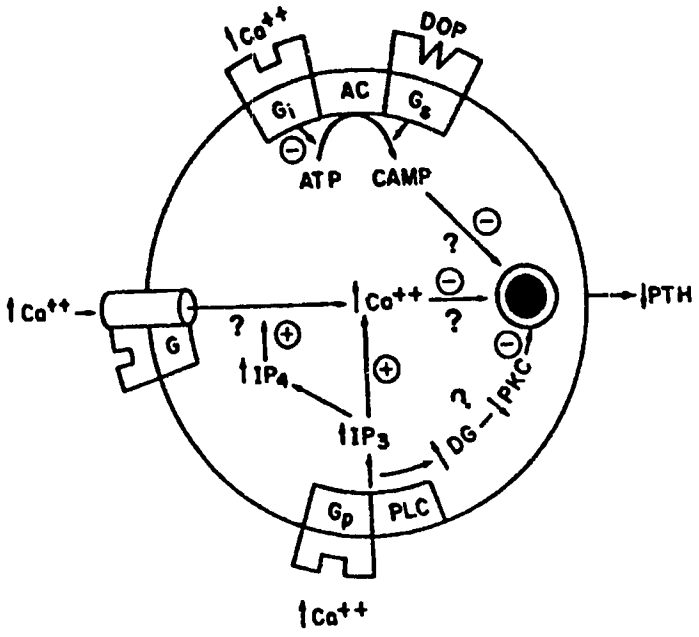

Figure 1.4. Model of the possible coupling of cell surface Ca^{2+} receptors to intracellular mediators and PTH (Brown, 1990).

1.13. REFERENCES

Agus, Z.S., Pushcett, J.G., Senesky, D. and Goldberg, M. Mode of action of parathyroid hormone and cyclic adenosine 3', 5'-monophosphate. J Clin Invest 50:617-626, 1971.

Akmal, M., Goldstein, D.A., Tuma, S.A., Fanti, R., Pattabhiraman, R. and Massry, S.G. The effect of parathyroid hormone (PTH) on the blood-brain barrier. Clin Res 31:52A, 1983.

Allen, E.M., Singer, F.R. and Melamed, D. Electorencephalographic abnormalities in hypercalcemia. Neurology 20:15-20, 1970.

Arnaud, C.D., Goldsmith, R.S., Bordier, P.J. and Sizemore, G.W. Influence of immunoheterogeneity of circulating parathyroid hormone on results of radioimmunoassays of serum in man. Am J Med 56:785-793, 1974.

Atkinson, M.J., Hesch, R.D., Cade, C., Wadwah, M. and Perris, A.D. Parathyroid hormone stimulation of mitosis in rat thymic lymphocytes is independent of cyclic AMP. J Bone Min Res 2:303-309, 1987.

Au, W.Y. Inhibition by 1,25 dihydroxycholecalciferol of hormonal secretion of rat parathyoid gland in organ culture. Calcif Tiss Int 36:384-391, 1984.

Aziz, N. and Munro, H.N. Both subunits of rat liver ferritin are regulated at a translational level by iron induction. Nucl Acids Res 14:915-927, 1986.

Balabanova, S., Tollerner, U., Richter, H.P., Pohlandt, F., Gaedicke, G. and Teller, W.M. Immunoreactive parathyroid hormone, calcium and magnesium in human cerebrospinal fluid. Acta Endocrinol 106:223-227, 1984.

Balabanova, S., King, O., Teller, W.M. and Reinhard, G. Distribution and concentration of immunoreactive parathyroid hormone, calcium and magnesium in human cerebrospinal fluid. Klinesche Wochrift 64:173-176, 1985.

Balabanova, S., Hartmann, J., Tollerner, U., Nowak, R. and Kirkpatrick, U.C. Effect of stress on calcium homeostasis in sheep. Horm Res 24:302-306, 1986.

Balabanova, S. and Helmer, B. Capacity of brain of sheep to degrade parathyroid hormone in vitro. Neuro Let 14:91-95, 1992.

Bartus, R.T., Dean, L.R., Beer, B. and Lippa, J. The cholinergic hypothesis of geriatric memory dysfunction. Science 217:408-417, 1982.

Bennett, M.K. and Scheller, R.H. The molecular machinery for secretion is conserved from yeast to neuron. Proc. Natl Acad. Sci. (USA) 90:1559-1563, 1993.

Bertoni, T.E. The skeletal muscle in hyperparathyroidism. In: First Intl Conf on New Actions of Parathyroid Hormone. Kobe, Japan, October 35 (abstr.) :1987.

Bogin, E., Massry, S.G. and Harary, I. Effect of parathyroid hormone on rat heart cells. J. Clin Invest 67:1215-1227, 1981.

Boustein, M.P., Johnson, M.E. and Needleman, P. Calcium dependent norepinephrine release from presynaptic nerve endings in vitro. Proc Natl Acad Sci (USA) 69:2237-2240, 1972.

Brent, G.A., Leboff, M.S., Seely, E.W., Conlin, P.R. and Brown, E.M. Relationship between the concentration and rate of change of calcium and serum intact parathyroid hormone levels in normal humans. J Clin Endocrinol Metab 67:944-950, 1988.

Bringhurst, F.R., Stern, A.M., Yotts, M., Mizrahi,N., Segre, G.V., Potts, J.T.Jr. Peripheral metabolism of [35S] parathyroid hormone in vivo: influence of alterations in calcium availability and parathyroid status. J Endocrinol 122:237-245,1989.

Broadus, A.E., Lang, R. and Kliger, A.S. The influence of calcium intake and the status of intestinal calcium absorption on the diagnostic utility of measurements of 24-hour cyclic adenosine 3', 5'-monophosphate excretion. J Clin Endocrinol Metab 52:1085-1089, 1981.

Bronner, F., Pansk, D. and Stein, W.D. Analysis of intestinal calcium transport across the rat intestine. Am J Physiol 250:G561-564, 1986.

Brookman, J.J., Farrow, S.M., Nicholoson, L., O'Riordan, J.L.H. and Henry, G.N. Regulation by calcium of parathyroid hormone mRNA in cultured parathyroid tissue. J Bone Min Res 1:529-537, 1986.

Brown, E.M., Gardner, D.G., Windneck, R.A. and Aurbach, G.A. Relationship of intracellular 3',5'-adenosine monophosphate accumulation to parathyroid hormone release from dispersed bovine parathyroid cells. Endocrinology 103:2323-2333, 1978.

Brown, E.M., Gardner, D.G., Brennan, M.F., Marx, S.J., Spiegel, A.M., Attie, M.F., Downs, R.W. Jr. Doppman, J.L., Aurbach, C.D. Calcium-regulated parathyroid hormone release in primary hyperparathyroidism. Am J Med 66:932-931, 1979.

Brown, E.M., Wilson, R.E., Thatcher, J.G. and Marynick, S.P. Abnormal calcium-regulated PTH release in normal parathyroid glands from pateints with an adenoma. Am J Med 71:565-570, 1981.

Brown, E.M. PTH secretion in vivo and in vitro regulation by calcium and other secretagogues. Miner Electrolyte Metab 8:130-150, 1982.

Brown, E.M. Four-parameter model of the sigmoidal relationship between parathyroid hormone release and extracellular calcium concentration in normal and abnormal parathyroid tissue. J Clin Endocrinol Metab 56:572-581, 1983.

Brown, E.M. Extracellular Ca++ sensing regulation of parathyroid cell function, and role of Ca++ and other ions as extracellular (first) messengers. Physiol Revs 71:371-411, 1991.

Brumbaugh, P.F. and Haussler, M.R. Nuclear and cytoplasmic binding components for vitamin D metabolites. Life Sci 16:353-362, 1975.

Burguyne, R.D. Control of exocytosis in adrenal chromaffin cells. Biochem Biophys Acta 1071:174-202, 1991.

Calvo, M.S., Kumar, R. and Heath, I Persistently elevated parathyriod secretion and action in young women after four weeks of ingesting high phosphorous, low calcium diets. J Clin Endocrinol Metab 70:1334-1340, 1990.

Calvo, M.S., Eastall, R., Offord, K.P., Bergstralh, E.J. and Burrit, M.F. Circadian variation in ionized calcium and intact parathyroid hormone: evidence for sex differences in calcium homeostasis. J Clin Endocrinol Metab 72:69-76, 1991.

Cantley, L.K., Russell, J., Lettieri, D. and Sherwood, L.M. 1,25-Dihydroxyvitamin D3 suppresses parathyroid hormone secretion from bovine parathyroid cells in tissue culture. Endocrinology 117:2114-2119, 1985.

Care, S.D, and Bell, J. Evidence that parathyroid hormone crosses the blood-brain barrier. Proceedings of the IX International conference of Calcium Regulating Hormones (abstract):181, 1986.

Castro, J.H., Caro, J.F., Kim, H.J. and Glenon, J.A. Effects of parathyroid hormone infusion and primary hypoparathyroidism on serum prolactin in man. J Clin Endocrinol Metab 51:397-398, 1980.

Clementi, G., Drago, F., Prato, A. Effects of calcitonin, parathyroid hormone and its related fragments on acquisition of active avoidance behavior. Physiol Behav 33:913-916, 1984.

Clementi, G., Drago, F., Amico-Roxas, M. Central actions of calcitonin and parathyroid hormone. In Calcitonin: Chemistry, Physiology and Clinical Aspectspp 275-286, 1985. Ed A Pecile Amsterdam, Elsevier Science Publishers BV.

Cloix, J.F., Cueille, G. and Funck-Brentano, J.L. Inhibition of bovine renal adenylate cyclase by urinary products. Biomedicine 25:215-218, 1976.

Cogan, M.G., Covey, C., Arieff, A.I. Central nervous system manifestaions of hyperparathyroidism. Am J Med 65:963-970, 1978.

Collipp J.B and Clark E.P Further studies of the physiological action of a parathyroid hormone. J Biol Chem 66, 133-138, 1925.

Copper, J.D., Lazarowitz, V.C. and Arieff, A.I. Neurodiagnostic abnormalities in patients with acute renal failure: evidence for neurotoxicity of parathyroid hormone. J Clin Invest 61:1448-1455, 1978.

Crass, M.F. and Pang, P.K.T. Parathyroid hormone: a coronary artery vasodilator. Science 207:1087-1089, 1980.

Crass, M.F., Moor, P.L., Strickland, M.L. and Citak, M.L. Cardiovascular response to human PTH-(1-34) in the dog. Proc West Parmacol Soc 25:269, 1982.

D, Amour,P., Palandy, J., Bahsali, G., Mallette, L.E., DeLean, A. and Lepage, R. The modulation of circulating parathyroid hormone immunoheterogeneity in man by ionized calcium concentration. J Clin Endocrinol Metab 74:525-532, 1992.

DeFeudis, F.V. Preferential "binding" of gamma-aminobutyric acid and glycine to synaptosome-enriched fractions fo rat cerebral cortex and spinal cord. Can J Physiol Pharmacol 52:138-147, 1974.

DeMay, M.G., Kiernan, M.S., Deluca, H.F. and Kronenberg, H.M. Sequences in the human parathyroid hormone gene that bind to the 1,25-dihydroxyvitamin D3 receptor and mediate transcriptional repression in response to 1,25-dihydroxyvitamin D3. Proc Natl Acad Sci (USA) 89:8097-8101, 1992.

Desir, D., Van Cauter, E., L'Hermite, M., Refetoff, S., Jadot, C., Caufriez, A., Copinschi, G., Robyn, C. Effects of "jet lag" on hormonal patterns. III. Demonstration of an intrinsic circadian rhythmicity in plasma prolactin. J Clin Endocrinol Metab 55:849-857, 1982.

Diamond, D.J. and Goodman, H.M. Regulation of growth hormone messenger RNA synthesis by dexamethasone and triiodothyronine. Transcription rate and mRNA stability changes in pituitary tumor cells. J Mol Biol 81:41-62, 1985.

Drueke, T., Zingraff, J., Bourdeau, A., Clair, F., Kindermans, C., Buisson, C., Manganella, G., Sachs, C. Hypocalcaemic effect of WR-2721, S-2 (3-aminopropylamino) ethly-phosphorothic acid in an anuric haemodialysis patient. Nephrol Dial Transplant 2(1) :48-52, 1987.

Dubovsky, S.L. and Franks, R.D. Intracellular calcium ions in affective disorders: a review and an hypothesis. Biol Psychiat 18:781-797, 1983.

Farrow, S.M., Karmali, R., Gleed, J.H., Hendy, G.N. and O'Riordan, J.L. Regulation of preproparthyroid hormone messenger RNA and hormone synthesis in human parathyroid adenomata. J Endocrinol 117:133-128, 1988.

Fioretti, P., Melis, G.B., Ciardella, F., Barsotti, G., Orlandi, M.C., Paoletti, A.M., Giovannetti., S. Parathyroid function and pituitary-gonadal axis in male uremics; effects of dietary treatment and of maintenance hemodialysis. Clin Nephrol 25:155-158, 1986.

Ford, D.H. Blood-brain barrier: a regulatory mechanism. Rev Neurol 2:1-42, 1976.

Fraser, C.L. and Arieff, A.I. Nervous system complications in uremia. Ann Int Med 109:142-153, 1988.

Fraser, R.A., Sarnaki, P. and Budayr, A. Evidence that parathyroid hormone-mediated calcium transport in rat brain synaptosomes is independent of cyclic adenosine monophosphate. J Clin Invest 81:982-988, 1988.

Fraser, R.A. PTH and PTH-like peptides in neural tissues. Ph.D. Thesis, University of Alberta. 1991.

Fraser, R.A., Kronenberg, H.M., Pang, P.K.T. and Harvey, S. Parathyroid hormone messenger ribonucleic acid in the rat hypothalamus. Endocrinology 127:2517-2522, 1990.

Gennari, C. Parathyroid hormone and pain. In New Actions of Parathyroid Hormone. Eds. S. G. Massry & T. Fujita. New York: Plenum Press. pp. 335-344, 1988.

Gennari, C., Agnusdei, D., Gonnelli, S. Hyperalgesic activity of parathyroid hormone. J. Endocrinol Invest 14:34, 1991.

Gilmour, J.R. The embryology of the parathyroid glands. J Pathol Bact 45:507-522, 1937.

Goldstein, D., Feinstein, E.I., Chui, L.A., Pattabhiraman, R. and Zacchei, F. The relationship between the abnormalities in electroencephalogram and blood levels of PTH in dialysis patients. J Clin Endocrinol Metab 51:130-134, 1980.

Goltzman, D., Huang, S.N., Browne, C. and Solomon, S. Adrenalcorticotropin and calcitonin in medullary thyroid carcinoma: frequency of occurence and localization in the same cell type immunocytochemistry. J Clin Endocrinol Metab 49:364-369, 1979.

Goltzman, D., Henderson, B. and Loveridge, N. Cytochemical bioassay of parathyroid hormone: characteristics of the assay and analysis of circulating hormonal forms. J Clin Invest 65:1309-1312, 1980.

Goltzman, D., Gomolin, H., DeLean, A., Wexler, M. and Meakins, J.L. Discordant disappearance of bioactive and immunoreactive parathyroid hormone after parathyroidectomy. J Clin Endocrinol Metab 58:70-75, 1984.

Guisado, R., Arieff, A.I. and Massry, S.G. Changes in the electroencephalogram in acute uraemia. Effects of parathyroid hormone and brain electrolytes. J Clin Invest 55:738-748, 1975.

Habener, J.F., Kemper, B., Potts, J.T.Jr. and Rich, A. Parathyroid mRNA directs the synthesis of pre-proparathyroid hormone and proparathyroid hormone in the Krebs ascites cell-free system. Biochem Biophys Res Comm 67:1114-1121, 1975.

Habener, J.K. and Potts, J.T.Jr. Relative effectiveness of magnesium and calcium on the secretion and biosynthesis of parathyroid hormone in vitro. Endocrinology 98:197-202, 1976.

Habener, J.F. Regulation of polypeptide-hormone biosythesis at the level of the genome. Am J Physiol 249:C191-C199, 1985.

Harvey, S., Zeng, Y. and Pang, P.K.T. Parathyroid hormone-like immunoreactivity in fish plasma and tissues. Gen Comp Endocrinol 68:136-146, 1987.

Harvey, S. and Fraser, R.A. Parathyroid hormone: neural and neuroendocrine perspectives. J Endocrinol 139:353-361, 1993a.

Harvey, S., Hayer, S. and Sloley, B.D. Dopaminergic actions of parathyroid hormone in the rat medial basal hypothalamus in vitro. Regul Pept 43:49-56, 1993b.

Harvey, S., Hayer, S. and Sloley, B.D. Parathyroid hormone-induced dopamine turnover in the rat medial basal hypothalamus. Peptides 14:269-274, 1993c.

Harvey, S. and Hayer, S Parathyroid hormone binding sites in the brain. Peptides 14:1187-1191, 1993.

Harvey,S., and Fraser,R.A Parathyroid hormone: neural and neuroendocrine perspectives (Review). J Endocrinol 139:353-361, 1993

Hawa, N.S., O'Riordan, J.L. and Farrow, S.M. Post-translational regulation of bovine parathyroid hormone synthesis. J Mol Endocrinol 10:43-49, 1993.

Heinrich, G., Kroneneberg, H.M., Potts, J.T.Jr. and Habener, J.F. Gene encoding parathyroid hormone. Nucleotide sequence of the rat gene and deduced amino acid sequence of rat preproparathyroid hormone. J Biol Chem 259:3320-3329, 1984.

Helwig, J.J., Pang, P.K.T., Krill, J. and Friedmann, E. Parathyroid hormone stimulation of renal adenylate cyclase in various vertebrate species: evidence for an effect in the frog. Comp Biochem Physiol 88 :349-354, 1987a.

Helwig, J.J., Yang, M.C., Bollack, C., Judes, C. and Pang, P.K.T. Structure-activity relationship of parathyriod hormone: relative sensitivity of rabbit renal microvessel and tubule adenylate cyclases to oxidize PTH and PTH inhibitors. Eur J Pharmacol 140:247-257, 1987b.

Isacc, R., Merceron, R.E., Caillens, G., Raymond, J.P. and Ardaillon, R. Effect of parathyroid hormone on plasma prolactin in man. J Clin Endocrinol Metab 47:18-23, 1978.

Itoh, N. and Okamoto, H. Translational control of proinsulin synthesis by glucose. Nature 286:100-102, 1980.

Joborn, C., Hetta, J., Niklasson, F., Rastad, J., Wide, L., Agren, H., Akerstrom, G., Ljunghall, S. Cerebrospinal fluid, calcium, parathyroid hormone and monoamine and purin metabolites and the blood-b function in primary hyperparathyroidism. Psychoneuroendocrinology 16:311-.

Kaneko, T. and Pang, P.K.T. Immunocytochemical detection of parathyroid hormone like substance in the goldfish brain and pituitary gland. Gen Comp Endocrinol 68:147-152, 1987.

Karmali, R., Farrow, S., Hewison, M., Barker, S. and O'Riordan, J.L.H. Effects of 1,25-dihydroxyvitamin D and cortisol on bovine and human parathyroid cells. J Endocrinol 123:137-172, 1989.

Katoh, Y., Klein, K.L., Kaplan, R.A., Sanborn, W.G. and Kurokawa, K. Parathyroid hormone has a positive inotropic action in the rat. Endocrinology 109:2252-2257, 1981.

Khudaverdyan, D.N., Astratyan, A.A., Vladimirov, S.V. and Svishchev, A.V. The morphofunction state of the supraopticoneurohypophyseal system of rats in hypoparathyroidism. Neural Behav Physiol 23:112-114, 1993.

Kostyuk, P.G., Lukyanetz, E.A. and Ter-Markosyan, A.S. Parathyroid hormone enhances calcium current in snail neurones stimulation of the effect by phorbol esters. Eur J Physiol 420:146-152, 1992.

Kruse, K., Gutekunst, B., Kracht, U. and Schwerda, K. Deficient prolactin response to parathyroid hormone in hypocalcemic and normocalcemic pseudohypoparathyroidism. J. Clin Endocrinol Metab 52:1099-1105, 1981.

Lalley, P.M., Rossi, G.V. and Baker, W.W. Analysis of local cholinergic tremor mechanisms following selective neurochemical lessions. Exp Neurol 27:258-275, 1970.

Liebold, E.A. and Munro, H.N. Cytoplamic protein binds in vitro to a highly conserved sequence in the 5'-untranslated region of ferritin heavy-and light-subunit mRNAs. Proc Natl Acad Sci (USA) 85:2171-2175, 1988.

Linkhart, T.A., Mohan, S. Parathyroid hormone stimulates release of insulin-like growth factor-I (IGF-I) and IGF-II from neonatal mouse calvaria in organ culture. Endocrinology 125:1484-1491, 1989.

Loffer, F., Van Calker, D. and Hamprecht, B. Parathyrin and calcitonin stimulate cyclic AMP accumulation in cultured murine brain cells. EMBO J. 1:297-302, 1982.

Logue, F.C., Fraser, W.C., O'Reilly, D.J. and Beastall, G.H. The circadian rhythm of intact parathyroid hormone and nephrogenous cyclic adenosine monophosphate in normal men. J Endocrinol 121:R1-R3, 1989.

Logue, F.C., Fraser, W.D., O'Reilly, D.J., Cameron, D.A., Kelly, A.J. and Beastall, G.H. The circadian rhythm of intact parathyroid hormone (1-84): temporal correlation with prolactin secretion in normal men. J Clin Endocrinol Metab 71:1556-1560, 1990.

Lougue, F.C., Fraser, W.D., O'Reilly, D.J., et al. Sleep shift dissociates the nocturnal peaks of parathyroid hormone (1-84), nephrogenous cyclic adenosine monophosphate, and prolactin in normal men. J Clin Endocrinol Metab 75:25-29, 1992.

Lui, J., Kahri, A., Temppo, A.M. and Voutilainen, R. Regulation of parathyroid hormone gene expression and peptide secretion in human parathyroid cells. Eur J Endocrinol 130:394-401, 1994.

MacGregor, R.R., Hinton, D.A. and Ridgeway, R.D. Effects of calcium on synthesis and secretion of parathyroid hormone and secretory protein I. Am J Physiol 255:E299-E305, 1988.

Mallete, L.E., Tuma, D.N., Berger, R.E. and Kirkland, J.L. Radioimmunoassay for the middle region of human parathyroid hormone using a homologous antiserum with a carboxy-terminal fragment of bovine parathyroid hormone as radioligand. J Clin Endocrinol Metab 54:1017-1024, 1982.

Marotta, S.F. The role of parathyroid hormone and thyrocalcitonin in altering plasma calcium levels and adrenocortical secretory rates. Horm Metab Res 3:344-348, 1971.

Massry, S.G., Smogorzewski, M. and Islam, A. Derangements in brain synaptosomes functions in chronic renal failure: role of parathyroid hormone. In: New Actions of Parathyroid Hormone. Eds. S.G.Massry & T.Fujita.New York: pp 301-316, 1988.

Mattia, E., den Blaauewn, J., Ashwell, G. and van Renswoude, J. Multiple post-translational regulatory mechanisms in ferritin gene expression. Proc Natl Acad Sci (USA) 86:1809-1805, 1989.

Mayer, G.P. and Hurst, J.G. Sigmoidal relationship between parathyriod hormone secretion rate and plasma calcium concentration in calves. Endocrinology 102:1036-1042, 1978.

Miller, F.R., Stavrakey, G.W. and Wonnton, G.A. Effects of serine, acetylcholine and atropine on electrocorticogram. J Neurosci 3:131-138, 1940.

Morimoto, S., Masugi, F., Hironaka, T., Saito, H., Tabuchi, Y. Relation of serum parathyroid hormone to cognitive function in elderly females. In: New Action of Parathyroid Hormone, pp 327-334, 1988. Eds.S.G.Massry & T.Fujita, New York, Plenum Press.

Morrissey, J.J., Hamilton, J.J., MacGregor, R.R. and Cohn, D.V. The secretion of parathormone fragments 34-84 and 37-84 by dispersed porcine parathyroid cells. Endocrinology 107:164-171, 1980.

Naveh-Many, T., Almogi, G., Livni, N. and Silver, J. Estrogen receptors and biologic response in rat parathyroid tissue and C cells. J Clin Invest 90:2434-2438, 1992.

Ni, Z., Smogorzewski, M. and Massry, S.G. Derangements in acetylcholine metabolism in brain synaptosomes in chronic renal failure. Kidney Int 44 :630-637, 1993.

Nickols, G.A., Metz, M.A. and Cline, W.H. Endothelium-independent linkage of parathyroid hormone receptors of rat vascular tissue with increased adenosine 3',5'-monophosphate and relaxation of vascular smooth muscle. Endocrinology 119:349-356, 1986.

Nickols, G.A., Nickols, M.A. and Helwig, J.J. Binding of parathyroid hormone and parathyroid hormone-related protein to vascular smooth muscle of rabbit renal microvessels. Endocrinology 126:721-729, 1990.

Okazaki, T., Igarashi, T. and Kronenberg, H.M. 5'-flanking region of the parathyroid hormone gene mediates negative regulation by 1,25-(OH)2 vitamin D3. J Biol Chem 263:2203-2208, 1988.

Okazaki, T., Zajac, J.D., Igarashi, T., Ogata, E. and Kronenberg, H.M. Negative regulatory elements in the human parathyroid hormone gene. J Biol Chem 266:21903-21910, 1991.

Orloff, J.B., Wu, T.L. and Stewart, A.F. Parathyroid hormone-like proteins: biochemical responses and receptor interaction. Endocr Rev 610:476-495, 1989.

Pang, P.K.T. and Sawyer, W.K. Parathyroid hormone preparations, salmon calcitonin, and urine flow in the South American Lungfish, Lepidosiren Paradoxa. J Exp Zool 193:407-412, 1975.

Pang, P.K.T., Yang, M., Oguro, C., Phillips, J.G. and Yee, J.A. Hypotensive action of parathyroid hormone preparations in vertebrates. Gen Comp Endocrinol 41:135-138, 1980.

Pang, P.K., Shew, R.L. and Sawyer, W.H. Inhibition of uterine contraction by synthetic parathyroid hormone fragment. Life Sci 58:1317-1321, 1981.

Pang, P.K., Yang, M.C., Keutmann, H.T. and Kenny, A.D. Structure activity relationship of parathyroid hormone: separation of the hypotensive and the hypercalcemic properties. Endocrinology 112:284-289, 1983.

Pang, P.K.T., Yang, M.C.M. and Tenner, T.D.J. Beta-adrenergic like actions of parathryoid hormone. Trend Pharm Sci 7:340-341, 1986.

Pang, P.K.T. Recent advances in the study of the vascular action of parathyroid hormone. In: New Actions of Parathyroid Hormone, 127-134, 1988a. Eds. S.G. Massry & T. Fugita, New York, Plenum Press.

Pang, P.K.T., Harvey, S., Fraser, R.S and Kaneko, T. Parathyroid hormone-like immunoreactivity in vertebrate brains. Am J Physiol 255:R635-R647, 1988b.

Pang, P.K.T., Kaneko, T. and Harvey, S. Immunocytochemical distribution of PTH immunoreactivity in vertebrate brains. Am J Physiol 255:R643-R647, 1988c.

Pang, P.K.T., Wang, R., Shan, J., Karpinski, E. and Benishin, C.G. Specific inhibition of long-lasting L-type calcium channels by synthetic parathyroid hormone. Proc Natl Acad Sci (USA) 87:623-627, 1990.

Parsons, J.A., Rafferty, B., Gray, D. Pharmacology of parathyroid hormone and some of its fragments and analogues. In: Calcium-regulating hormones: Proceedings of the Fifth Parathyroid Conference, 21-26, 1975. Amsterdam, Exerpta Medica.

Peraldi, M.N., Rondeau, E. and Jousset, V. Dexamethasone increases preparathyroid hormone messenger RNA in human hyperplastic parathyroid cell in vitro. Eur J Clin Invest 20:392-397, 1990.

Peris, A.D., Weiss, L.A. and Whitfield, J.F. Parathyriodectomy and the induction of thymic atrophy in normal, adrenalectomised and orchidectimised rats. J Cell Physiol 76:141-150, 1970.

Pocotte, S.L., Ehrenstein, G. and Fitzpatric, L.A. Regulation of parathyroid hormone secretion. Endocr Rev 12:291-301, 1991.

Potts, J.T. Parathyroid hormone: sequence, synthesis, immunoassay studies. Am J Med 50:639-642, 1971.

Potts, J.T., Kronenberg, H.M. and Rosenblatt, M. Parathyroid hormone: chemistry, biosynthesis, and mode of action. Adv Prot Chem. 35:323-396, 1982.

Quamme, G., Pfeilshifter, J. and Murer, M. Parathyroid hormone inhibition of Na+/phosphate co-transport in OK cells: generation of second messengers in the regulatory cascade. Biochem Biophys Res Comm 158:951-956, 1989.

Rasmussen, H. and Barett, P.Q. Calcium messenger system: An integrated view. Physiol Rev 64:938, 1984.

Raymond, J.P., Isaac, R., Merceron, R.E. and Wahbe, E.F. Comparison between the plasma concentrations of prolactin and parathyroid hormone in normal subjects and in patients with hyperparathyroidism or hyperprolactinemia. J Clin Endocrinol Metab 55:1222-1225, 1982.

Reis, A., Hecht, W., Groger, R., Bohm, I., Cooper, D.N. and Lindenmainer, W. Cloning and sequence analysis of the human parathyroid hormone gene region. Human Genet 85:119-124, 1990.

Rinaldi, F. and Himwich. H.E. Alerting response and actions of atropine and cholinergic drugs. Arch Neurol Psychiat 73:387-395, 1955a.

Rinaldi, F. and Himwich, H.E. Cholinergic mechanism involved in fuction of mesodiencephalic activating system. Arch Neurol Psychiat 73:396-402, 1955b.

Rodjmark, S., Edstrom, E. and Nordlund, M. Effect of chronic endogenous hypercalcemia on prolactin and thyrotropin responsiveness in man. J Endocrinol Invest 7:635-63[illegible], 1984.

Rosenblatt, M., Kronenberg, H.M. and Potts, J.T.Jr. Parathyroid hormone physiology, chemistry, biosynthesis, secretion, metabolism and mode of action. In Endocrinology, Vol 2 L.J.De Groot.Philadelphia: W.B.Saunders Company. pp884-891, 1989.

Rouault, T.A., Tang, C.K., Kaptain, S., Burgess, W.H., Haile, D.J., Samaniego, F. Cloning of the cDNA encoding an RNA regulatory protein-the human iron-responsive element-binding protein. Proc Natl Acad Sci (USA) 87:7958-7962, 1990.

Rupp, E., Mayer, H. and Wingender, E. The promoter of the human parathyroid hormone gene contains a functional cyclic AMP response element. Nucl Acid Res 18:5677-5683, 1990.

Russel, J., Lettieri, D. and Sherwood, L.M. Direct regulation by calcium of cytoplasmic messenger ribonucleic acid coding for pre-pro-parathyroid hormone in isolated bovine parathyroid cells. J Clin Invest 72:1851-1855, 1983.

Segre, G.V., Habener, J.R., Powell, D., Tregear, G.W. and Potts, J.T.Jr. Parathroid hormonem in human plasma: imunochemical characterization and biologic implications. J Clin Invest 51:3163-3167, 1972.

Sherwood, I .M., Mayer, G. and Potts, J.T.Jr. Regulation of parathyroid hormone secretion: proportional control by calcium, lack of effect of phosphate. Endocrinology 83:1329-1351, 1968.

Silver, J., Russell, J. and Sherwood, L.M. Regulation by vitamin D metabolites of messenger ribonucleic acid for preproparathyroid hormone in isolated bovine parathyroid cells. Proc Natl Acad Sci. (USA) 82:4270-4273, 1985.

Sollner, T., Whiteheart, S.W. and Brunner, M. SNAP receptors implicated in vesicle targeting and fusion. Nature 362:318-320, 1993.

Spiegel, A.M. Signal transduction by guanine nucleotide binding proteins. Mol Cell Endocrinol 49:1-16, 1987.

Sugimoto, T., Brown, A.J., Ritter, C., Morrisey, J., Slatopolsky, E. and Martin, K.J. Combined effects of dexamethasone and 1,25-Dihydroxyvitamin D3 on parathyroid hormone secretion in cultured bovine parathyroid cells. Endocrinology 125:638-641, 1989.

Tanguay, K.E., Mortimer, S.T., Wood, P.H. and Hanley, D.A. The effects of phorbol myristate acetate on the intracellular degradation of bovine parathyroid hormone. Endocrinology 128:1863-1868, 1991.

Tenner, T.E., Ramanadham, S., Yang, M.C.M. and Pang, P.K.T. Chronotropic actions of bPTH (1-34) in the right atrium of the rat. Can J Physiol Pharmacol 61:1162-1168, 1983.

Tregear, G.W., Van Rietschoten, J., Greene, E., Niall, H.D., Keutmann, H.T., Parsons, J.A., O'Riordan, J.L., Potts, J.T. Jr. Bovine parathyroid hormone: minimum chain length of synthetic peptide required for biological activity. Endocrinology 93:1349-1353, 1973.

Urena, P., Kong, X.F., Abou-Samra, A.B., Juppner, H., Kroneneberg, H.M., Potts, J.T.Jr., Segre, G.V. Parathyroid hormone (PTH)/PTH-related peptide receptor messenger ribonucleic acids are widely distributed in rat tissues. Endocrinology 133:617-623, 1993.

Walden, W.E., Daniels-Queen, S., Brown, P.H., et al. Translational repression in eukaryotes: partial purification and characterization of a repressor of ferritin mRNA translation. Biochemistry 5:9503-9507, 1988.

Werner, S., Low, H. Stimulation of lipolysis and calcium accumulation by parathyroid hormone in rat adipose tissue in vitro after adrenalectomy and administration of high doses of cortisone acetate. Horm Metab Res 5 :292-296, 1973.

Wang, R., Pang, P.K., Wu, L., Shipley, A., Karpinski, E., Harvey, S., Berdan, R.C. Neural effects of parathyroid hormone: modulation of the calcium channel current and metabolism of monoamines in identified Helisoma snail neurones. Can J Physiol Pharmcol 71 :582-591, 1993.

Wendellar Bonga, S.E., Lafeber, F.P.J.G., Flik, G., Kaneko, T. and Pang, P. Immunochtochemical demonstration of a novel system of neuroendocrine peptidergic neurons in the pond snail Lymnaea stagnalis, with antisera to the teleostean hormone hypcalcin and mammalian parathyroid hormone. Gen Comp Endocrinol 75:29-38, 1989.

Yamamoto, I., Potts, J.T. and Segre, G.V. Circulating bovine lymphocytes contain receptors for parathyroid hormone. J Clin Invest 71:404-407, 1983.

Yamamoto, Y., Fukase, M., Fujii, Y. and Fujita, T. The effects of human parathyroid hormone-related peptide on cytosolic free calcium and cAMP production in oppossum kidney cell. Bone Min 7:221-231, 1989.

Yao, C.Z., Ishizuka, J., Courtney, M.T.J. and Thompson, J.C. Successful intracerebroventricular allotransplantation of parathyroid tissue in rats without immunosuppression. Transplantation 55:251-253, 1993.

Zabel, B.U., Kronenberg, H.M., Bell, G.I. and Shows, T.B. Chromosome mapping of genes on the short arm of human chromosome 11: parathyroid hormone gene is at 11p15 together with the genes for insulin, c-harvey-ras 1, and beta-hemoglobin. Cytogenet & Cell Genet 39:200-208, 1985.

CHAPTER 2. SEQUENCE ANALYSIS OF PARATHYROID HORMONE (PTH)

2.1. INTRODUCTION

Parathyroid hormone (PTH)-like immunoreactivity is present in extracts of the rat hypothalamus (Pang *et al.* 1988) and sheep brain (Balabanova *et al.* 1985, Balabanova *et al.* 1986). The immunoreactivity in these tissues is associated with a heat-stable, non-dialazable peptide that co-elutes with authentic PTH_{1-84} after HPLC fractionation (Pang *et al.* 1988). The blood-brain barrier has been considered to be impermeable to PTH in systemic circulation (Akmal *et al.* 1984, Balabanova *et al.* 1984) and the brain may therefore be an extra-parathyroidal site of PTH production. This possibility is supported by the localisation of PTH-immunoreactivity within perikarya in discrete rat hypothalamic nuclei (Pang *et al.* 1988), in which PTH-like mRNA was detected by *in situ* hybridization (Fraser *et al.* 1990). It is, however, still uncertain if the PTH-like immunoreactivity and PTH-like mRNA in the brain reflects the extra-parathyroidal transcription of the PTH gene or the expression of a closely related gene.

The PTH-like mRNA in the rat hypothalamus was found, by Northern blotting with a PTH riboprobe, to be of comparable size to the RNA moiety in parathyroidal tissue (Fraser *et al.* 1990). Some sequence homology with the parathyroidal transcript was also indicated by the

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Foot Note. A version of this chapter, *Sequence Analysis of Hypothalamic Parathyroid Hormone Messenger Ribonucleic Acid,* was submitted to *Endocrinology* for publication. Dr Parimi gave advice on the cloning of the PCR fragments.

generation of a 384 bp RT-PCR (reverse transcriptase-polymerase chain reaction) cDNA moiety of predicted size, identical to parathyroidal PTH cDNA (Fraser *et al.* 1990). This PTH-like transcript was, however, of low abundance and the cDNA fragment generated by RT-PCR was only distinguishable after a "booster" PCR re-amplification. It is therefore possible that this cDNA moiety was a PCR artefact reflecting the "illegitimate transcription" of a constitutively expressed gene (Cally et al. 1989). It may also be possible that the "sticky end" PCR primers chosen for this study (Fraser et al. 1990) simply lacked sufficient homology to the PTH gene, thus reducing the efficiency of PCR amplification. The abundance of PTH-like mRNA in the rat brain and its sequence homology with parathyroidal PTH mRNA has therefore been determined in the present study.

2.2. MATERIALS AND METHODS

2.2.1 Tissue preparation

Parathyroid glands, hypothalami, extra-hypothalamic brain, pituitary glands and liver tissues were rapidly dissected from 3 week old (200 - 250g), male Sprague Dawley rats and immediately frozen in liquid nitrogen and stored at -80°C prior to analysis. The hypothalami were excised from the rest of the brain through four perpendicular cuts in the shape of a diamond, each approximately 2.4 mm lateral and 3.5 mm dorsal of the midline (Paxinos et al. 1986).

2.2.2. RNA preparation

Total cellular RNA was isolated from tissue extracts using RNA NOW™ (Bio/Can Scientific, Mississauga, ON, Canada). Briefly, following homogenisation in RNA NOW™, 0.2ml chloroform was added to each sample and then shaken for 20 seconds. After resting on ice for 5 min the samples were centrifuged (10,000 x g for 10 min at 4C°) and the RNA precipitates were washed twice with (1ml) 75% (v/v) ethanol and centrifuged (5000 x g for 5 min at 4°C) prior to resuspension in diethylpyrocarbonate (DEPC) treated water. The purity and amount of RNA extracted was assessed spectrophotometricly at 260 nm after electrophoresis in ethidium bromide stained 1% (w/v) agarose minigels (Maniatis). Total RNA was used to prepare poly A^+ mRNA using the polyATtract® mRNA isolation system (Promega, Madison, WI, USA).

2.2.3. RT-PCR

The cDNA sequence of the neural PTH transcripts was determined by RT-PCR (Kawasaki et al. 1990). Total (0.2 - 1.0 ug) or poly A^+ RNA (1ug) from extracted tissue was reverse transcribed with Superscript (100U; BRL, Burlington, ON, Canada) in the presence of a 50pmol 3'-oligomer rPTH antisense primer (MTN; 5'-GCACGGTCTAGAATACGT CAGCATTTA-3'), based on the known sequence of rat PTH cDNA (12,13), or a 100pmol oligodeoxythymine primer (Boehringer Mannheim, Montreal, Canada), excess deoxynucleotides (10mM each of dATP, dCTP, dGTP, dTTP; Boehringer Mannheim) and 5X RT buffer (BRL). The reactions were diluted with double distilled water (50:1 v/v) and an

aliquot of each (0.5% of total volume) was added to a PCR mixture containing one of three overlapping oligonucleotide primers (MTN1: 5'>AAGAGAGTCAGTGAAATACAGCTT<3'; MTN3: 5'>AGTCCAGTTCATCAGCTGTCTGGCTTA<3'; MTN2: 5'>ATGATGTC TGCAAGCACCATGGCTAAG<3, Nucleotide Synthesis Laboratory, University of Alberta, Edmonton, Alberta, Canada). These primer sets were based on the nucleotide sequence of the rat PTH gene and were designed to generate fragments of 459 base pairs (bp) (MTN3; spanning nucleotides 36 - 2082 and cDNA residues 36 - 494, from the first, second and third exons), 372 bp (MTN2; spanning nucleotides 1600 - 2082 of the PTH gene and cDNA residues 123 - 494, from the second and third exons) and 285 bp (MTN1; spanning nucleotides 1797 - 2082 of the gene and cDNA residues 210 - 494, from the third exon) (Fig. 1a). A common 3'-oligomer rat PTH antisense primer (MTN) was used in each primer set. The PCR mixtures contained 15 pmol of each oligonucleotide primer set, excess deoxynucleotides (200μmol of each) 1X PCR buffer (80mM KCl; 16mM Tris-Cl, pH 8.4; 1.5mM $MgCl_2$; and 0.1% Triton X-100), and *Thermus Aquaticus* (*Taq*) DNA polymerase (5U, Promega). The mixtures were overlaid with mineral oil (2 drops), and denatured at 94°C for 2 min prior to 35 cycles of denaturing (92°C for 1 min), annealing (50°C for 1.5 min) and extension (72°C for 2 min), ending with a final extension (72°C for 10 min) in a genetic thermal cycler (MJ Research, Watertown, MA, USA). Reaction products were analysed by electrophoresis in ethidium bromide stained 1.5% (w/v) agarose gels and compared with DNA molecular weight markers ϕX174 RF DNA/*Hinf I* (Promega).

2.2.4. Nested PCR

The large PCR fragment, generated by the MTN3 primer set, was excised from the agarose gel and purified using a cDNA gel purification kit, Gene Clean II (Bio/Can Scientific), according to manufacturers instructions. cDNA aliquots were then overlaid with equal volumes of mineral oil and re-amplified by heating to 94°C for 2 min before 30 cycles of annealing (50°C for 1 min), extension (72°C for 30 sec) and denaturation (94°C for 30 sec), followed by a final extension (72°C for 10 min) with the internal (MTN2) oligonucleotide primer set.

2.2.5. Restriction endonuclease digestion

Sequence homology with the PCR generated cDNA moieties (MTN3/MTN) was determined by restriction endonuclease digestion (1 h at 37° C) with *BstNI* (5U/μg DNA; Boehringer Mannheim), which would result in PTH cDNA residues of 217 bp and 242 bp (13). Digestion products were identified after electrophoresis in ethidium bromide stained 1.5% (w/v) agarose gels.

2.2.6. 3'RACE

3'RACE (14) was utilised to examine the untranslated 3' region of the neural PTH gene. Total RNA (5ug) was denatured for 10 min at 92°C and then reverse transcribed with 200 units of MMLV reverse transcriptase, in the presence of 4 ul of 5X reverse transcription buffer, 1.3 ul of dNTPs (from 15 mM stocks of each dNTP), 0.25 ul (10 units) of RNasin, and 0.5 ul of Q_T primer (5'>GACTCGAGTCGACATCGTTTTTTTTTTTTTTT<3'; 100 ng/ul).

After 2 h at 37°C the reaction was terminated by dilution to 1 ml with TE (10 mM Tris-HCl; pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA) and the cDNA was stored at 4°C until use.

The cDNA was subsequently amplified by semi-nested PCR, since a single round of PCR resulted in the production of numerous non-specific fragments (data not shown). First, an aliquot of the cDNA (1/200th of the total reaction) was amplified in the presence of oligonucleotide primers MTN2 and Qo (5'>GACTCGAGTCGACATCG<3'; 25 pmol of each), dNTP's (1.5 mM of each), 1 x Taq Polymerase buffer (Promega) and 10% DMSO. Taq polymerase (2.5U, Promega) was added after an initial 5 min denaturation, as described by Frohman (14). The mixture was subsequently overlaid with 30 ul mineral oil (Life Brand, Shoppers Drug Mart, Edmonton, Canada) and subjected to initial annealing (52°C, 2 min) and extension (72°C, 40 min) steps prior to 35 amplification cycles (94°C, 1 min; 52-60°C, 1 min; 72°C, 3 min), and a final extension (72°C, 15 min). An aliquot (1 ul) of the amplification reaction was diluted in TE (1:20) and amplified under identical conditions, in the presence of Q_o and an internal primer, MTN1. 3'RACE products and ϕX174 RF DNA/*Hinf I* (Promega) markers were then subjected to electrophoresis through 1.5% agarose gels, stained with ethidium bromide and viewed under ultraviolet light.

2.2.7. Southern blot analysis

Sequence homology between hypothalamic and parathyroidal PTH cDNA's was further assessed by Southern blotting, using a complementary [^{32}P] radiolabelled PTH cDNA

probe (corresponding to the cDNA region flanked by primer set MTN1). After electrophoresis in 1.5% (w/v) agarose, ethidium bromide stained gels, cDNA moieties were transferred by capillarity to nylon membranes, where they were rinsed in 6 x SSC (1 x SSC: 0.15 mol NaCl/l; 0.015 mol sodium citrate/l, pH 7.2), and baked at 80 C^{o} for 2 hours. The membranes were prehybridised for 2 h at 42°C in 30% (v/v) formamide containing 6 x SSC, 5 x Denhardts (0.1% (w/v) Ficoll, 0.1% (w/v) BSA, 0.2% (w/v) SDS and 0.1% (w/v) polyvinylpyrrolidone), 10% (w/v) dextran sulfate and salmon sperm DNA (50 ug/ml) (Sigma). The membranes were then hybridised under the same conditions for 18 h in the presence of a [^{32}P] αdCTP labelled (200 uCi/mmol; New England Nuclear, Mississauga, Ontario, Canada) random primer (BRL) generated (Maniatis et al. 1982) cDNA probe, homologous to the region of the PTH gene spanned by primer set MTN1 and MTN. The probe was generated by cloning a PTH RT-cDNA PCR product into a TA vector (pCR3^{TM}), as described below. Once this plasmid was sequenced, the cDNA region spanned by the MTN1/MTN fragment was cut from the plasmid with *EcoR1* and electrophoresed in 1.5% (w/v) agarose ethidium bromide stained minigels (Maniatis et al. 1982). The cDNA probe was excised from the gel and purified from agarose using Gene Clean II (BioCan Scientific). The cDNA probe was then resuspended in double distilled water prior to random primer labelling. Following a brief rinse in 2X SSC, the nylon membranes were washed at room temperature (15 min) in 0.1% (w/v) SDS containing 2X SSC and subsequently washed twice (15 min each) at 55°C in 1% (w/v) SDS containing 0.1X SSC. Blots were then exposed to Kodak (X-OMAT AR) X-ray film for 20-40 min.

2.2.8. Nucleotide cloning and sequencing

The identity of the hypothalamic PTH cDNA was determined by nucleotide sequencing. cDNA moieties were electrophoresed in 1.5% (w/v) agarose, ethidium bromide stained minigels (Maniatis et al. 1982). The visualised cDNA bands from PCR (459, 372 and 285 bp) and 3'RACE (499 bps) were excised from the gel and purified from excess nucleotides and agarose using Gene Clean II (BioCan Scientific) and the fragments were then resuspended in double distilled water prior to cloning into a TA vector (pCR3^{TM}), using a Eukaryotic TA Cloning Kit (Invitrogen, San Diego, CA, USA), according to manufactures instructions. Ten colonies were selected for plasmid isolation and restriction analysis (*EcoRI*) for the presence of the insert. Selected colonies were grown overnight in LB broth (50 ug/ml kanamycin) prior to plasmid purification, using a Mini Prep kit (Quiagen, Chatsworth, CA, USA) and resuspended in 60 ul of TE buffer. Automated sequencing (Biochemistry Sequencing Lab, University of Alberta) of plasmids containing PCR and 3'RACE fragments was performed from both 5' and 3' directions.

2.2.9. Northern blot analysis

The relative abundance of PTH mRNA in rat neural tissues was determined by Northern blotting. Total RNA from rat hypothalamus (20ug), extra-hypothalamic brain (20ug), liver (20ug) and parathyroid glands (0.25 ng to 0.0625 ng) was quantified using a fluorimeter and subjected to electrophoresis in a 1% (w/v) agarose and 3.1% (w/v) formaldehyde gel, stained with ethidium bromide, and transferred to a nylon membrane by capillarity. A 258 bp rat PTH cRNA probe was constructed from the same plasmid used to produce the cDNA probe

for Southern blotting. The plasmid pCR3^{TM} (Invitrogen) containing the probe was linerized by *Hind III* digestion and transcribed by Sp6 polymerase, using a riboprobe kit (Promega), in the presence of [^{32}P]αCTP (800 uCi/mmol; New England Nuclear, Mississauga, Ontario, Canada). The cRNA probe was then hybridised with the immobilised RNA in 60% (w/v) formamide containing 6x SSC. 0.2% (wt/vol) SDS,and 1X Denhart's Reagent (0.1% (w/v) ficoll, 0.1% (w/v) BSA, 0.1% (w/v) polyvinylpyrrolidine, 100ug denatured salmon sperm DNA/l, pH 6.8) for 12 h at 53°C, following a 3 h incubation in the absence of the probe. After a brief rinse in 2X SSC, the nylon membranes were washed at room temperature (15 min) in 0.1% (w/v) SDS containing 2X SSC and subsequently washed twice (15 min each) at 65°C in 1% (w/v) SDS containing 0.1X SSC. Membranes were then placed between intensifying screens and exposed to Kodak X-OMAT-AR film (Kodak, Rochester, New York, USA) for 1-7 days. The sizes of the hybridizing RNA moieties were determined by comparison to the 18S and the 28S ribosomal bands, visualised on ethidium bromide stained agrose gels using ultraviolet light. Laser densitometry was used to determine the relative abundance of the hybridising bands. For comparative purposes similar blots were also probed with a sense cRNA probe generated by transcription of the pCR3^{TM} plasmid (Invitrogen) with T7 polymerase.

2.2.10. Western blot analysis

Since cDNA sequences coding for PTH_{1-84} were found in the brain, the presence and relative abundance of this peptide in the hypothalamus were determined by Western blotting. Frozen tissues (hypothalamus and parathyroid) were homogenised (1 g/10 ml) in 1% (w/v)

SDS, 1 mmol phenylmethyl-sulphonylfluoride (PMSF)/L and 10 ug/ml aprotinin, using a Polytron homogeniser (Brinkman Instruments, IL, USA). Homogenates were centrifuged at 2000 x for 5 min at 4°C and 20 ug of hypothalamic and 0.1ug of parathyroidal protein (determined by the Bradford method (Bradford 1976)) was diluted 1:1 with loading buffer (0.06 mol Tris HCl/L, pH 6.8 (v/v) glycerol; 2% (w/v) SDS; 5% (v/v) 2-β-mercaptoethanol; 0.001% bromophenol blue) and heated to 55°C for 15 min prior to loading. Proteins were then separated by electrophoresis through a 15% SDS-polyacrylamide gel and transferred electrophoretically (30V, 4h at 4°C) to an Immobilon PVDF membrane (Millipore, Bedford, MA, USA). Non-specific binding sites on the membrane were blocked (1 h at room temperature) by incubation in 5% non fat dried milk, dissolved in Tris buffered saline (TBS: 25 mmol Tris. HCl/l , pH 7.5; 0.5 mol NaCl/l). PTH-immunoreactivity was detected using a polyclonal bovine antibody (Cooper et al. 1978). The antibody, specific for the carboxyl region of $rPTH_{1-84}$, was diluted 1:400 in TBS/5% non fat dried milk and incubated with the membrane for 6h at room temperature. Membranes were then incubated with a horseradish peroxidase (HRP) conjugated anti-guinea pig IgG (Amersham. Mississauga , Ontario) diluted 1:1000 in TBS/5% non fat dried milk. Membranes were exposed to Kodak X-OMAT-AR film (Kodak, Rochester, NY, USA) and visualisation was achieved using an enhanced chemiluminescence detection system (ECL kit, Amersham). Antibody specificity was determined using $bPTH_{1-84}$ (Peninsula Laboratories, California, USA) as a standard and by the preabsorbtion of the antibody with excess (> 10 ug/ml) $bPTH_{1-84}$ and by the substitution of the antibody with

immune serum. Laser densitometry was used to determine the relative abundance of the immunoreactive proteins.

2.3. RESULTS

2.3.1. PCR amplification

PCR amplification of reverse transcribed hypothalamic RNA in the presence of the oligonucleotide primer MTN and the oligonucleotides MTN1, MTN2 or MTN3 readily generated cDNA moieties of expected size (285 bp, 372 bp and 459 bp, respectively), identical to those generated from reverse transcribed parathyroidal total RNA (Figure 2). Similar moieties were also generated from reverse transcribed pituitary total RNA and from total RNA and polyA$^+$ mRNA from extra-hypothalamic brain and hypothalamic tissues (Figure 3a). These moieties were not, however, generated when reverse transcribed liver RNA was ed by 35 cycles of PCR (data not shown) or when hypothalamic RNA or polyA$^+$ mRNA was not transcribed by reverse transcriptase (Figure 2 and 3a, respectively).

2.3.2. Nested PCR

Re-amplification of the 459 bp hypothalamic cDNA (MTN3/MTN) fragment with the nested oligonucleotide primers MTN2/MTN produced a smaller moiety of predicted size (372 bp), identical to that generated from the corresponding parathyroidal template cDNA (Figure 4a).

2.3.3. Endonuclease digestion

Restriction endonuclease digestion of the 459 bp hypothalamic cDNA (MTN3/MTN) fragment generated smaller moieties of 217 bp and 242 bp (Figure 4b). Fragments of identical size were also produced after *BstN1* digestion of amplified parathyroidal cDNA (Figure 4b).

2.3.4. 3'RACE

Amplification of reverse transcribed brain tissue RNA with the oligonucleotide primers MTN2 and Q_o generated a 586 bp moiety (data not shown), which was identical in size to that generated from reverse transcribed parathyroidal RNA. Multiple weakly staining larger and smaller cDNA moieties were also generated. In the absence of reverse transcriptase, a cDNA fragment could not be generated from extra-hypothalamic RNA (data not shown).

Under similar conditions, re-amplification of the RACE mixture from primer set MTN2/Q_o with the oligonucleotide primer MTN1 and Q_o generated a cDNA moiety of 499 bp, identical to that generated from the RACE amplification of the corresponding parathyroidal template cDNA (Figure 5a).

2.3.5. Southern blot analysis

Hybridisation to the ^{32}P radiolabelled 285 bp hypothalamic cDNA (MTN1/MTN) probe was observed with the 372 bp cDNA PCR fragments derived from reverse transcribed pituitary, hypothalamic and extra-hypothalamic brain total RNA and from hypothalamic and extra-hypothalamic brain polyA$^+$ mRNA. Hybridization to the 372 bp parathyroidal cDNA was similarily observed (Figure 3b). Hybridisation of the probe to the 499 bp cDNA fragments

generated by 3'RACE amplification of the same samples was also demonstrated (Figure 5b). Hybridisation was not observed from reverse transcribed cDNA amplified in the absence of reverse transcriptase (data not shown).

2.3.6. Nucleotide Cloning and Sequencing

The PCR and 3'RACE fragments sequenced encompased 95% of the parathyroidal PTH cDNA (13) between residues 36 and 704, corresponding to nucleotides 36 to 2293 of the PTH gene. The nucleotide sequences of these products were all identical (100% homology) to those of parathyroidal PTH cDNA. This homology extended over 87 bp of the 5' noncoding region, through all of the coding region (345 bp) and through 239 bp of the 3' non-coding region (Figure 6).

2.3.7. Northern Blot Analysis

Hybridization of the [^{32}P] radiolabelled cRNA probe occured to a hypothalamic RNA moiety of approximately 800 bp, identical in size to a hybridising moiety in parathyroidal RNA (Figure 7). The relative abundance of this hypothalamic moiety was, however, < 0.01% of that in the parathyroid gland. Hybridization to a moiety of similar size was also detected in RNA derived from extra-hypothalamic brain tissue (data not shown). This probe did not however, hybridise to liver RNA (data not shown). Moreover, in contrast with the antisense probe, the PTH sense cRNA probe did not hybridise to hypothalamic or parathyroidal RNA (data not shown).

2.3.8. Western blot analysis

A single hypothalamic protein of approximately 10 kDa cross-reacted with antibodies directed against $bPTH_{1-84}$ (Figure 8), identical in size to that detected in the parathyroid gland, and comparable in size to bovine PTH_{1-84}. These proteins were not visualised when the primary antibody was replaced by preimmune serum (data not shown) or when it was preabsorbed with excess bovine PTH_{1-84}. The relative abundance of the immunoreactive protein in the hypothalamus was approximately 0.25% of that in the parathyroid gland.

2.4. Discussion

These results clearly demonstrate the presence of PTH cDNA, coding for PTH_{1-84}, within the brain. The brain is thus likely to be an extra-parathyroidal site of PTH gene expression, especially as neural PTH cDNA is at least 95% homologous to parathyroidal PTH cDNA and as neural PTH is of comparable size and antigenicity to parathyroidal PTH_{1-84}. The PTH gene in the brain is therefore likely to be the same as that expressed in the parathyroid gland, rather than a closely related gene. The neural PTH gene has, however, yet to be fully cloned and the possibility that it may differ from the parathyroidal PTH sequence in the 5' untranslated region remains.

The results of this study confirm and extend the preliminary findings reported by Fraser et al. (Fraser et al. 1990) and demonstrate the presence of a single PTH mRNA in hypothalamic and extra-hypothalamic brain tissue identical in size to parathyroidal mRNA. In contrast, Rubin *et al.* (Rubin et al. 1992) reported that human placental cells express a PTH-like mRNA that codes for a protein with homology to the carboxy-terminal residues of PTH,

but this transcript was found to be 200-250 nucleotides smaller than human parathyroid adenoma PTH mRNA.

In recent studies, ectopic transcription of the PTH gene has also been demonstrated in hepatocytes, lymphocytes and lymphoblastoid cells (Handt et al. 1992). However, as the detection of PTH gene transcripts in these tissues was dependent upon 'booster' PCR, Handt *et al.* (Handt et al. 1992) considered this reflected the phenomenon of 'illegitimate' transcription, especially as the abundance of these transcripts was not regulated by vitamin D_3 or phorbol esters. The occurrence of PTH gene transcripts in the brain is, however, unlikely to be due to illegitimate transcription (Chally *et al.* 1989). Although these transcripts were only detected by Fraser *et al.* (1990) after booster PCR, cDNA fragments were not similarly generated when reverse transcribed liver RNA was amplified by two cycles of PCR under identical conditions (Fraser *et. al.* 1990). Indeed we were similarly unable to generate any PCR fragments from reverse-transcribed liver RNA in the present study, even after 45 cycles of PCR (data not shown). Moreover, when the oligonucleotide primers used by Fraser *et al.* (Fraser *et. al.* 1990) were subsequently used in the present study without the *Eco*RI/*Hind*III linker sequences, cDNA moieties of predicted size and sequence were readily generated in after a single round (35 cycles) of PCR (Figures 2 and 3). Expression of the PTH gene in the brain, unlike in hepatocytes and immune cells (Handt *et al.* 1992), would thus appear to be physiological rather than a methodological artefact, even though PTH mRNA is a rare neural transcript.

The abundance of PTH mRNA in the hypothalamus was far less than that in the parathyroid gland, although the level of PTH gene expression in discrete nuclei was not determined. Indeed, Fraser *et al.* (Fraser *et. al.* 1990) were only able to localize, by *in situ*

hybridization, PTH mRNA in the supraoptic and paraventricular nuc[illegible]t hypothalamus. The transcription of the PTH gene in the brain may, the[illegible]e confined to a relatively small number of neural cells. These cells may not, however, be confined to the hypothalamus, since PTH mRNA was also detected in the present study in extrahypothalamic brain tissue. This finding contrasts with the *in situ* hybridization data of Fraser *et al.* (1990), but is consistent with the widespread distribution of PTH immunoreactivity in the mammalian brain (Balabanova *et al.*1985, Pang *et al.* 1988).

The presence of PTH immunoreactivity in the brain suggests the translation of the neural PTH message. This immunoreactivity is primarily associated with a protein identical in size to PTH_{1-84}, as demonstrated by Western blotting (Figure 8) and by HPLC fractionation (Pang *et al.* 1988). Thus, although PTH-degrading enzymes are present in neural tissues (Balabanova et al. 1992) the relative rate of PTH degradation into amino- and carboxy-terminal fragments may be far less than that in peripheral tissues. Moreover, as the relative abundance of brain PTH (approximately 0.25% of that in the parathyroid gland) was found to be greater than the relative abundance of brain PTH mRNA (approximately 0.01%), the relative rate of PTH degradation in the brain may be less than that in the parathyroid glands. The rate of PTH mRNA turnover may, alternatively, differ in hypothalamic tissue.

The low abundance of PTH immunoreactivity in the brain suggests it is unlikely to contribute to the pool of PTH in the systemic circulation, especially as some authors (Akmal et al. 1984, Balabanova et al. 1984, Rastad et al. 1991) consider the blood-brain barrier to be impermeable to PTH. The permeability of this barrier is, however, uncertain. Indeed the PTH concentration in cerebrospinal fluid (CSF) (Balabanova et al. 1984, Rastad et al. 1991) is

higher than that likely to result from local production (20-70% of the plasma concentration) and in some studies CSF PTH levels correlate with plasma PTH concentrations (Joborn et al. 1991, Gennari 1988, Care et al. 1986). The possibility that PTH of neural origin may, conversely, enter systemic circulation therefore exists, especially as the implantation of parathyroid glands in the brains of parathyroidectomized rats is able to maintain periperal plasma PTH levels within the normal range (Yao et al. 1993). It is therefore of interest that immunoreactive PTH (albeit at very low concentrations) has frequently been measured in the peripheral plasma of hypoparathyroid and parathyroidectomized patients (Manning et al. 1981, Mallette et al. 1982, Roos et al. 1981) and is measurable in the peripheral plasma of fish and amphibians that lack parathyroid glands but have PTH immunoreactivity in their central nervous system (Harvey et al. 1987). Under physiological conditions it is, however, unlikely that neural PTH would be present in the peripheral plasma of mammals at concentrations sufficient to induce biological effects in the traditional target sites of parathyroidal PTH (eg. in renal and osseous tissue). It is, therefore, possible that PTH produced within the brain has autocrine or paracrine actions to modulate neural function.

It is now well-established that PTH has neurological actions (Harvey and Fraser 1993, for review), modulating Ca^{++} flux (Harvey et al. 1988, Wang et al. 1994), phosphoinositol metabolism (Islam et al. 1989) and the synthesis, release and metabolism of neurotransmitters (Smogorzewski et al. 1989, Harvey et al. 1993). Indeed, actions of PTH within the brain may induce neurological dysfunction (Harvey et al. 1993) affect central behaviour (Clementi et al. 1984,). A central action of PTH within the rat brain has also recently been considered to participate in the regulation of peripheral Ca^{++} homeostasis (Matsui et al. 1995).

Intracerebroventricular injections of PTH have been shown to inhibit neuronal activity in the ventromedial nucleus (VMN) of the hypothalamus and to inhibit urethane-induced hypocalcemia (Matsui et al. 1995).

The neural actions of PTH are likely to be receptor-mediated, especially as membrane-binding sites for PTH and/or PTH-related protein (PTHrP) have been found in the cerebrum and cerebellum and particularly in the hypothalamus (Harvey et al. 1993). The demonstration of PTH/PTHrP receptor mRNA in the rat cerebral cortex and cerebellum (Urena et al. 1993) also suggests the brain is a target site for PTH action.

Within the brain, PTH would appear to affect both neuronal and glial cells. PTH, *in vitro*, has direct effects on isolated neurons (Wang et al 1994, Kostyuk et al. 1992) and in brain cell cultures that do not contain neurons (Loffler et al. 1982, Hashimoto et al. 1994), in which PTH/PTHrP receptor mRNA is expressed in astrocytes (Hashimoto et al. 1994). These neural receptors may, however, normally mediate actions of PTHrP rather than PTH since its expression in neural tissue is greater than the expression of PTH (Weir et al. 1990,). The recent discovery of PTH-specific (PTH2) receptors within the brain (Usdin et al. 1995) nevertheless suggests that PTH has physiological roles in neural regulation. Indeed, PTH2 receptors are more abundant in the brain than in kidney or osseous tissues (Usdin et al. 1995), suggesting the brain is a major target site for PTH action. Furthermore, these PTH-specific receptors may be responsible for the demonstrated effects of PTH on the firing of VMN neurons (Matsui et al. 1995), since both PTHrP and PTH/PTHrP receptor mRNA are not present within this nucleus (Weaver et al. 1994). Similarly, while PTH/PTHrP receptor mRNA levels are relatively low in the paraventricular and supraoptic nuclei (Weaver et al.

1994), these are areas of the rat brain in which PTH mRNA is most clearly demonstrated (Fraser et al. 1990). These results therefore suggest that PTH may have autocrine or paracrine effects within the brain, particularly within the hypothalamus.

In summary, these results demonstrate the presence of PTH cDNA, coding for PTH_{1-84}, within neural tissues and suggest local roles for PTH within the central nervous system.

2.5. Figures

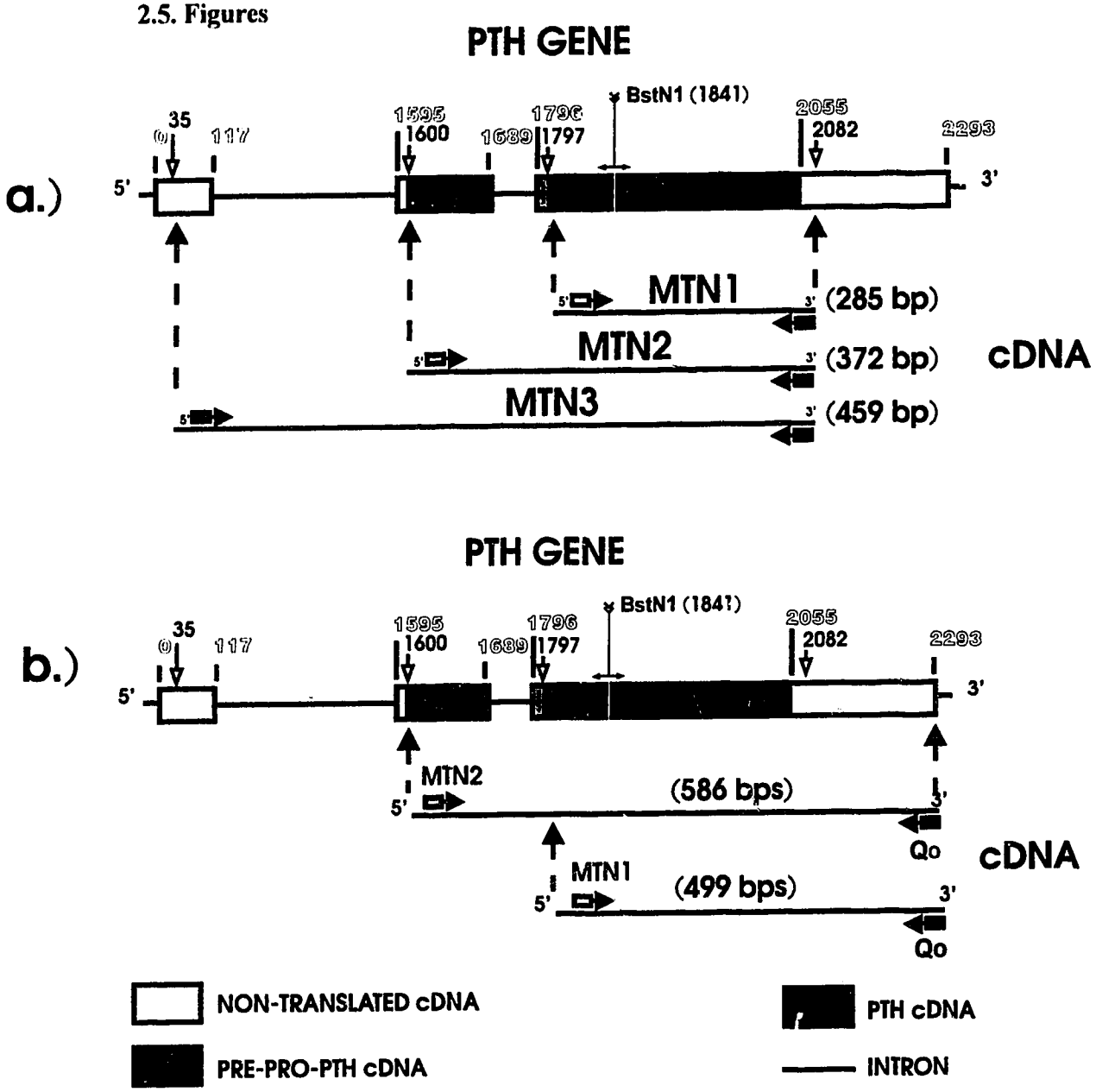

Figure 2.1. PCR and 3'RACE primer map demonstrating the sites of primer annealing and endonuclease digestion on the PTH gene. Primer sets were based on the nucleotidesequence of rPTH gene (12). Three overlapping PCR primers (panel a) generate fragments of 459 base pairs (bp) (MTN3; spanning 35 - 2082 bp, 1st, 2nd and 3rd exons) 372 bps(MTN2; spanning 1600 - 2082 bp, 2nd and 3rd exons) and 285 bp (MTN1; spanning 1803 - 2082 bp, 3rd exon only). All primer sets use a common 3'-oligomer rPTH antisense primer (MTN). 3'RACE primers (panel b) generate fragments of 586 bps(MTN2 + Q_T; spanning 1600 - 2082 bp, 2nd and 3rd exons) and 499 bp (MTN1 + Q_b; spanning 1803 - 2082 bp, 3rd exon only).

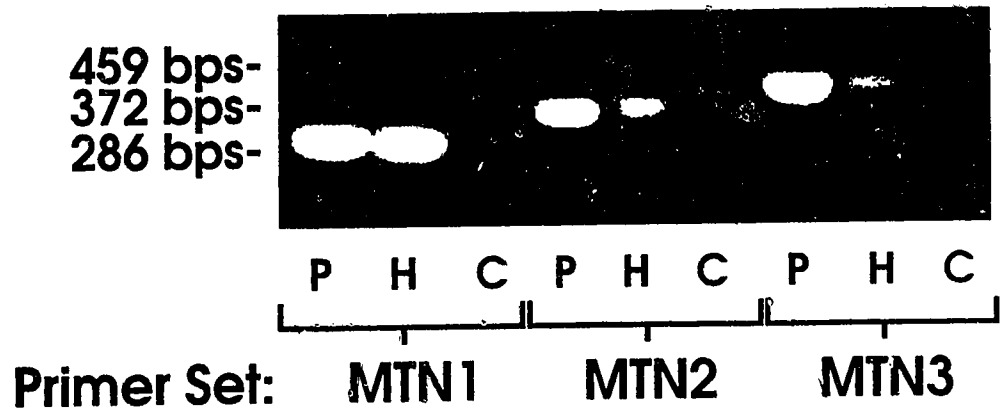

Figure 2.2. Analysis of neural PTH transcripts by the polymerase chain reaction (PCR) in the presence of PTH primer sets MTN1/MTN, MTN2/MTN, and MTN3/MTN. RNA from rat parathyroid gland, (P) and hypothalamus, (H) was reverse transcribed and amplified in the presence of each oligonucleotide primer set. The amplified cDNA was visualised by electrophoresis in ethidium bromide-stained 1.5 % minigels, and the size of the fragments was determined by ϕX174RF DNA/*HaeIII* size markers. Lane C shows hypothalamic RNA in the absence of reverse transcriptase.

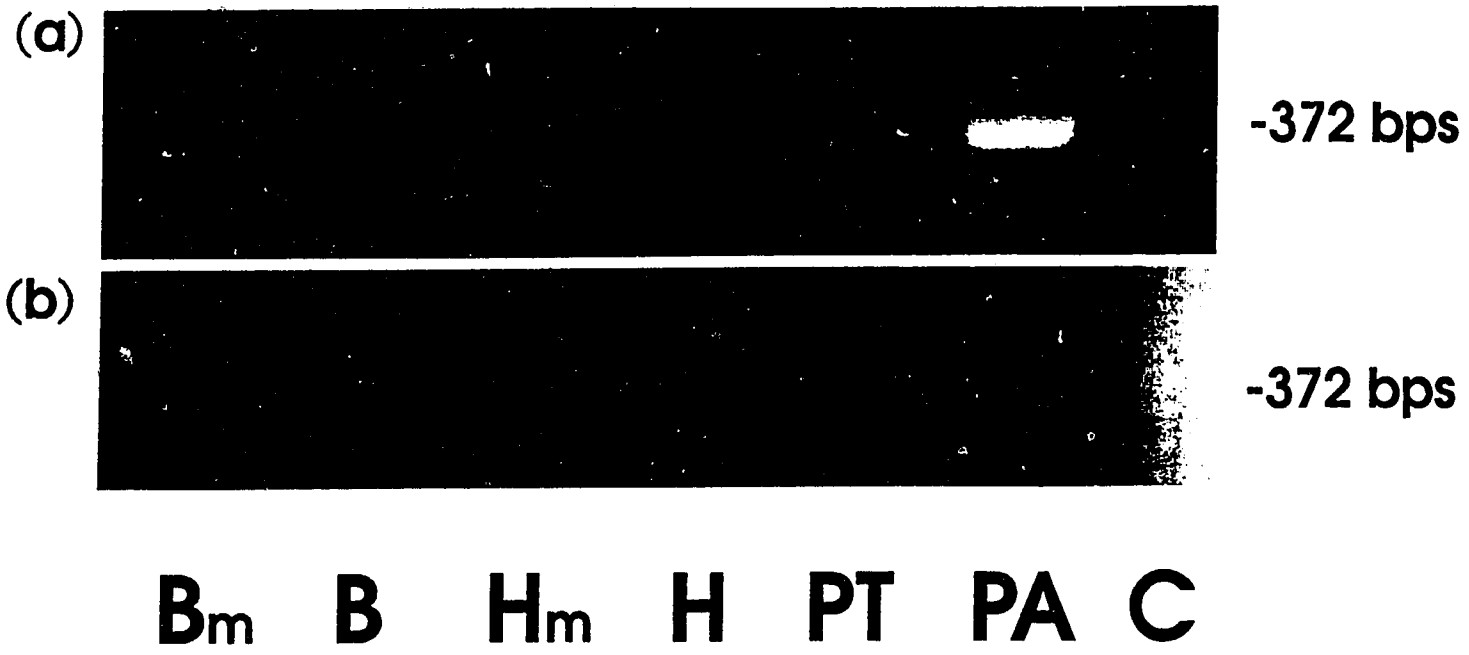

Figure 2.3. (a.) Analysis of neural PTH transcripts by PCR in the presence of PTH primer set MTN2/MTN. Poly A^+ mRNA from extra-hypothalamic, (B_m) and hypothalamic, (H_m) tissue and total RNA from extra-hypothalamic, (B); hypothalamic, (H); pituitary, (PT) and parathyroidal, (P) tissue was transcribed and amplified in the presence of oligonucleotide primer set MTN2/MTN. The amplified cDNA was visualised by electrophoresis in 1.5 % ethidium bromide-stained minigels, and the size of the fragments was determined by ϕX174 RF DNA/*HaeIII* size markers. Lane C is a negative control containing hypothalamic RNA in the absence of reverse transcriptase. (b.) Southern analysis of the RT-PCR transcripts (a.) with a [^{32}P] radiolabelled rPTH cDNA fragment.

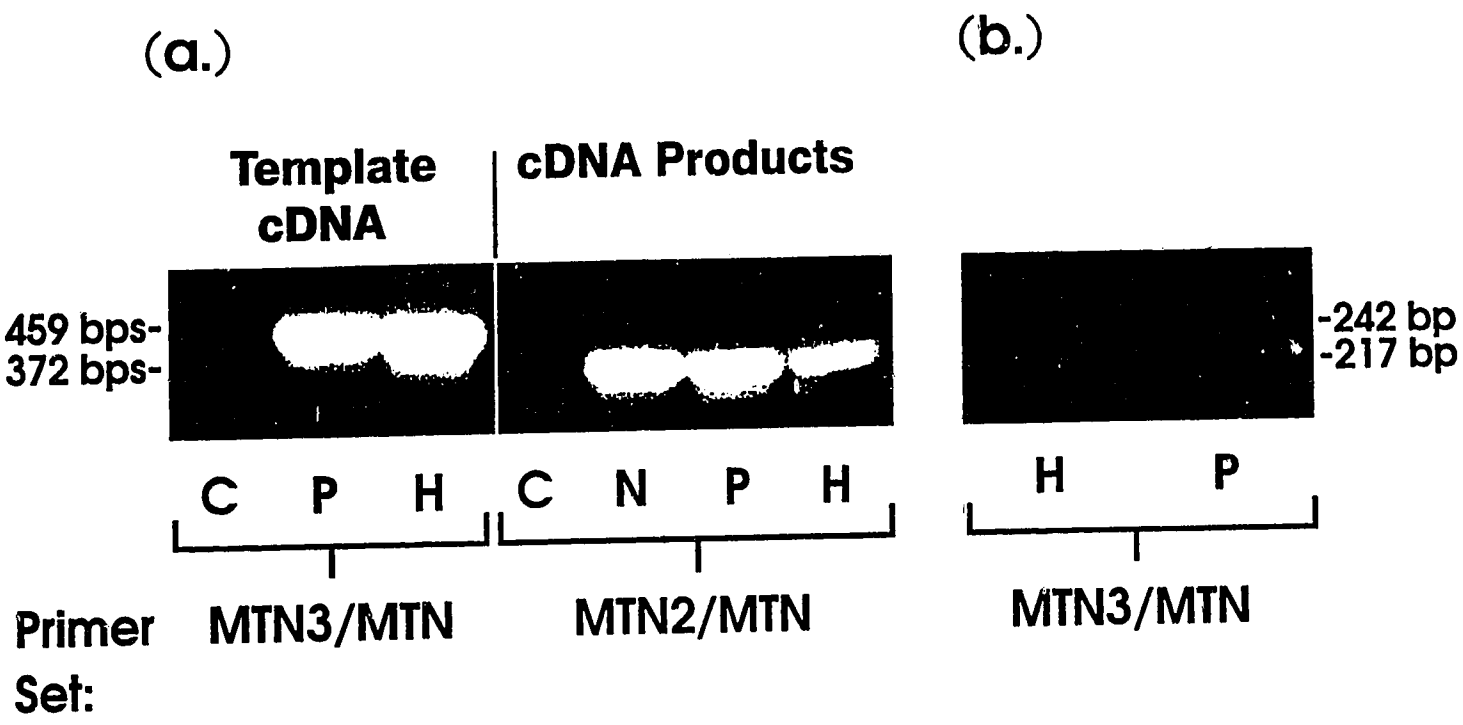

Figure 2.4. (a.) Semi-Nested PCR cDNA fragments generated from parathyroidal, (P) and hypothalamic, (H) RNA by RT-PCR using primer set MTN3/MTN were excised from the agarose gel and re-amplified with the internal primer set MTN2/MTN, generating hypothalamic and parathyroidal cDNA fragments (H) and (P) respectively. A positive control, using RT-cDNA from parathyroidal RNA (N) was also amplified using primer set MTN2/MTN and compared in size to the reamplified fragments. Negative controls (C) of hypothalamic cDNA lacked reverse transcriptase. (b.) *BstN1* digestion of hypothalamic, (H) and parathyroidal, (P) MTN2/MTN1 cDNA fragments. The amplified cDNA was visualised by electrophoresis in 1.5 % ethidium bromide-stained minigels, and the size of the fragments was determined by ϕX174RF DNA/*HaeIII* size markers.

Figure 2.5. Analysis of 3' neural PTH transcripts by the Rapid Amplification of cDNA Ends (3'RACE) in the presence of primer set MTN2 + Q_o followed by a second round of 3'RACE amplification with internal primer set MTN2 + Qo. RNA from rat whole brain, (W) hypothalamus, (H) and parathyroid gland, (P) was reverse transcribed with oligonucleotide primer Q_T and amplified with two subsequent RACE oligonucleotide primer sets. The amplified cDNA was visualised by electrophoresis in 1.5 % ethidium bromide-stained minigels, and the size of the fragments was determined by ϕX174RF DNA/*HaeIII* size markers. Hypothalamic RNA in the absence of reverse transcriptase acted as negative control (C). (b) Southern analysis of the 3'RACE transcripts (a.) with a [^{32}P] radiolabelled rPTH cDNA fragment.

(36)
AGTCCAGTTC ATCAGCTGTC TGGCTTACTC CAGCTTAATA CAGGGTCACT

CCTGAAGGAT CCTCTCTGAG AGTCATTGTA TGTGAAGATG ATGTCTGCAA

GCACCATGGC TAAGGTGATG ATCCTCATGC TGGCAGTTTG TCTCCTTACC

CAGGCAGATG GGAAACCCGT TAAGAAGAGA GCTGTCAGTG AAATACAGCT

TATGCACAAC CTGGGCAAAC ACCTGGCCTC TGTGGAGAGG ATGCAATGGC

TGAGAAAAAA GCTGCAAGAT GTACACAATT TTGTTAGTCT TGGAGTCCAA

ATGGCTGCCA GAGAAGGCAG TTACCAGAGG CCCACCAAGA AGGAGGAAAA

TGTCCTTGTT GATGGCAATT CAAAAAGTCT TGGCGAGGGG GACAAAGCTG

ATGTGGATGT ATTAGTTAAG GCTAAATCTC AGTAAATGCT GACGTATTCT

AGACCGTGCT GAGCAATAAC ATATGCTGCT ATCCTTTCAA GCTCCACGAA

GATCACCAGT GCTAATTCTT CTACTGTAAT AAAAGTTTGA AATTTGATTC

CACTTTTGCT CTTTAAGGTC TCTTCCAATG ATTCCATTTC AATATATTCT

TCTTTTTAAA GTATTACACA TTTCCACTTC TCTCCTTAAA TATAAATAAA

(709)
GTTTAATGAT CATGAACCAA A

Figure 2.6. Automated nucleotide sequencing of the cloned PCR and 3' RACE products between cDNA residues 36 to 704 (corresponding to nucleotides 36 to 2293 of the PTHgene) (12)(13) were identical (100% homology) to those of parathyroidal PTH cDNA. The homology of these sequenced 673 nucleotides extended over 87 bp of the 5' noncoding region, throughout all of the coding region (372 bp) and through 214 bp of the 3' non-coding region. Underline (_) represents amino acid coding region.

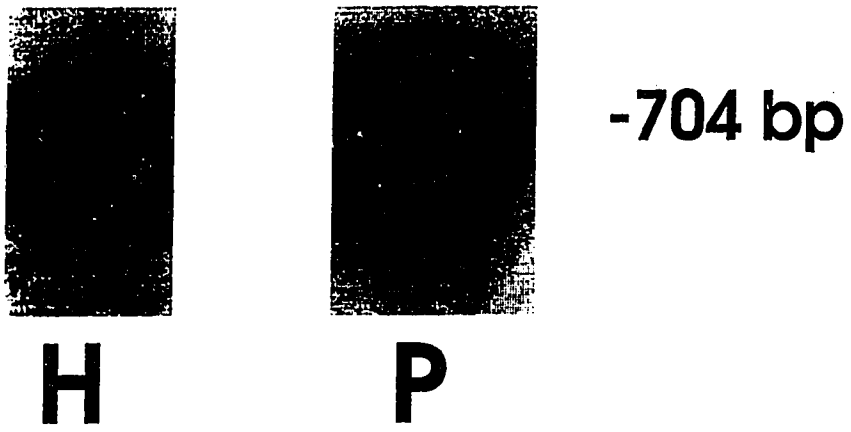

Figure 2.7. Northern analysis of PTH mRNA in neural tissues. Total cellular RNA extracted from hypothalamic, (H) and parathyroidal, (P) tissue was subjected to electrophoresis and immobilised on nylon membranes. PTH mRNA-like species were visualised by hybridisation with a [^{32}P]CTP-labelled rPTH cDNA probe and autoradiography.

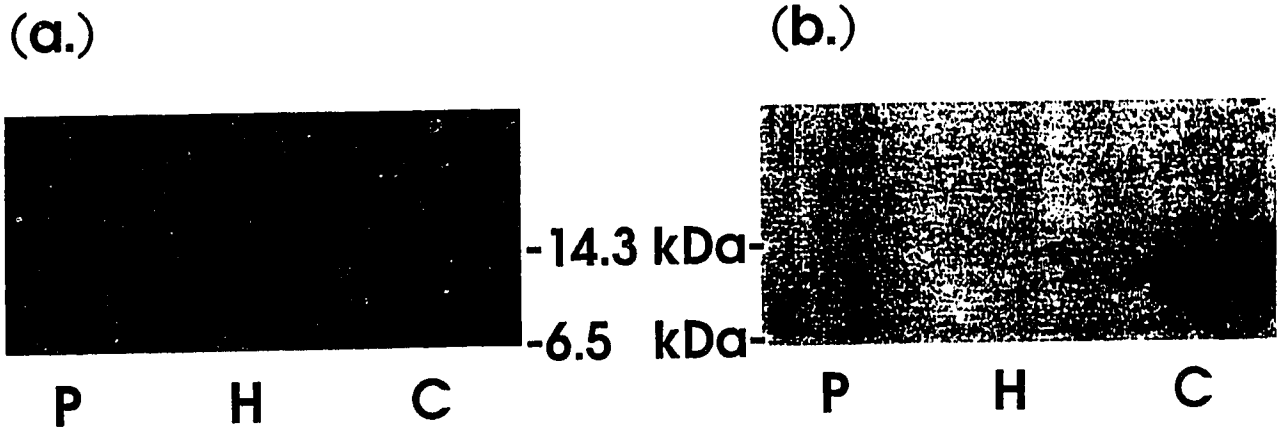

Figure 2.8. Immunoblot detection of PTH in rat neural tissue. Crude tissue homogenates (parathyroid gland, P; hypothalamus, H) and $bPTH_{1-84}$, (C) were subjected to reducing SDS-PAGE electrophoresis, transferred to PVDF membranes and incubated with rat PTH antiserum (αrPTH) (panel a). Identical blots were also incubated with αrPTH preabsorbed with excess bPTH (panel b). Immunoreactive proteins were visualised using HRP-labelled anti- guinea pig IgG and enhanced chemiluminescence.

2.6. References

Akmal M, Tuma DA, Goldstein DA, Pattabhiraman R, Bernstein L, Massry SG 1984 Intact and carboxy-terminal PTH do not cross the blood-cerebrospinal fluid barrier. Proc Soc Exp Biol Med 176: 434-437

Balabanova S, Helmer B 1992 Capacity of brain of sheep to degrade parathyroid hormone in vitro. Neurosci Lett 14: 91-95

Balabanova S, King O, Teller WN, Renhardt G 1985 Distribution and concentration of immunoreactive parathyroid hormone in brain and pituitary of sheep. Klinische Wochenschrift 63: 419-422

Balabanova S, Peter J, Reinhardt G 1986 Parathyroid hormone secretion by brain and pituitary of sheep. Klinische Wochenschrift 64: 173-176

Balabanova S, Toller U, Richter HP, Pohlandt F, Gaedicke G, Teller WM 1984 Immunoreactive parathyroid hormone, calcium and magnesium in human cerebrospinal fluid. Acta Endocrinol 106: 223-227

Bradford MM 1976 Protein assay utilizing coomassie brilliant blue G-250. Anal Biochem 86: 2617-2621

Cantley LK, Scott DL, Cooper CW, Mahaffee DD, Leight GS, Thomas CG, Ontjes DA 1984 Divergent effects on forskolin on 3', 5' cyclic adenosine monophosphate production and parathyroid hormone seretion. Calcif Tiss Int 36: 87-94

Care AD, Bell NH 1986 Evidence that parathyroid hormone crosses the blood-brain barrier. Proc Ixth Int Conf Calcium Reg Horm Bone Metab, Nice, p.181 (Abstract)

Chally J, Concordet JP, Kaplan JC, Kahn A 1989 Illegitimate transcription: transcription of any gene in any cell type. Proc Natl Acad Sci (USA)86: 2617-2621

Clementi GF, Caruso A, Fiore CE, Leone MG, Prato A 1984 Hyperalgesic activity of parathyroid hormone and its fragments in male rats. Brain Res 295: 376-377

Clementi GF, Drago A, Prato S, Cavalier S, Di Benedetto A, Leone F, Scapagnini U, Rodolico G 1984 Effects of calcitonin, parathyroid hormone and its related fragments on acquisition of active avoidance behaviour. Physiol Behav 33:913-916

Fraser CL, Sarnachi P, Budayr A 1988 Evidence that parathyroid hormone-mediated calcium transport in rat brain synaptosomes is independent of cyclic adenosine monophosphate. J Clin Invest 81:982-988

Fraser RA, Kronenberg HM, Pang PKT, Harvey S 1990 Parathyroid hormone messenger ribonucleic acid in the rat hypothalamus. Endocrinology 127: 2517-2522

Frohman M 1993 Rapid amplification of complementary DNA ends for generation of full-lengh complementary DNAs: Thermal RACE. Method Enzymol 218: 340-357

Gennari C 1988 Parathyroid hormone and pain. In: Massry SG, Fujita T (eds) New Actions of Parathyroid Hormone. Plenum Press, New York pp. 335-344

Handt O, Reis A, Schmidtke J 1992 Ectopic transcription of the parathyroid hormone gene in lympocytes, lympoblastoid cells and tumour tissue. J Endocrinol 135: 259-256

Harvey S, Fraser RA 1993 Parathyroid hormone; neural and neuroendocrine perspectives. J Endocrinol 139: 353-361

Harvey S, Hayer S 1993 Parathyroid hormone binding sites in the brain. Peptides 14, 1187-1191

Harvey S, Hayer S, Sloley BD 1993 Dopaminergic actions of parathyroid hormone in the rat medial basal hypothalamus in vitro. Reg Pept 43: 49-56

Harvey S, Hayer S, Sloley BD 1993 Parathyroid hormone-induced dopamine turnover in the rat medial basal hypothalamus. Peptides 14: 269-274

Harvey S, Zeng Y-Y, Pang PKT 1987 Parathyroid hormone-like immunoreactivity in fish plasma and tissues. Gen Comp Endocrinol 68: 136-146

Hashimoto H, Arnott T, Ogawa N, Nagata S, Baba A 1994 Identification and characterization of parathyroid hormone/parathyroid hormone related peptide receptor in cultured astrocytes. Biochem Biophy Res Commun 200: 1042-1046

Heinrich G, Kronenberg HM, Potts JTJr, Habener JF 1984 Gene encoding parathyroid hormone. Nucleotide sequence of the rat gene and deduced amino acid sequence of rat preproparathyroid hormone. J Biol Chem 259: 3320-3329

Islam A, Smogorzewski M, Massry SG 1989 Effect of chronic renal failure and parathyroid hormone on phospholipid content of brain synaptosomes. Am J Physiol 256: F705-F710

Joborn C, Hetta J, Niklasson F, Rastad J, Wide L, Agren H, Akerstrom G, Ljunghall S 1991 Cerebrospinal fluid calcium, parathyroid hormone and monoamine and purine metabolites and the blood-brain barrier function in primary hyperparathyroidism. Psychoneuroendocrinology 16: 311-322

Kawasaki ES 1990 Amplification of RNA. In: Innis MA, Gelfand DH, Sninsky JJ, White RTJ (Eds) PCR Protocols: A Guide to Methods and Applications. Academic Press, New York. pp. 21-27

Kostyuk PG, Lukyanetz EA, Ter-Markosyan AS 1992 Parathyroid hormone enhances calcium current in snail neurones: stimulation of the effect by phorbol esters. Pflugers Archiv 420: 146-152

Loffler F, Van Calker D, Hamprecht B 1982 Parathyrin and calcitonin stimulate cyclic AMP accumulation in cultured murine brain cells. EMBO J 1:297-302

Mallette LE, Tuma SN, Berger RE, Kirkland JL 1982 Radioimmunoassay for the middle region of human parathyroid hormone using an homologous antiserum with a carboxy-terminal fragment of bovine parathyroid hormone as radioligand. J Clin Endocrinol Metab 54: 1017-1024.

Maniatis T, Fritch EF, Sambrook J 1982 Molecular Cloning. A Laboratory Manual. Cold Spring Harbor, New York

Manning RM, Adami S, Papapoulos SE, Gleed JH, Hendy GN, Rosenblatt M, O'Riordan JLH 1981 A carboxy-terminal specific assay for human parathyroid hormone. Clin Endocrinol 15:439-449

Matsui H, Aou S, Ma J, Hori T 1995 Central actions of parathyroid hormone on blood calcium and hypothalamic neuronal activity in the rat. Am J Physiol 268: R21-R27

Pang PKT, Harvey S, Fraser RA, Kaneko T 1988 Parathyroid hormone-like immunoreactivity in vertebrate brains. Am J Physiol 255: R635-R642

Pang PKT, Kaneko T, Harvey S 1988 Immunocytochemical distribution of PTH immunoreactivity in vertebrate brains. Am J Physiol 255: R643-R647

Paxinos G, Watson C 1986 The Rat Brain in Stereotaxic Co-ordinates. Second Edition. Academic Press, London

Rastad J, Joborn C, Akerstrom G, Ljunghall S 1991 Incidence, type and severity of psychic symptoms in patients with sporadic primary hyperparathyroidism. J Endocrinol Invest 19, 149-156

Roos BA, Lindall AW, Aron DC, Orf JW, Yoon M, Huber MB, Pensky JC, Ells J, Lambert PW 1981 Detection and characterization of small midregion parathyroid hormone fragments in normal and hyperparathyroid glands and sera by immunoextraction and region-specific radioimmunoassays. J Clin Endocrinol Metab 53: 709-720

Rubin LP, Robbinson BG, Arbiser JL 1992 Human placenta expresses a parathyroid hormone-like mRNA. Program Abstracts 71st Ann Meet Endocrine Soc, Seattle, WA, pp. 320 (Abstract 1192)

Schmelzer HJ, Gross G, Widera G, Mayer H 1987 Nucleotide sequence of a full-length cDNA clone encoding preparathyroid hormone from pig and rat. Nucleic Acid Res 15: 6740

Selvanayagam P, Graves K, Cooper C, Rajaraman S 1991 Expression of parathyroid hormone-related peptide gene in rat tissues. Lab Invest 64:713-717

Smogorzewski M, Campese VM, Massry SG 1989 Abnormal norepinephrine uptake and release in brain synaptosomes in chronic renal failure. Kidney Int 36:458-465

Urena P, Kong XF, Abou-Samra AB, Juppner H, Kronenberg HM, Potts JT Jr., Segre GV 1993 Parathyroid hormone (PTH)/PTH-related peptide receptor messenger ribonucleic acids are widely distributed in rat tissues. Endocrinology 133: 617-623

Usdin TB, Gruber C, Bonner TI 1995 Identification and functional expression of a receptor selectively recogninzing parathyroid hormone, the PTH2 receptor. J Biol Chem 270: 15455-15458

Wang R, Pang PKT, Wu L, Karpinski E, Harvey S, Berdan RC 1994 Enhanced calcium influx by parathyroid hormone in identified *Helisoma trivolvis* snail neurons. Cell Calcium 15: 89-98

Weaver DR, Deeds JD, Lee K, Segre GV 1994 Localization of parathyroid hormone-related peptide (PTHrP) receptor mRNAs in rat brain. Mol Brain Res 28:296-310

Weir EC, Brines ML, Ikeda K, Burtis WJ, Broadus AE, Robbins RJ 1990 Parathyroid hormone-related peptide gene is expressed in the mammalian central nervous system. Proc Soc Natl Acad Sci 87:108-112

Yao CZ, Ishizuka J, Townsend M Jr., Thompson JC 1993 Successful intracerebroventricular allotransplantation of parathyroid tissue in rats without immunosuppression. Transplantation 55: 251-253

CHAPTER 3. GENERAL DISCUSSION

3.1 SUMMARY

Parathyroid hormone (PTH) is a peptide hormone released from the parathyroid gland in response to varying se[illegible] calcium and phosphate concentrations. The possibility that PTH may have neural actions was first demonstrated by Collipp and Clark (1925), who showed that PTH had a dose-related neuromuscular effect on vascular smooth muscle. Since then, PTH has been shown to have central effects, such as acquisition of learning and behavoiral modification (Clementi *et al.* 1984). Furthermore, the suggestion of a role for PTH in the brain has been supported by the wide distribution of PTH receptors throughout the central nervous system (CNS) of the rat (Urena *et al.* 1993).

The objective of this thesis was to examine the expression and the translation of neural PTH in the CNS of the rat. It was hypothesized that: 1.) the PTH gene is expressed in the rat brain, specifically in the hypothalamus 2.) the neural PTH gene is very similar to that found in the parathyroid gland, and, 3.) the neural PTH gene is transcribed to an immunoreactive protein in the CNS.

In the second chapter, the expression of the neural PTH gene and its possible translation was examined in pituitary, hypothalamic and extra-hypothalamic rat brain. Although previous studies have suggested the expression of a PTH-like gene and the presence of PTH-like immunoreactivity in mammalian brain (Fraser *et al.* 1991), this thesis has clearly demonstrated that the region of the gene encoding the neural PTH peptide is identical to that found in the parathyroid gland. Therefore, in addition to its similarity in size and immunoreactivity to authentic PTH, it is likely that the PTH peptide

sequence resulting from the neural PTH gene is identical to that found in the parathyroid gland.

This work also demonstrated that in addition to the coding region, the entire 3' non-coding region of neural PTH mRNA is also identical to that of authentic PTH mRNA. Most of the nucleotide sequence of the non-coding 5' region of the neural PTH gene (80 bps) is however, still undetermined, although at least 27 bps are identical to parathyroidal PTH cDNA.

3.2 EVOLUTIONARY IMPLICATIONS

Although the parathyroid gland phylogentically appeared in tetrapods in response to the transition from aquatic to terrestrial environment, PTH-like peptides exist in more primitive vertebrates and invertebrates (Fraser et al. 1991). Even though these primitive species lack parathyroid glands, PTH-like peptides are found in both their central and peripheral nervous systems. It has been suggested, therefore, that parathyroidal PTH may have evolved from an ancestral neuropeptide. Sequencing data presented in this thesis provides additional support for this evolutionary theory.

PTH has been demonstrated to have a number of Ca^{++} dependent neurological actions in vertebrate and invertebrate species (Fraser and Arieff, 1988; Fraser *et al.* 1988; 1991; Kostyuk *et al.* 1990; 1992; Wang *et al.* 1993). Therefore, the function of PTH ancestrally may have been to regulate neural Ca^{++} rather than systemic Ca^{++} concentration. Embryological evidence supporting the prospect of PTH having a neurological origin comes from the fact that the frog parathyroid gland develops from the

same layer of neural ectoderm as that giving rise to the hypothalamo-hypophyseal axis. It has also been suggested that the mammalian parathyroid gland is derived from neuroectoderm (Pearse and Takor, 1976).

The occurrence of PTH-like peptides in the CNS has now been well established in mammalian, avian, reptilian and amphibian brains and pituitary glands (Pang *et al.* 1988; Harvey *et al.* 1993) which are similar in immunoreactivity to that found within the brain of fish (Balabanova *et al.* 1985) and snails (Wendellar Bonga *et al.* 1989). It is therefore possible that PTH gene expression in the parathyroid gland may reflect the ectopic transcription of the neural PTH gene.

3.3 LIMITATIONS OF PCR

The reliability of PCR as a technique has been criticised for the occurrence of what has been described as the phenomenon of "illegitimate" (Chelley *et al.* 1988) or "ectopic" transcription (Sarkar and Sommer, 1989). This theory suggests that the sensitivity of PCR is such that it amplifies rare transcripts in tissues where previously it was thought the gene sequences were not expressed. No physiological role has been attributed to the illegitimate transcription, but it has been suggested that it may be due to a permanent basal level of transcription of "any gene in any cell" (Chelley *et al.* 1989). This phenomenon however, requires the use of a technique called "Booster PCR" (Ruano *et al.* 1989). This process requires an initial PCR of 30-35 cycles which is then followed by an additional round of 30-35 cycles of PCR. This was therefore a major criticism brought against preliminary neural PTH expression data by Fraser *et al.* (1990) where re-amplification of PTH cDNA by a

second round of PCR was necessary as a result of suboptimal PCR primer selection. In this study, PCR amplified cDNA was easily visualised after a single round of PCR amplification of 35 cycles suggesting the occurrence of a physiologically significant amount of PTH-like mRNA in these tissues.

During this project, cloning and sequencing of a number of additional PCR and RACE moieties illustrated the limitations of these cDNA amplification techniques. Sequencing of a number of cDNAs from PCR and 3'RACE moieties revealed that the primers used were amplifying, in addition to neural PTH, other gene sequences found in high abundance (e.g. pyruvate dehydrogenase kinase and glyceraldehyde-3-phosphate) in neural tissues . Amplification of these genes occurred despite low primer homology (< 60%), and highly stringent primer annealing conditions. The problem of generating non-specific cDNA products was most apparent with the 3'RACE protocol, as only one of the two primers is specific for the PTH gene sequence. This problem was, however, circumvented by reamplification of the initial RACE cDNA products with nested primers. A cDNA band of smaller size (~400 bp) was still observed on ethidium bromide gels. This band may be of some interest, as it also hybridised to a radiolabelled PTH probe suggesting some level of homology to authentic PTH. Unfortunately, due to limiting time constraints, this fragment was never isolated for sequencing.

Elucidation of the neural PTH nucleotide sequence would seem to be best achieved by screening a DNA library. This approach was not considered, due to the low copy number of neural PTH mRNA. The low copy number would make detection of positive neural PTH clones difficult and time consuming.

3.4 NEURAL PTH mRNA: RELATIVE ABUNDANCE

The discrepancy between the relative amounts of immunoreactive PTH and PTH mRNA detected in the parathyroid gland were not reflected by a corresponding relationship in the rat hypothalamus. This suggests, therefore, that the turnover of neural PTH differs from that in the parathyroid gland. One explanation for this discrepancey may be that neural PTH differs from peripheral PTH in its stability within neural tissues. Since neural immunoreactive PTH is readily detectable in the brain, neural PTH mRNA may undergo more rapid turnover in the brain. This could occur if the neural PTH mRNA had a shorter 3' poly A tail than PTH mRNA from the parathyroid gland, thus making neural PTH mRNA less resistant to RNase enzymatic degradation. Another explanation for this discrepancy is the suggestion that the PTH peptide may penetrate the blood-brain barrier from systemic circulation (Akmal et al. 1983; Care and Bell 1986). This would account for the greater that expected amount of PTH peptide in the brain.

3.5. CONCLUSION

In this thesis, characterization of neural PTH RNA was established by PCR, 3'RACE, endonuclease digestion, Northern blotting, Southern blotting, Western blotting and nucleotide sequencing. Results demonstrate that the PTH gene expressed in neural tissues has an identical peptide coding region to that of the parathyroid gland PTH gene. The neural PTH gene is therefore unlikely to be a variant or a closely related PTH-like gene, although its 5' flanking DNA sequence may differ from the parathyroidal gene in a tissue specific manner. This data may also have evolutionary significance, since PTH-like

peptides are present in the brains of primitive invertebrate gastropods (Wendellar Bonga *et al.* 1989) and vertebrates lacking parathyroid glands (Balabanova *et al.* 1985 and Pang *et al.* 1988), implicating the brain an the ancestral site of PTH production. Although a definitive role for neural PTH has yet to be determined, these results suggest that PTH may be involved in a non-classical autocrine or paracrine neuroendocrine role.

3.6. REFERENCES

Akmal, M., Goldstein, D.A., Tuma, S.A., Fanti, R., Pattabhiraman, R. and Massry, S.G. The effect of parathyroid hormone (PTH) on the blood brain barrier. Clin Res 31:52A, 1983.

Balabanova S, King O, Teller W N and Reinhardt G. Distribution and concentration of immunoreactive parathyroid hormone in brain and pituitary of sheep. Klinische Wochenschrift 63 419-422, 1985.

Care, S.D, and Bell, J. Evidence that parathyroid hormone crosses the blood-brain barrier. Proceedings of the IX International conference of Calcium Regulating Hormones (abstract):181, 1986.

Chelly,J., Concordet, J.P., Kaplan, J.C., and Kahn, A. An Illegitimate transcription: transcription of any gene in any cell type. Proc Natl Acad Sci USA 86:2617-2621, 1988.

Clementi, G., Drago, F., Prato, A.,Caruso, A., Pratti, F., Pataine, S., Drago, F. Effects of calcitonin, parathyroid hormone and its related fragments on acquisition of active avoidance behavior. Physiol Behav 33:913-916, 1984.

Collipp J.B and Clark E.P Further studies of the physiological action of a parathyroid hormone. J Biol Chem 66, 133-138, 1925.

Fraser C L & Arieff A .I. (1988) PTH increases Ca^{++} transport in rat brain synaptosomes in uremia. In: New Actions of Parathyroid Hormone pp 317-328. Eds S G Massry & F Fujita. New York: Plenum Press.

Fraser, R.A., Sarnaki, P. and Budayr, A. Evidence that parathyroid hormone-mediated calcium transport in rat brain synaptosomes is independent of cyclic adenosine monophosphate. J. Clin. Invest. 81:982-988, 1988.

Fraser, R.A., Kronenberg, H.M., Pang, P.K.T. and Harvey, S. Parathyroid hormone messenger ribonucleic acid in the rat hypothalamus. Endocrinology 127:2517-2522, 1990.

Fraser R.A, Kaneko T, Pang P.K.T and Harvey S. Hypo- and hypercalcemic peptides in fish pituitary glands. Am J Physiol 260 R622-R626, 1991.

Harvey,S., and Fraser,R.A Parathyroid hormone: neural and neuroendocrine perspectives. J Endocrinol 139:353-361, 1993

Kostyuk P.G, Lukyanetz E.A, Ter-Markosyan A.S and Khudaverdyan D.N. Stimulation of neuronal calcium conductance by parathyroid hormone. Neirofiziologiya 22:373-380, 1990.

Pang, P.K.T., Yang, M.C.M. and Tenner, T.E.Jr. Beta-adrenergic like actions of parathryoid hormone. Trend Pharm Sci 54:340-341, 1986.

Pang, P.K.T., Harvey, S., Fraser, R.S and Kaneko, T. Parathyroid hormone-like immunoreactivity in vertebrate brains. Am J Physiol 255:R635-R647, 1988.

Pearse, A.G.E., Takor, T.T. Neuroendocrine embryology and the APUD concept. Clin Endocrinol 5 (Suppl) 2295-2445, 1976.

Ruano,G., Fenton,W and Kidd, K.K. Biophasic amplification of very dilute DNA samples via "booster" PCR. Nucl Acid Res 17:5407-5411, 1989.

Sakar,G and Sommer,S.S. Access to a messenger RNA sequence or its protein product is not limited by tissue or species specificity. Science 224:331-334, 1989.

Urena, P., Kong, X.F., Abou-Samra, A.B., Juppner, H., Kronenberg, H.M., Potts, J.T.Jr. Sergre, G.V. Parathyroid hormone (PTH)/PTH-related peptide receptor messenger ribonucleic acids are widely distributed in rat tissues. Endocrinology 133:617-623, 1993.

Wang, R., Pang, P.K., Wu, L., Shipley, A., Karpinski, E., Harvey, S., Berdan, R.C. Neural effects of parathyroid hormone: modulation of the calcium channel current and metabolism of monoamines in identified Helisoma snail neurones. Can J Physiol Pharmacol 71 :582-591, 1993.

Wendellar Bonga, S.E., Lafeber, F.P.J.G., Flik, G., Kaneko, T. and Pang, P.K.T. Immunocytochemical demonstration of a novel system of neuroendocrine peptidergic neurons in the pond snail Lymnaea stagnalis, with antisera to the teleostean hormone hypocalcin and mammalian parathyroid hormone. Gen Comp Endocrinol 75:29-38, 1989.

APPENDIX: PARATHYROID HORMONE AND HYPOTHALAMO-FUNCTION

A.1.1 INTRODUCTION

Although parathyroid hormone (PTH) was first isolated from parathyroid glands and thought to be only synthesized in this tissue (Selvanayagan *et al.* 1991), the PTH gene is also ectopically expressed in human placental cells (Rubin *et al.* 1989) and in lympocytes, lymphoblastiod cells and tumor tissue (Handt *et al.* 1992). PTH-like peptides, mRNA and degradative enzymes are also present in the brain, even in primary vertebrates lacking encapsulated parathyroid glands (Harvey and Fraser 1993). The central nervous system (CNS) may therefore be an ancestral site of PTH production, especially as PTH-like peptides are also found in the sensory ganglia of invertebrates (Wendellar Bonga *et al.* 1989). Thus, in addition to its traditional role in whole-body calcium homeostasis, PTH may have roles in neural or neuroendocrine function (Harvey and Fraser 1993). In this brief review the possibility that PTH participates in hypothalamo-pituitary regulation is discussed.

A.1.2. PTH IN THE HYPOTHALAMO-PITUITARY AXIS

Expression of the PTH gene occurs in hypophysiotropic regions of the rat hypothalamus, particularly in the supraoptic and paraventricular nuclei (Fraser *et al.* 1990). The perikarya in these nuclei also possess PTH immunoreactivity (Pang *et al.*

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1988). Fiber tracts from these cell bodies terminate in the external zone of the rat median eminence, close to hypophysial portal blood vessels (Pang *et al.* 1988), and, in teleosts, in the neuropars intermedia, close to adenohypophyseal cell membranes (Kaneko *et al.* 1987). Axons with PTH immunoreactivity (PTH-IR) do not, however, appear to terminate in the tetrapod pituitary gland, which lacks immunoreactive cells (Pang *et al.* 1988). This may, however, merely reflect the poor sensitivity of immunocytochemistry (Kaneko *et al.* 1987), since the pituitary glands of amphibian, reptilian, avian and mammalian species do contain radioimmunoassayable PTH-like peptides that co-elute with authentic PTH (1-84) following reverse-phase high pressure liquid chromatography (Pang *et al.* 1988; Fraser *et al.* 1991). The possibility that the pituitary is a site of PTH synthesis is also indicated by the presence of PTH-like mRNA in extracts of the rat pituitary gland (Fig. 4.1.). The expression of the PTH gene in the pituitary gland may, however, be tissue-specific. A 372-bp cDNA fragment is generated by the polymerase chain reaction using oligonucleotide primers for rat parathyroid cDNA (Heinrich *et al.* 1984) and reverse-transcribed parathyroidal RNA, whereas a 440 bp fragment is generated from reverse-transcribed pituitary RNA by the same oligonucleotide primers. Translation of pituitary PTH mRNA may thus produce a peptide that differs from parathyroidal PTH and be of larger molecular size.

A.1.3. HYPOPHYSIOTROPIC ACTIONS OF PTH

The possibility that PTH may regulate pituitary function has rarely been considered, although PTH may modulate prolactin release. Hyperparathyroidism has

been cited as a possible cause of hyperprolactinaemia (Fioretti *et al.* 1986), since parathyroidectomy may restore circulating prolactin concentrations in some hyperprolactinaemic patients (Raymond *et al.* 1982). Pseudohypoparathyroidism, a disease characterized by defective PTH receptors, is conversely characterized by isolated prolactin deficiency (Kruse *et al.* 1981; Marx *et al.* 1977; Furlong *et al.* 1986). Plasma prolactin levels are also elevated in normal subjects and in patients with primary hypoparathyroidism following systemic injections of PTH of parathyroidal extracts (Kruse *et al.* 1981; Furlong *et al.* 1986; Isaac *et al.* 1978; Castro *et al.* 1980; Brickman *et al.* 1981). A physiological role for PTH in prolactin release is also suggested by its blockade by dopamine (Isaac *et al.* 1978), an acknowledged inhibitor of prolactin secretion. PTH may also induce prolactin secretion in fish (Srivaster *et al.* 1987), in which hypothalamic PTH-IR nerve fibers terminate near prolactin-secreting cells (Kaneko *et al.* 1987). Circulating prolactin concentrations in conscious and anaesthetized rats are not, however, elevated by intravenous injections of PTH (Wendellar Bonga *et al.* 1989). Prolactin release from monolayers of rat pituitary cells is also unaffected by exposure to bovine (b) PTH (1-84) (at 0.1 or 1.0 μmol/l for 60 min) (Harvey and Fraser 1993), even though these cells release prolactin in response to other secretagogues (vasoactive intestinal peptide and thyrotropin-releasing hormone) (unpubl. data). The basal release of growth hormone, thyrotropin, adrenocorticotropin, luteinizing hormone and follicle-stimulating hormone from these cells (Table 4.1.) and the stimulated release induced by GH-releasing factor, thyrotropin-releasing hormone, corticotropin-releasing factor and gonadotropin-releasing factor, respectively, are also unaffected by 1 μmol/l

bPTH (1-34). However, while plasma levels of growth hormone, thyrotropin, adrenocorticotropin, luteinizing hormone and follicle-stimulating hormone are also unaffected by intracerebroventricular injections of 1 or 10 μg bPTH (1-34), prolactin concentrations are elevated within 30 min (Harvey and Fraser 1993).

A.1.4. MECHANISMS OF PTH-INDUCED PROLACTIN SECRETION

Although PTH stimulates prolactin secretion in man and in rats, this is unlikely due to direct pituitary action (Table 4.1.). This conclusion is also supported by the absence of binding sites for radioiodinated PTH on pituitary plasma membranes (unpubl. observations). Indeed, in recent studies Urena *et al.* (1993) demonstrated that the PTH/PTH-related peptide receptor gene was not expressed in the rat pituitary gland (Urena *et al.* 1993). A hypophysiotropic action of hypothalamic PTH is also unlikely since the concentration of PTH-IR in hypophysial portal blood is no higher than that in peripheral concentration (Harvey and Fraser 1993). The inability of PTH to enhance prolactin secretion in rats when systematically administered (unpublished observations) also suggests the prolactin response induced by intracerebraventricular injection is not mediated by increased vitamin D production nor by changes in the plasma calcium concentration. The prolactin response to PTH in man is, furthermore, independent of plasma calcium levels (Isaac *et al.* 1978) and contrasts with the inhibitory effect of hypercalcaemia on prolactin secretion (Rodjmark *et al.* 1984). The prolactin-releasing effect of PTH is thus likely to be mediated at CNS sites, in which PTH/PTH-related peptide receptor gene expression and PTH-binding sites have been detected (Urena *et al.*

1993; Harvey and Hayer 1993). PTH may therefore stimulate the release of a hypothalamic prolactin-releasing factor and/or inhibit the release of a prolactin-inhibiting factor. The increased *in vivo* metabolism of hypothalamic dopamine and *in vitro* suppression of hypothalamic dopamine release following PTH stimulation (Harvey *et al.* 1993a; Harvey *et al.* 1993b) suggests the prolactin-releasing effect of PTH results from dopamine disinhibition.

The presence of PTH in the median eminence may therefore reflect a neuromodulatory role that results in reduced dopamine secretion into hypophyseal circulation, rather than indicating a hypophysiotropic role in pituitary regulation. A similar role has also been described for many other neuropeptides that have recently been found in the median eminence (Clarke *et al.* 1993). Such a role is also indicated by the ability of radiolabelled PTH to bind to synaptosomes prepared from the rat medial basal hypothalamus (Fig. 4.2.). Actions of PTH on brain synaptosome function are well established and include an inhibition of norepinephrine release, mediated by a cyclic AMP-independent mechanism triggered by increased Ca^{2+} flux (Smogorzewski *et al.* 1989; Massry *et al.* 1988; Fraser *et al.* 1988). Moreover, while the blood-brain barrier may restrict the access of peripheral PTH to CNS target sites (Balabanova *et al.* 1984; Akmal *et al.* 1983), the median eminence lies outside this barrier. The increased prolactin secretion in hyperparathyroid patients and in patients given systemic PTH injections may thus reflect PTH actions within this circumventricular organ. Indeed, the ability of systemic PTH to modify dopamine metabolism within the rat medial basal hypothalamus is indicated by the increased DOPAC:dopamine ratio 60 minutes after PTH

administration (Fig. 4.3). It is, therefore, surprising that prolactin secretion in these rats is not elevated at this time, although this may indicate that the prolactin response is less sensitive than dopamine turnover to PTH stimulation or has a different time course of action.

A1.5. CONCLUSION

PTH is synthesized in hypothalamic nuclei and has CNS receptors and actions on hypothalamic dopamine turnover. Dopamine disinhibition may thus account for PTH-induced prolactin secretion, since PTH has no direct effects on pituitary function. These conclusions are shown schematically in Figure 4.4 Since PTH is produced in the pituitary gland, which lacks PTH receptors, the pituitary is unlikely to be an endocrine, paracrine or autocrine target site of PTH action. The pituitary gland may thus be an extraparathyroidal source of plasma PTH, especially as low levels of immunoreactive PTH have been observed in some parathyroidectomized patients (Mallet *et al.* 1982; Manning *et al.* 1981).

A.1.6. TABLES

Table 4.1. Influence of PTH on adenohypophyseal hormone release in vitro and in vivo

Hormone	Pituitary effect[1]	CNS effect[2]
Growth hormone	0	0
Luteinizing hormone	0	0
Follicle-stimulating hormone	0	0
Thyrotropin	0	0
Adrenocorticotropin	0	0
Prolactin	0	↑

0 = No effect; ↑ = increase.

[1] Release of radioimmunoassayable hormones from monolayers of rat pituitary cells incubated for 60 min with bPTH(1–34) (at concentrations of 0.1–1.0 µmol/l); in comparison with controls.

[2] Concentrations of pituitary hormones in peripheral plasma 10–60 min after an intracerebroventricular injection of 1.0 or 10.0 µg of bPTH(1–34) in 0.4 µl 0.9% NaCl, in comparison with controls.

A.1.7. FIGURES

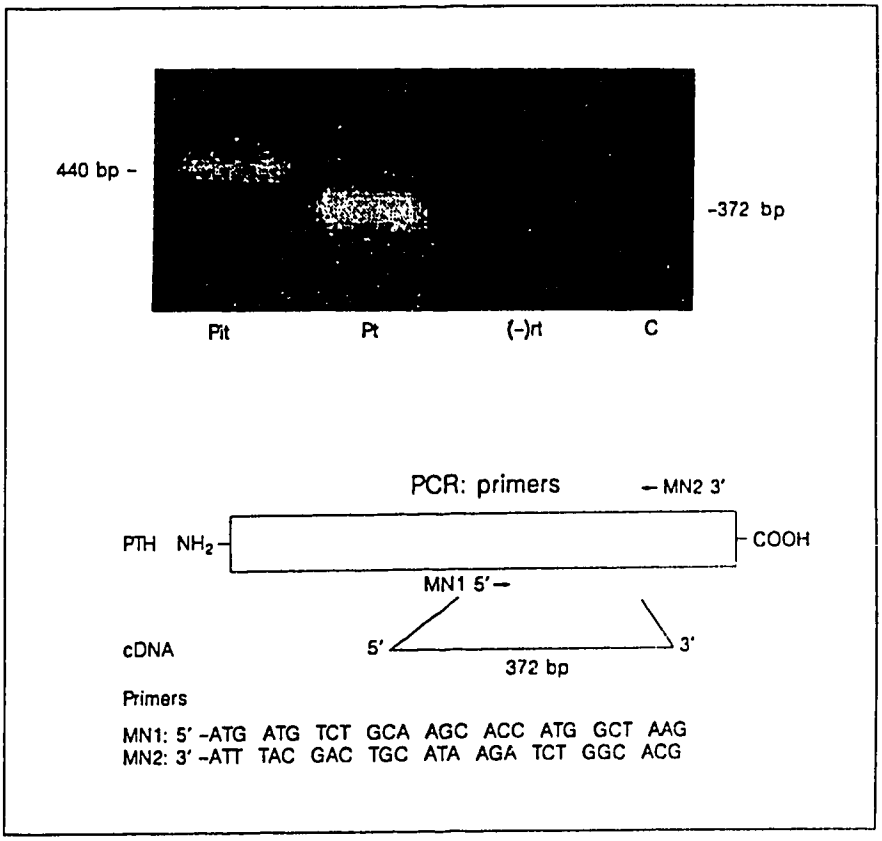

Figure 4.1. Pituitary expression of the PTH gene. The upper panel shows an ethidium bromide-stained 1.4% agarose gel through which cDNA was electrophoresed. The cDNA was reverse-transcribed from total RNA extracted from the pituitary gland (Pit) and the parathyroid gland (Pt) and amplified over 35 cycles by the polymerase chain reaction (PCR) in the presence of oligonucleotide primers MN1 and MN2. An amplified fragment was not observed using reverse-transcribed hepatic or ovarian mRNA (data not shown). Pretreatment of pituitary mRNA with DNase prior to reverse transcription did not eliminate the PCR fragment generated. PCR fragment size was determined by comparison with the migration of *Hae*III-digested pUC18 and *Eco*RI/*Bam*HI-digested lambda size markers. In the absence (-) of reverse transcriptase (rt) pituitary mRNA did not produce a PCR fragment. Lane C is a negative control, lacking cDNA and mRNA.

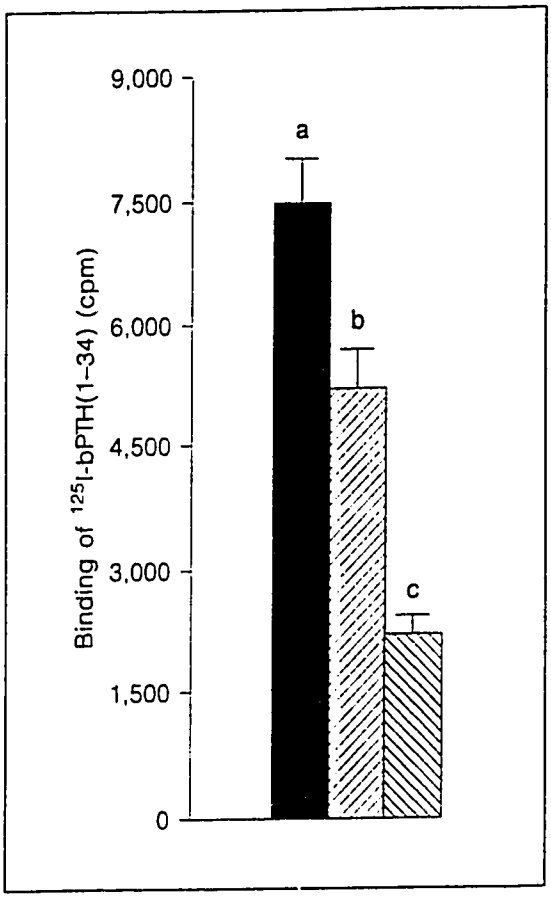

Fig. 4. 2. PTH binding sites in hypothalamic synaptosomes. Synaptosomes were prepared from rat medial basal hypothalamic nuclei and aliquots were incubated for 4 h with [Nle^{8,18}, Tyr^{34}] PTH(1–34) in the presence or absence of 3 µmol/l bPTH(1–34), to determine non-specific binding (▧). Bound and free tracer were separated by centrifugation [25]. Specific binding (▨) accounted for approximately 30% of total binding (■). Means ± SEM (n = 4). Groups with different letters are significantly different ($p < 0.01$ analysis of variance).

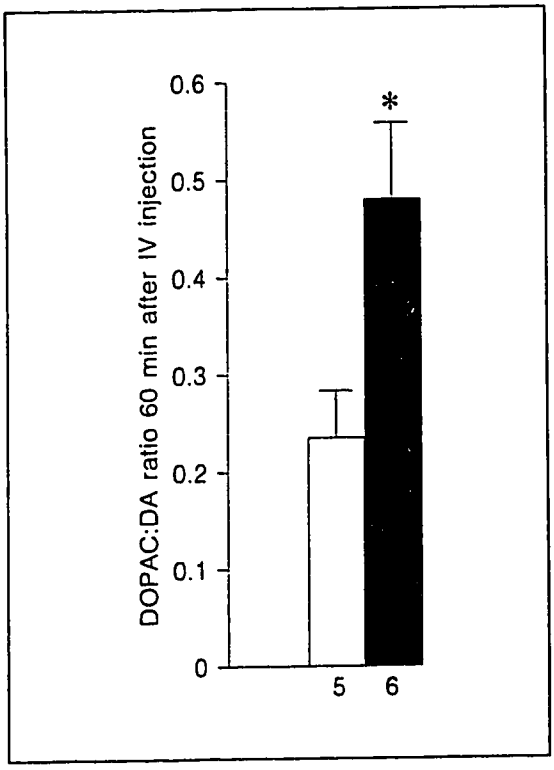

Figure 4.3. PTH-induced dopamine turnover in the rat medial basal hypothalamus following an intravenous (IV) injection of bovine PTH(1–34) (10 µg/kg ■): in comparasion with controls (□) injected with saline (0.5 ml/kg). Means ± SEM (n = 5 or 6). Dopamine turnover is expressed as the tissue ratio of dihydroxyphenylacetic acid (DOPAC) to dopamine (DA). *Significantly different from controls, $p < 0.05$ (students *t* test).

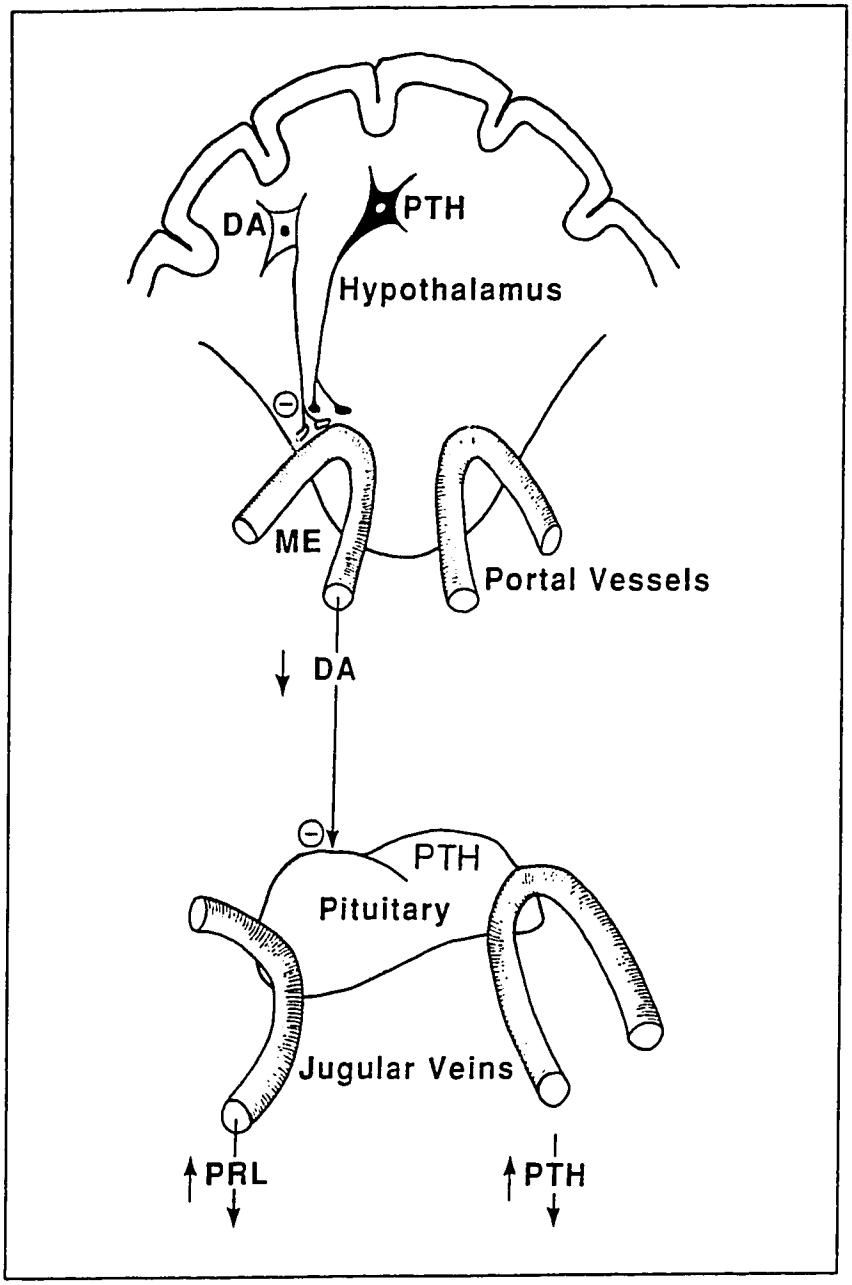

Figure 4.4. Schematic illustration of the participation of PTH in hypothalamo-pituitary regulation. PTH is expressed in paraventricular nuclei with axon terminals in the median eminence (ME). It is suggested that PTH inhibits the release of dopamine (DA) into hypophyseal blood vessels, and thereby removes pituitary lactotrophs from inhibitory dopaminergic control, elevating prolactin (PRL) concentrations in peripheral plasma. PTH does not appear to be released into portal circulation nor to act directly at pituitary sites. PTH is also expressed in the pituitary gland and may be released into systemec circulation.

A.1.8. REFERENCES

Akmal, M., Goldstein, D.A., Tuma, S.A., Fanti, R., Pattabhiramen, R., Massry, S.G.The effect of parathyroid hormone (PTH) on the blood-brain barrier. Clin Res 31:51A, 1982.

Balabanova, S., Toller, U., Richter, H.P., Pohlandt, F., Gaedicke, G., Teller, W.M. Immunoreactive parathyroid hormone, calcium and magnesium in human cerebrospinal fluid. Acta Endo 106:223-227, 1984.

Brickman, A.S., Carlson, H.E., Deftos, L.J. Prolactin and calcitonin responses to parathyroid hormone in hypoparathyroid, pseudohypoparathyroid and normal subjects. J Clin Endocrinol Metab 53:661-664, 1981.

Castro, J.H., Caro, J.F., Kim, H.J., Glenon, J.A. Effects of parathyroid hormone infusion and primary hypoparathyroidism on serum prolactin in man. J Clin Endocrinol Metab 51:397-398, 1980.

Clark, I., Jessop, D., Millar, R., Morris, M., Bloom, S., Lightman, S., Coen, C.W., Lew, R., Smith, I. Many peptides that are present in the external zone of the median eminence are not secreted into the hypophysial portal blood of sheep. Neuroendocrinology 57:765-775, 1989.

Clementi, G., Drago, F., Prato, A. Effects of calcitonin, parathyroid hormone and its related fragments on acquisition of active avoidance behavior. Physiol Behav 33:913-916, 1984.

Fioretti, P., Melis, G.B., Gardells, F. Parathyroid function and pituitary-gonadal axis in male uremics; effects of dietary treatment and of maintenance hemodialysis. Clin Nephrol 25:155-158, 1986.

Fraser, R.A., Sarnaki, P. and Budayr, A. Evidence that parathyroid hormone-mediated calcium transport in rat brain synaptosomes is independent of cyclic adenosine monophosphate. J Clin Invest 81:982-988, 1988.

Fraser, R.A., Kronenberg, H.M., Pang, P.K.T. Harvey, S. Parathyroid hormone messenger ribonucleic acid in the rat hypothalamus. Endocrinology 127:2517-2522, 1990.

Fraser, R.A., Kaneko, T., Pang, P.K.T., Harvey, S. Hypo- and hypercalcemic peptides in fish pituitary glands. Am J Physiol 160: R622-R626, 1991.

Furlong, T.J., Seshadri, M.S., Wilkinson, M.R., Cornish, C.J., Posen, S. Clinical experiences with human parathyroid hormone 1-34. Aust N Z J Med 16:794-798, 1986.

Handt, O., Reis, A., Schmidtke, J. Ectopic transcription of the parathyroid hormone gene in lympocytes, lympoblastoid cells and tumour tissue. J Endocrinol 135(2):259-256, 1992.

Harvey, S., Fraser, R. Parathyroid hormone: neural and neuroendocrine perspectives. J. Endocrinology 139:353-361, 1993.

Harvey, S., Hayer, S. Parathyroid hormone binding sites in the brain. Peptides 14:1187-1191, 1993.

Harvey, S., Hayer, S., Sloley, B.D. Dopaminergic actions of parathyroid hormone in the rat medial basal hypothalamus in vitro. Regul Pept 43(1-2):49-56, 1993.

Harvey, S., Hayer, S., Sloley, B.D. Parathyroid hormone-induced dopamine turnover in the rat medial basal hypothalamus. Peptides 14(2):269-274, 1993.

Heinrich, G., Kroneneberg, H.M., Potts, J.T.Jr. Habener, J.F. Gene encoding parathyroid hormone. Nucleotide sequence of the rat gene and deduced amino acid sequence of rat preproparathyroid hormone. J Bio Chem 259(5):3320-3329, 1984.

Isacc, R., Merceron, R.E., Caillens, G., Raymond, J.P. Ardaillon, R. Effect of parathyroid hormone on plasma prolactin in man. J Clin Endocrinol Metab 47:18-23, 1978.

Kaneko, T., Pang, P.K.T Immnunocytochemical detection of parathyroid hormone like substance in the goldfish brain and pituitary gland. Gen Comp Endocrinol 68:147-152, 1987.

Kruse, K., Gutekunst, B., Kracht, U., Schwerda. K. Deficient prolactin response to parathyroid hormone in hypocalcemic and normocalcemic pseudohypoparathyroidism. J. Clin Endocinol Metab 52:1099-1105, 1981.

Mallete, L.E., Tuma, D.N., Berger, R.E., Kirkland, J.L. Radioimmunoassay for the middle region of human parathyroid hormone using a homologous antiderum with a carboxy-terminal fragment of bovine parathyroid hormone as radioligand. J Clin Endocrinol Metab 54:1017-1024, 1982.

Manning, R.M., Adami, S., Papapoulos, S.E., Giled, J.H., Hendy, G.N., Rosenblatt, M., O'Riordan, J.L.H A carboxyl-terminal specific assay for human parathyroid hormone. Clin Endocrinol 15:439-449, 1981.

Marx, S.J., Aurbach, G.D Heterogenous hormonal disorder in pseudo-hypoparathyroidism. New Engl J Med 296:169-170, 1990.

Massry, S.G., Smogorzewski, M., Islam, A. Derangements in brain synaptosomes functions in chronic renal failure: role of parathyroid hormone. In New Actions of Parathyroid Hormone,Eds.S.G.Massry & T.Fujita.New York: P 301-316, 1988.

Pang, P.K.T., Harvey, S., Fraser, R.S,. Kaneko, T. Parathyroid hormone-like immunoreactivity in vertebrate brains. Am J Physiol 255:R635-R647, 1988.

Pang, P.K.T., Kaneko, T., Harvey, S. Immunocytochemical distribution of PTH immunoreactivity in vertebrate brains. Am J Physiol 255:R643-R647, 1988.

Raymond, J.P., Isaac, R., Merceron, R.E., Wahbe, E.F. Comparison between the plasma concentrations of prolactin and parathyroid hormone in normal subjects and in patients with hyperparathyroidism or hyperprolactinemia. J Clin Endocrinol Metab 55:1222-1225, 1982.

Rodjmark, S., Edstrom, E., Nordlund, M. Effect of chronic endogenous hypercalcemia on prolactin and thyrotropin responsiveness in man. J Endocrinol Invest 7:635-639, 1984.

Rubin, L.P., Robinson, B.G., Arbiser, J.L Human placenta expresses a parathyroid hormone-like mRNA. 71st Annu Meet Endocr Soc, Seattle, p 320, 1989.

Selvanayagam, P.E., Graves, K., Copper,C., Rajaraman, S. Expression of the parathyroid hormone-related gene in rat tissues. Lab Invest 64:713-717, 1991.

Srivaster, S.P., Swarup, K., Srivastar, A.K. Prolactin cells of *Clarium Batiachus* in response to corpuscles of Stannius extract administration. Zool Sci 4:201-204, 1987.

Urena, P., Kong, X.F., Abou-Samra, A.B. Parathyroid hormone (PTH)/PTH-related peptide receptor messenger ribonucleic acids are widely distributed in rat tissues. Endocrinology 133:617-623, 1993.

Wendellar Bonga, S.E., Lafeber, F.P.J.G., Flik, G., Kaneko, T. Pang, P. Immunochtochemical demonstration of a novel system of neuroendocrine peptidergic neurons in the pond snail Lymnaea stagnalis, with antisera to the teleostean hormone hypocalcin and mammalian parathyroid hormone. Gen Comp Endocinol 75:29-38, 1989.