Order Number 9517553

The ontogeny of peptidases involved in the post-transitional processing of cholecystokinin

Oakes, Mary Geraldine, Ph.D.

The University of Arizona, 1994

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

THE ONTOGENY OF PEPTIDASES INVOLVED IN THE POST-TRANSLATIONAL PROCESSING OF CHOLECYSTOKININ

by

Mary Geraldine Oakes

A Dissertation Submitted to the Faculty of the

COMMITTEE ON PHARMACOLOGY AND TOXICOLOGY (GRADUATE)

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1994

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Mary Geraldine Oakes

entitled The Ontogeny of Peptidases Involved in the Post-translational Processing of Cholecystokinin

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Hugh E. Evans II	9-16-94 Date
Frank Fonseca	9-20-94 Date
Clifton D. Gutteridge	9-20-94 Date
Radhakrishna	10-27-94 Date
	Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

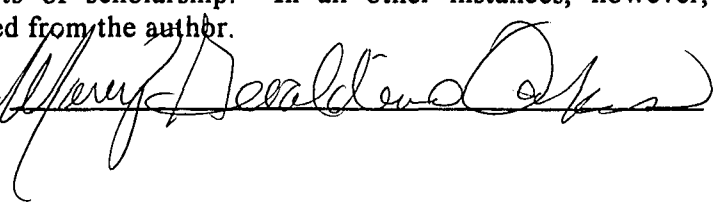
Thomas P. O'Connor — Dissertation Director

September 15, 1994 — Date

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: 

ACKNOWLEDGMENTS

I would like to extend my sincere appreciation to my advisor, Dr. Thomas P. Davis, for his patience and his ability to guide me through the storms during the past five years. In addition, I would like to thank the members of my advisory committee including Dr. Frank Porreca, Dr. Hugh Laird II, Dr. Klaus Brendel and Dr. Hank Yamamura, Dr. David Kreulen, Dr. Clifford Crutchfield, and Dr. Rao Radhakrishna for their enthusiasm, guidance and responsiveness. A special thanks to Dr. Mark Van Ert for their assistance throughout the program. It is with the aid of these outstanding researchers that I have been able to overcome my own mental and physical barriers to obtain the skills needed for acquiring future goals.

I would like to thank Mr. Terry Gillespie for his assistance and expertise in laboratory procedures as well as his unconditional friendship. I wish the best of luck to all of the members of the laboratory including the graduate students, Steve Waters, Craig Myers and Tom Abruscatto. A special thanks to Richmond Sarpong for his excellent technical assistance during the past few months.

I also wish to thank Mrs. Rebecca Papineau and the Akwesasne Mohawk Board of Education for supporting me through all of my endeavors.

DEDICATION

I dedicate this dissertation to my best friend and mother, Helen Oakes, who has given me the strength to forge above or around any obstacle that I have encountered. Sharing in this dedication are my three courageous children, Amanda, Katherine and Stephanie Morrill who provided me with three motives and the love to go on.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

There are three levels at which biologically active peptides may be regulated: transcription, translation, and post-translational processing. The data presented in this study focused on post-translational processing to illustrate the presence and significance of neuropeptide processing and metabolic enzymes in regulating levels and forms of cholecystokinin detected by radioimmunoassay.

The activity of the processing enzyme, carboxypeptidase H (EC 3.4.17.10, CPH) and the metabolic enzyme, neutral endopeptidase (EC 3.4.24.11, NEP), were altered by postnatal age 4 days (P4) in central nervous system (CNS) regions. Metallo endopeptidase (EC 3.4.24.15), another metabolic enzyme of cholecystokinin (CCK) has a more constant activity throughout development of the CNS than did CPH or NEP. For enteric nervous system regions, CPH activity showed no change in the more proximal tissues (stomach, duodenum and jejunum) but decreased in distal tissues (midjejunum and ileum) with development. NEP activity decreased with age around P30 in the proximal regions and P4 in more distal regions of the enteric nervous system.

Dramatic changes in CCK-like immunoreactivity appear to occur from P4 to P7 in the central nervous system and vary considerably by region. In general, large molecular weight forms of cholecystokinin (L8D-IR) did not change with development in either the central or enteric nervous system regions. Moderate molecular weight forms (I11H-IR) show a regional alteration in the central

nervous system whereas these forms decreased in the enteric nervous system. Carboxy-extended (D10Y-IR) forms showed regional alterations in both the central and enteric nervous systems. Bioactive cholecystokinin forms (G17-IR) increased in the enteric nervous system but showed regional alterations in the central nervous system with development.

These data suggest that post-translational enzymes aid in regulating the levels and forms of cholecystokinin detected in the central and enteric nervous systems.

INTRODUCTION

Only 42 papers were published from 1966 to 1968 which dealt with neuroactive peptides. By 1974, a total of 400 papers were published and since then an explosion of peptide research continued until 1992 when approximately 1800 articles were published (Myers, 1994). Many peptides during this time have met the traditional definition of a neurotransmitter and thus have stimulated curiosity in the peptide field. For example, cholecystokinin has been shown to be synthesized and released presynaptically. Purified or synthesized cholecystokinin is able to mimic the actions of the endogenous compound that is released via nerve stimulation both in the central and enteric nervous systems where cholecystokinin appears to have several functions. Specific inhibitors and antibodies block postsynaptic responses to the endogenously released and exogenously administered cholecystokinin. Cholecystokinin also meets the criteria to be labeled a neuromodulator and a neurohormone. Thus, the possibility that peptides may also play a major role in the central and enteric nervous system has placed them as important as some of the classical neurotransmitters such as acetylcholine, dopamine and norepinephrine.

The **Hypothesis** that I will test in this dissertation is that neuropeptide development is linked to neuropeptidase development in the rat enteric and central nervous systems. To test this hypothesis, I wish to determine if there are regional variations of post-translational enzyme activity and/or regional variations of

cholecystokinin forms detected during development in the enteric and central nervous systems. These data will aid in determining if a relationship exists between peptidase activity and levels of cholecystokinin-forms detected during development.

Peptidergic Systems

One of the major differences between peptides and classical transmitters is in the synthesis of the biologically active transmitter. For instance, acetylcholine is synthesized in a reaction catalyzed by choline acetyltransferase that transfers an acetyl group from acetyl-CoA to choline to produce the biologically active neurotransmitter. This reaction occurs in the cytoplasm of the nerve terminal and/or in the area of the cell body where choline acetyltransferase is abundant. The choline is obtained from dietary sources and is transported into the neuron whereas the acetylCoA is produced in the cell by several processes including glycolysis. Peptides on the other hand, are synthesized by mRNA on ribosomes, packaged in golgi vesicles and therefore synthesis can only take place in the perikaryon or dendrites of a neuron. Once translated, this peptide is known as a much larger precursor form called a propeptide or prohormone. Propeptides require further enzymatic processing to produce the biologically active form of the peptide. Once synthesized, classical andpeptide transmitters are packaged in synaptic vesicles to be released upon stimulation of the neuron.

Once released another major difference between classical and peptide transmitters is seen. For example, once released into the synapse, acetylcholine may bind to its receptor and/or be cleaved by acetylcholinesterase to produce acetate and choline. These inactive molecules and a quantity of the active transmitter may be taken back up into the cells which release them. Peptide transmitters are released from a cell upon stimulation but no mechanism is present for the reuptake of the intact peptide. The only method to stop the action of the peptide is to enzymatically metabolize it with specific peptidases.

Advantage can be taken of these differences between classical neurotransmitters and peptide transmitters in that peptidases involved in processing of propeptides and metabolism of the active peptide allow possible clinical manipulations. This has been previously shown by the clinical use of captopril to inhibit angiotensin converting enzyme (ACE). Inhibition of ACE results in a decreased production of angiotensin from its precursor. This decreased availability of angiotensin results in the lowering of blood pressure in hypertensive patients.

Distribution

Many peptides have been detected in the central and enteric nervous systems including: angiotensin, calcitonin gene-related peptide, cholecystokinin, dynorphin, enkephalin, gastrin releasing peptide, neuropeptide Y, neurotensin, somatostatin and substance P to name a few. Not only are these peptides found in the central and enteric nervous systems but they may also be colocalized with other transmitters in the same cell. For example, vasoactive intestinal peptide (VIP) and acetylcholine are found in postganglionic parasympathetic neurons whereas neuropeptide Y and norepinephrine coexist in postganglionic sympathetic neurons. Both sets of peptide and classical transmitter interactions are involved in the control of blood flow and salivary secretions (Lundberg, 1994). Cholecystokinin has been colocalized with dopamine in mesolimbic neurons, specifically the A10 neurons of the ventral tegmental area of the rat. This colocalization with dopamine suggests a role for cholecystokinin in various nervous disorders such as Parkinson's disease and schizophrenia (Hokfelt, 1980).

Not only are peptides found within the central and enteric nervous system but there are varying peptide levels in specific regions as exemplified by the central nervous system (Crawley, 1985). For instance, very high levels of cholecystokinin (CCK) were detected in the cortex. Met-enkephalin and somatostatin were detected in moderate levels whereas gastrin releasing peptide was detected in low levels in the cortex as well. The predominant peptides found in the hypothalamus were thyroid releasing hormone, somatostatin and α-

melanocyte stimulating hormone. Moderate levels of substance P, gastrin releasing peptide and cholecystokinin were detected in the hypothalamus. Interestingly, the cerebellum contains only moderate levels of met-enkephalin, somatostatin, corticotropin releasing factor and very low levels of cholecystokinin. Why are cholecystokinin levels so high in the cerebral cortex, moderate in the hypothalamus and nondetectable in the cerebellum? We believe that the main regulators of peptide levels are due to the activity of peptidases involved in the post-translational processing and metabolism of the peptide.

Post-translational Processing

The synthesis of a peptide transmitter occurs along similar pathways to all peptides produced in a cell and is described extensively in most classical biochemistry texts. First the transcription of a DNA template and translation of the mRNA results in the production of a prepropeptide. The signal sequence on the amino terminus of the prepropeptide is cleaved in the endoplasmic reticulum and the propeptide is then directed to the golgi apparatus. Further processing of the propeptide may include sulfation and glycosylation. The propeptide is then packaged into vesicles to travel down the axon to the neuronal terminal. Further processing occurs in the vesicles where the propeptide is cleaved to smaller forms that may be amidated to produce the biologically active peptide. Once released at the synapse, further processing or metabolism of the active peptide by degradative enzymes occurs and results in the production of inactive fragments.

Thus, post-translational processing may be considered as two processes: one considered as synthesis of the biologically active peptide and the other as the metabolism or destruction of the active peptide. Once formed by pretranslational events, the level of biologically active peptide may be regulated by the activity of peptidases involved in post-translational processing.

In general, the processing of a propeptide involves several processing enzymes including carboxypeptidase H (EC 3.4.17.10, CPH). CPH sequentially removes basic amino acids from the carboxyl terminal end (Fricker, 1982) of a prohormone after they have been exposed by subtilisn-like (i.e., kex-2 gene) enzymes known as prohormone convertases (Barr, 1981). The cleaved peptide when containing a terminal glycine is then amidated by peptidyl-glycine-α-amidating mono-oxygenase (PAM) (Bradbury, 1982). Amidation may lead to the biologically active form of the peptide as speculated for cholecystokinin (CCK) and many other (i.e., 30% of all) neuropeptides.

The metabolic process of post-translational processing involves specific peptidases for the degradation of particular peptides. These peptidases are of a wide variety and have a wide specificity (Turner, 1987) and in some cases may produce a fragment of the active peptide with biological activity (e.g., CCK-8). A further discussion of metabolic profiles for specific peptides is included since this is an area where peptide processing differs substantially.

Differences in levels of peptides and metabolic profiles have been previously reported. For instance, in post-mortem brain samples of schizophrenic patients,

levels of several neuropeptides have been found to vary by region. One such peptide, β-endorphin, serves as an active transmitter but also as a precursor for the production of various pharmacologically active fragments. One active fragment, des-enkephalin-γ-endorphin [DEγE or β-E-(6-17)], is a major product formed upon incubation of β-endorphin with rat brain slices of the pituitary, hypothalamus and hippocampus (Li, 1988). When β-endorphin was incubated with twice-washed membrane homogenates of the frontal cortex there was a decreased production of DEγE in schizophrenic post-mortem samples (Davis, 1986). This DEγE fragment of β-endorphin is considered an endogenous "neuroleptic-like" agent in the brain since it produces similar responses as classical antipsychotics (DeWeid, 1978). Chronic treatment of rats with haloperidol and chlorpromazine has resulted in an increased production of DEγE when whole brain membranes were incubated with β-endorphin (Davis, 1984). There were no differences observed in the production of α-type fragments suggesting that antipsychotic treatment may have altered specific peptidase activity involved in the production of DEγE. Interestingly, neutral endopeptidase (EC 3.4.24.11) breaks down DEγE thereby halting this fragment's activity. Chronic treatment of rats with haloperidol has shown an increase of neutral endopeptidase (NEP) activity in the nucleus accumbens whereas chronic treatment with chlorpromazine increases NEP activity in the caudate putamen (Konkoy, 1993). In this same study, treatment with apomorphine resulted in a decreased neutral endopeptidase activity in both

the nucleus accumbens and caudate putamen. No change of NEP activity occurred in the hypothalamus upon treatment with either haloperidol, chlorpromazine or apomorphine. This suggests that drugs may alter peptidase activity in specific regions of the brain that may result in changes of biologically active peptide levels. These alterations of peptide levels may result in a different physiological response in specific regions of the central nervous system.

Differences in metabolic profiles of peptides in the enteric nervous system have also been described. For instance, β-endorphin has been shown to increase gut motility when vascularly perfused in isolated segments of the dog gastrointestinal tract (Burks, 1982). Various forms of α- and γ-type endorphins were detected in the venous effluent including moderate levels of DEγE. β-endorphin was incubated with either the muscularis or the mucosal region of the dog small intestine. The muscularis contained the longitudinal, circular and deep muscle with associated nerve tissue and the mucosal region contained the mucosa, submucosa and lamina propria (Hynes, 1983). The mucosal region produced a higher number and quantity of peptide fragments than did the muscularis region suggesting a variation in post-translational enzyme activity of these two regions.

Similarly, other peptides such as neurotensin have different metabolic profiles as well. Incubation of brain regions with neurotensin showed a favored cleavage between the basic amino acid residues Arg^8 and Arg^9 to yield the octapeptide NT-(1-8) and the C-terminal fragment NT-(9-13) by metallo endopeptidase (Checkler,

1985). The NT-(9-13) fragment produced by metallo endopeptidase has been shown to bind to the neurotensin receptor and to produce antinociception (Furuta, 1984). The NT-(9-13) fragment is rapidly degraded once formed so its role for antinociception in *vivo* is uncertain. Neutral endopeptidase has also been implicated in the degradation of neurotensin with cleavage between the Tyr^{11}-Ile^{12} bond resulting in a NT-(1-11) fragment (Emson, 1985). Treatment with neuroleptics increased the production of NT-(1-8) and NT-(9-13) (Davis, 1987). Increased production of these fragments may suggest an increased metallo endopeptidase activity and therefore a theoretically increased activity at the receptor due to the NT-(9-13) fragment.

The above discussion of β-endorphin and neurotensin was meant to describe several points of interest. Metabolic enzymes are specific for one peptide but may be involved in the degradation of other peptides. For example, neutral endopeptidase can process both β-endorphin and neurotensin. Metabolic enzymes may also produce bioactive fragments as exemplified by the cleavage of neurotensin by metallo endopeptidase (EC 3.2.24.15) to produce the bioactive fragment NT-(9-13). Therefore, there are regional variations of peptide and fragment concentrations in both the central and enteric nervous systems.

Development

The morphogenesis of the germ layers produced at gastrulation are a series of complex events. The mesodermal layer ultimately produces the muscles, kidneys,

heart, blood, dermis, skeleton and smooth muscle. The endodermal layer develops into the pancreas, liver, bladder, intestine and the respiratory system. The ectodermal layer is responsible for producing external coverings such as the inner ear, milk secreting glands, hair, sweat glands and the development of the brain, spinal nerves and cord, and sympathetic nerves.

The maturation of the neuroepithelial cells of the neural tube lumen and the ependymal cells into highly organized structural units to produce the functional brain is a dramatic developmental event as well. Neuroblast proliferation, migration and differentiation into mature neurons with axons, dendrites, synaptic networks, and the production of neurotransmitters illustrate the complexity of the central nervous system.

The gastrointestinal tract of humans is mature at birth whereas in the rat it is immature up to the first two postnatal weeks of life. From birth until early suckling age the primary diet of the pup is milk. Weaning of the rat begins at postnatal age 17 days and is usually complete by postnatal age 28 days (Henning, 1979). The change from milk to solid food requires a change in the gastrointestinal tract to handle larger amounts of carbohydrates. Milk contains low concentrations of carbohydrates as lactose while solid food contains high concentrations of starch and sucrose. Thus the weanling period would require an increase of specific disaccharidase activities to handle the increase of starch in the diet. By the fourth postnatal week, the gastrointestinal system of the rat is mature and levels of gastrointestinal enzymes are stabilized (Henning, 1987).

Not only do the brain and gastrointestinal system of the rat develop differently but so do the peptides produced in these areas as well. Peptidergic systems in discrete areas of the rat brain are variable in their onset of synthesis and release (Strittmatter, 1986). For example, endorphins (e.g., β-endorphin, dynorphin) appear at embryonic day 16 (E16) and enkephalins are detectable at postnatal day 6 (P6) (Bayon, 1979). Other peptides such as cholecystokinin (CCK) (Brand, 1982) and gastrin-releasing peptide (GRP) (McGregor, 1982) do not appear in whole brain samples until well after birth. Interestingly, most gastrointestinal peptides do not appear in the gastrointestinal tract until the third postnatal week when extensive developmental changes occur.

Cholecystokinin

Cholecystokinin was first discovered in 1928 when an unknown substance released from the upper section of the small intestine produced gall bladder contraction (Ivy, 1928). Later, a substance termed pancreozymin was shown to stimulate pancreatic enzyme secretion (Harper, 1943). Purification of cholecystokinin occurred in 1964 (Jorpes, 1964) and two years later it was suggested that cholecystokinin and pancreozymin were the same molecule (Jorpes, 1966). Research into the physiological effects of cholecystokinin then resumed in the gastrointestinal system. A gastrin-like immunoreactive peptide was discovered in the brain in 1975 (Vanderhaeghen, 1975) but further analysis found that this immunoreactive peptide was the octapeptide of cholecystokinin or CCK-8 (Dockray, 1976). The recent use of molecular biology techniques allowed the gene sequence of the cholecystokinin peptide to be described and generated much interest in the post-translational processing of the peptide to the biologically active form.

Structure

The cDNA sequence of rat preproCCK indicates a single copy of CCK with a characteristic signal sequence and a peptide of 115 amino acids (Deschenes, 1984). Later, these authors identified three exons separated by two relatively large (1-5 kbp) introns (Deschenes, 1985). The first exon corresponds to the 5' untranslated region, the second to the signal sequence, and the third to most of

the CCK-33 region and the remaining C-terminal portion of proCCK. A transcription start site was located 29 bp 3' to a TATA sequence. A promoter region was found near the transcription start site.

Post-translational Processing

The post-translational processing of cholecystokinin is depicted in Figure 1. Processing begins with cleavage of the signal sequence in the endoplasmic reticulum. A signal peptidase cleaves the signal fragment at an Alanine residue to produce CCK-58 (Rehfeld, 1985). ProCCK then enters the Golgi where sulfation of the tyrosine residues occur (Vargas, 1985). There are four tyrosine residues found in proCCK including one in the C-terminus region. Three of these tyrosine residues are sulfated and are preceded by one or two acidic residues that act as a consensus sequence for sulfation. The fourth tyrosine residue located in the N-terminus of CCK-39 is not sulfated and is not preceded by acidic residues (Eng, 1986).

Pro-CCK is cleaved at single Arg residues by an endopeptidase (i.e., prohormone convertase) on the N-terminal side of CCK-8, -33, -39 and -58. The presence of large molecular weight peptides (e.g., CCK-58) in synaptic terminals suggests that further proteolytic cleavage may occur in synaptic vesicles. Their presence also suggests that large forms may be released along with CCK-8 to exert an influence on synaptic transmission in the rat brain (Beinfeld, 1985).

Large forms of CCK such as CCK-58 containing a C-terminal extension may then be cleaved by prohormone convertases (i.e., PC1, PC2) to produce carboxy extended CCK-8 forms depicted in figure 1. The N-terminal fragments produced after cleavage by prohormone convertase have been identified in rat brain (Beinfeld, 1985).

The amino acid sequence of carboxy-extended CCK-8 is shown in figure 1. Cleavage of the C-terminal serine residue by prohormone convertase (PC) and removal of arginine residues by carboxypeptidase H (CPH) produces CCK forms containing a C-terminal glycine (i.e., glycine-extended) forms. These glycine-extended forms were characterized in the porcine cerebral cortex (Rehfeld, 1986; Allard, 1985). The C-terminus is then amidated by peptidyl glycine α-amidating monooxygenase (PAM) which amidates the phenylalanine with the amine group of the glycine. The biologically active forms of cholecystokinin are present in synaptic vesicles and are released into the synaptic cleft upon stimulation of the neuron.

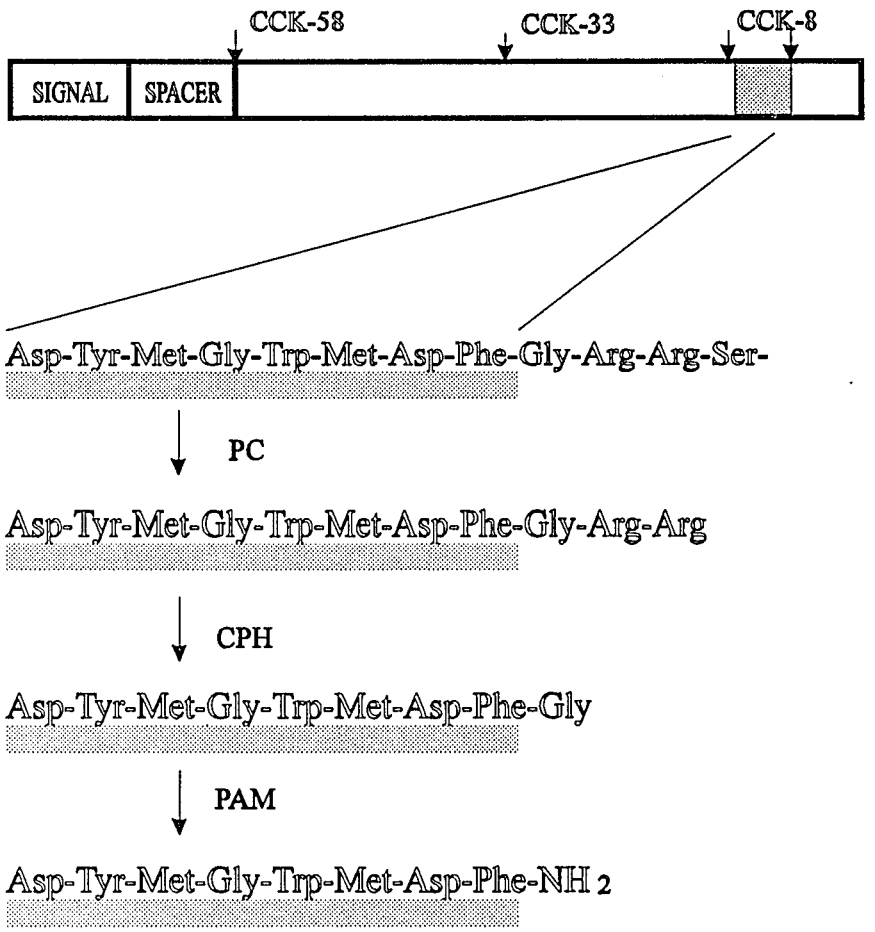


Figure 1. Post-translational Processing of Preprocholecystokinin

The cleavage pattern of bioactive cholecystokinin is depicted in Figure 2. Degradation of CCK-8 and smaller CCK fragments by plasma and brain synaptic membrane peptidases have been studied in detail (Deschodt-Lanckman, 1981, 1983; Koulischer, 1982). Cleavage sites leading to degradation of CCK include cleavage of the Trp^5-Met^6 bond by metallo endopeptidase (EC 3.4.24.15) and cleavage of the Asp^7-Phe^8 bond by neutral endopeptidase (NEP, EC 3.4.24.11). Neutral endopeptidase also produces an active CCK-4 fragment by cleavage at the Gly^4-Trp^5 bond (Bunnett, 1988; Deschodt-Lanckman, 1984; Zuzel, 1985).

Peptides shorter than CCK-8 have shorter half-lives than the naturally occurring form and peptides containing a sulfated tyrosine have longer half-lives than nonsulfated cholecystokinin. The major degrading activity in plasma appears to be an aminopeptidase (EC 3.4.11.1). Several types of enzymatic activity in tissue other than those mentioned above are able to degrade CCK-8. These were postulated to include a membrane-bound aminopeptidase (EC 3.4.11.2) and a non-specific thiol protease. Another activity has been found in brain that produced a C-terminal pentapeptide and was sensitive to inhibitors of serine protease (McDermott, 1983).

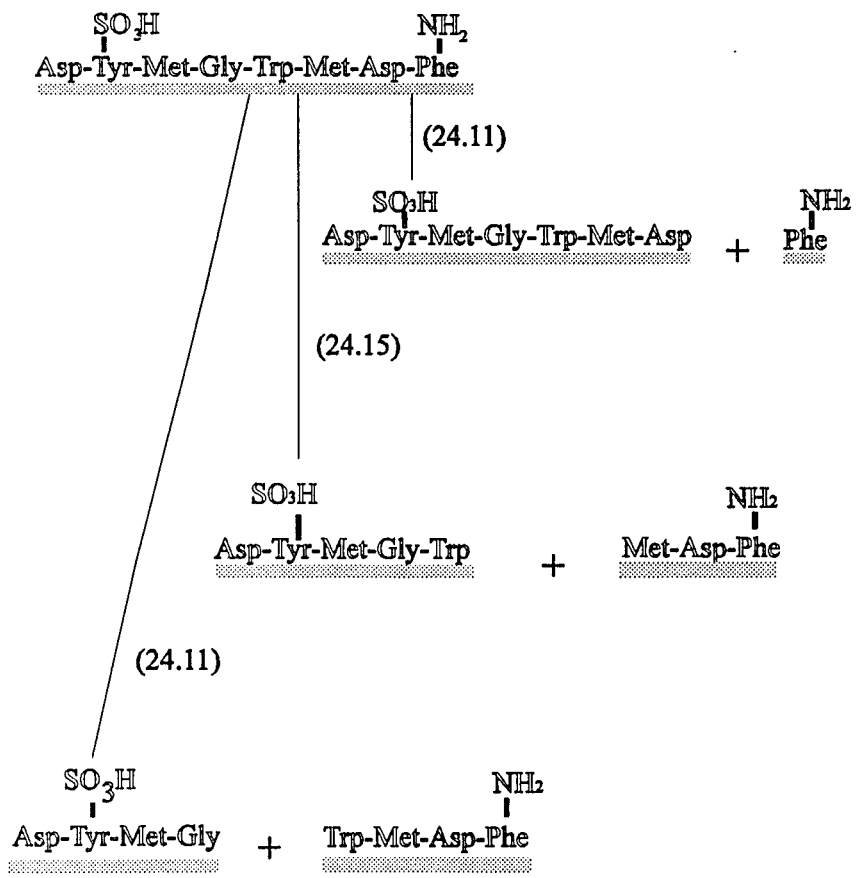


Figure 2. Enzymatic Cleavage Sites of Cholecystokinin

Actions

Cholecystokinin has many actions in both the enteric and central nervous systems. When food is placed in the stomach or the proximal intestine, cholecystokinin is released from the small intestinal mucosa cells resulting in an increase of circulating plasma cholecystokinin. Cholecystokinin released from the gastrointestinal mucosa in response to food in the lumen has been shown to inhibit gastric acid secretion by releasing somatostatin. Cholecystokinin acting through a vagal pathway may regulate satiety to decrease food consumption. Cholecystokinin directly regulates the secretion of digestive enzymes from the pancreas and gallbladder. Cholecystokinin also increases arterial blood flow to the upper small intestine (Chou, 1977) and liver (Richardson, 1977) of dogs thereby aiding in the digestion of food. Cholecystokinin alters smooth muscle contraction involved in propulsion of food down the gastrointestinal tract. Besides digestive functions, cholecystokinin has been implicated to be an antipsychotic, analgesic, anticonvulsant, sedative and is implicated in a number of disease states such as Parkinson's, Alzheimer's, Schizophrenia and Depression (Crawley, 1983).

A discussion of cholecystokinin levels in the plasma and physiological actions will be discussed in order to illustrate the complexity of cholecystokinin actions.

Plasma Concentrations

When food enters the duodenum a release of cholecystokinin from proximal small intestine mucosal cells into the peripheral circulation occurs and a dramatic increase of cholecystokinin immunoreactivity in the blood is found (Phillip, 1991). Low levels of cholecystokinin either present in the circulation after a meal or given *ip* appear to physiologically decrease appetite and food intake in humans (Stacher, 1986). Large (e.g., CCK-58, -33) and small forms (e.g., CCK-8, -4) of cholecystokinin are released into the plasma by the proximal small intestine of normal human subjects (Calam, 1982). CCK-58 is the major circulating large form of cholecystokinin in humans (Eysselein, 1987) and dogs (Eberlein, 1987). These large circulating forms of cholecystokinin may ultimately be found to modulate the enteric nervous system or have a role as a storage form for CCK-8.

Cholecystokinin levels in the plasma can vary under a variety of different conditions such as disease states and stress. Patients with anorexia nervosa appear to have elevated baseline cholecystokinin levels in their plasma with a three fold higher postprandial (following a meal) concentration. The increased plasma concentrations of cholecystokinin may be responsible for the early satiety and delayed gastric emptying noted in these patients (Phillipp, 1991). Interestingly, rats fed ethanol chronically show a higher response to endogenously released cholecystokinin resulting in reduced feeding without an alteration of plasma CCK levels. Decreased cholecystokinin levels in the plasma were found for patients with celiac disease manifested as a destruction of upper small

intestinal mucosa. A normal patient produces 50% large forms of cholecystokinin for release into the plasma. In celiac patients there is a lower quantity of peptide released and a decreased release of large forms of CCK into the plasma which upon treatment returned to normal (Calam, 1982). Plasma concentrations of cholecystokinin also vary under conditions of stress. For example, an increased release of cholecystokinin was found in the blood stream of marathon runners before and after a run (Philipp, 1992).

Satiety

Cholecystokinin mediates the satiety response that leads to cessation of feeding when food is placed in the stomach or intestine. The satiety response includes a reduction of food intake and appetite behaviors such as decreased exploration and social interactions. The possibility that cholecystokinin has sedative effects leading to decreased appetite behaviors was rejected (Crawley, 1982). Endogenous CCK released from the small intestine by the intragastric administration of soybean trypsin inhibitor also decreased feeding in neonatal rats (Weller, 1990). Exogenous CCK inhibits food intake in rats, monkeys (Gibbs, 1977), mice (Schneider, 1979) and humans (Stacher, 1982). The intraperitoneal administration of CCK-33 and CCK-8 results in a similar inhibition of food intake in rats (Melville, 1993). Continuous infusion of CCK in rats for 2 days resulted in a decreased total food intake (Hsiao, 1983). Continued infusion resulted in

tolerance and no detectable change in food intake or body weight occurred (Crawley, 1983).

It has been suggested that the release of CCK from the duodenum during a meal activates a CCK-A receptor mediated vagal message. This vagal message relays information via the nucleus tractus solitarius to the hypothalamus where CCK is released upon stimulation of CCK-B receptors. Vagotomy blocks the satiety response to peripherally administered cholecystokinin suggesting that CCK acts on peripheral nerves containing CCK-A receptors (Garlicki, 1990). In addition, devazepide, a CCK-A receptor antagonist increased the size of the first meal in free feeding rats (Grignaschi, 1993). Interestingly, ligation of the cervical vagus in the rat indicates that CCK binding sites or receptors are transported down the vagus to sub-diaphragmatic vagal fibers including those that serve the stomach. These afferent neurons have cell bodies in the nodose ganglia that synthesize CCK-A receptors for intra-axonal transport to peripheral terminals and to central nucleus tractus solitarius terminals (Moran, 1987).

Cholecystokinin may also act on CCK-B receptors of the ventromedial hypothalamus since the CCK-B antagonist, L-365,260, increased food intake and postponed the onset of satiety in partially satiated rats (Dourish, 1989). CCK-like immunoreactivity was released by the hypothalamus during the intake of food (Schick, 1987). CCK-8 has also been shown to suppress feeding when injected directly into the lateral hypothalamus (Schick, 1990). To further demonstrate the

effect of cholecystokinin, an increased food intake and weight gain occurred in rats injected with CCK antibodies (McLaughlin, 1985).

Thus, cholecystokinin may act on either CCK-A enteric receptors or CCK-B central receptors to produce satiety. The satiety response may be modulated by a variety of factors including estradiol (Butera, 1993), serotonin (Grignaschi, 1993), and ethanol (Weatherford, 1993).

Pancreatic Secretions

Cholecystokinin, gastrin, bombesin, and acetylcholine all stimulate pancreatic enzyme secretions including juice volume, bicarbonate and amylase (Shiratori, 1993). Cholecystokinin causes changes in calcium movement and turnover of phosphoinositols (Calderon, 1980). The pancreatic response appears to correspond with plasma CCK levels after endogenous release and exogenous infusion in humans (Hildebrand, 1990; Beglinger, 1985) with a lower response shown in dog (Dale, 1989).

A controversy exists on the form of cholecystokinin that is more potent at stimulating pancreatic enzyme secretions. A recent study suggests that smaller forms of cholecystokinin (i.e., CCK-8) were more active in stimulating rat pancreatic acini cell secretion than were larger forms such as CCK-58 (Hocker, 1990). Another study suggests that CCK-58 (Eysselein, 1983) and CCK-33 (Solomon, 1984) were of equal potency to CCK-8 for pancreatic stimulation. In addition, a sulfated tyrosine residue on CCK-8 was required for full pancreatic

stimulating activity (Jensen, 1981). Interestingly, shorter fragments of CCK-8 lacking the tyrosine residue possess pancreatic stimulating activity but were much less potent than the longer sulfated forms (Villaneuva, 1982). CCK-(30-33) has been shown to inhibit pancreatic secretion.

Although one physiological role of CCK is in stimulating pancreatic secretion it has been shown that the Sandoz Inc somatostatin analog, SDZ, inhibits cholecystokinin induced pancreatic secretions suggesting a feedback loop utilizing somatostatin (Shiratori, 1993). In the calf, pancreatic receptors were predominantly CCK-A at birth but converted during weanling age to CCK-B receptors (Le Meuth, 1993). This CCK receptor conversion occurred during the weanling period. This suggests that CCK may play a predominant role for proliferation and secretion of the pancreas early in development whereas gastrin acting at CCK-B receptors may predominate after weanling age.

Gallbladder Contraction

Circulating plasma cholecystokinin also induces gallbladder contraction. In man, gallbladder contraction can be induced after the administration of cholecystokinin at doses similar to those found in the plasma after ingestion of a meal (Kerstens, 85). CCK-33 and CCK-like peptides are much more potent than gastrin in stimulating gallbladder contraction in muscle preparations of guinea pigs, rabbit (Amer, 1969), cat (Chowdhury, 1975), dog (Rubin, 1969), and man (Mack,1968). Interestingly, cholecystokinin- and gastrin-like peptides appear to

be equipotent in muscle preparations of the salmon suggesting a more primitive receptor in this species (Vigna, 1977). In mammals, previous authors have shown that CCK-8, CCK-33, and CCK-58 are equipotent for gallbladder muscle contracting activity in the dog (Eysselein, 1983). Gallbladder contraction stimulated by cholecystokinin can be antagonized either transiently or prolonged by the iv bolus or continuous infusion of proglumide in the conscious dog (Fujimura, 1992). Similarly, loxiglumide in the human will abolish the gall bladder response to a meal (Malesci, 1990)

Distribution

Cholecystokinin was originally isolated from hog intestine as a 33 amino-acid peptide (Mutt, 1968). Cholecystokinin-like peptides have been detected in gastrointestinal tissues of invertebrates such as gastropods and crustaceans (Larson, 1983) and vertebrate tissues of the Coho salmon, leopard frog (Vigna, 1979), Dungeness crab (Larson, 1983) and Bluegill (Rajjo, 1988). Cholecystokinin-like peptides were isolated in the brain of the *aplysia californica* (Vigna, 1984), the rainbow trout (Vigna, 1985) and in chickens (Vigna, 1984). In mammals, cholecystokinin-like peptides have been found in the central and the enteric nervous systems.

CCK-8 appears to be the predominant form of cholecystokinin isolated from human (Reeve, 1984) and rat (Dockray, 1980) brain. Cholecystokinin is present in high concentrations in several brain regions. Cholecystokinin was detected at

levels greater than 4000 pmoles/gram tissue in the cerebral cortex, hippocampus, amygdala, septum, olfactory tubercle. Moderate levels were found in the arcuate nucleus, hypothalamus, median eminence but were nondetectable in the caudate nucleus and cerebellum (Crawley, 1985). Cholecystokinin levels in the cortex are approximately four times higher than in the jejunum (Dockray, 1980). Layer I neurons, bipolar cells and either multipolar or bitufted dendritic trees of the cortex have been shown to have CCK-like immunoreactivity as examined by an immunoperoxidase technique and electron microscopy (Peters, 1983). Cholecystokinin also coexists with several other neurotransmitters including dopamine in meso-limbic neurons (Hokfelt, 1980), oxytocin in the neurohypophysis (Vanderhaeghen, 1981), and substance P in the mesencephalic periaqueductal central gray (Skirboll, 1982).

The major form of cholecystokinin present in human duodenal and jejununal mucosal extracts was sequenced and shown to be CCK-58 (Eysselein, 1990). The sequence is identical to that predicted from the cDNA of human preprocholecystokinin and suggests an initial cleavage after the basic arginine residue to produce CCK-58. In humans, fifty percent of the released cholecystokinin from proximal small intestinal mucosa was composed of large forms including CCK-58, -39 and -33. The distal portion of the jejunum produced mainly large forms (Calam, 1982). Cholecystokinin of the rat gastrointestinal tract is believed to be similar to human in that CCK-58 has been characterized

from rat small intestine (Turkelson, 1989). In addition, CCK-22 was also purified and sequenced from the rat intestine (Eng, 1984).

Cholecystokinin was first localized to gut endocrine cells using an immunostaining technique with an antibody to CCK-33. Immunostaining appeared in the villi and crypt cells of the human duodenum. Staining occurred in the I cells and the G cells with this antisera that crossreacted with gastrin. Since that time, electron immunohistochemistry has shown that the I cell of the gastrointestinal mucosa produces cholecystokinin and that the G cell produces gastrin (Buchan, 1978). In situ hybridization confirmed that CCK mRNA cells are confined to the small intestine and that these cells do not contain gastrin mRNA (Yabu, 1989). The CCK-8 form predominates in gastrointestinal neurons located in the myenteric plexus and longitudinal muscle of the jejunum representing 15% of the immunoreactivity (Dockray, 1977). The innervation of the gastrointestinal system is quite complex in that some CCK nerves are afferent vagal fibers of the myenteric plexus. Intrinsic neurons are also present in the submucosal plexus that project to the mucosa and myenteric plexus. CCK in the submucosa occurs in nerves that also contain several other transmitters such as somatostatin, substance P, neuropeptide Y and acetylcholine (Schultzberg, 1980).

Tissue Extraction Techniques for Analytical Level Analysis

Previous extraction techniques have led to conflicting radioimmunoassay results. For example, extraction in methanol results in an excellent extraction of

low molecular weight forms of CCK but only a partial extraction of larger forms. This extraction method led previous authors to suggest that CCK-8 is the predominant form of cholecystokinin in the brain. CCK-8s may be modified during extraction either due to partial oxidation of methionine residues to sulphoxide forms or conversion of aspartate residues to aspartoyl configurations (Marley, 1984). The oxidation of methionine was shown to decrease the activity of CCK markedly (Bacarese-Hamilton, 1985) and thus may alter its binding to the receptor.

Extraction methods such as in methanol or boiling in water at neutral pH result in a high yield of CCK-8 but low extraction of larger cholecysokinin forms. Boiling in water and acetic acid extraction allowed a high recovery of low and high molecular weight forms of CCK. The authors conclude that extraction in dilute acetic acid after boiling is simple and releases all forms and fragments without significant damage to the peptide (Rehfeld, 1986).

Radioimmunoassay

The amino acid sequence shown in the figure 3 is that of rat pro-CCK. The enclosed or bracketed amino acid sequences are the sequences for which each specific antisera were produced against. Sequence analysis of cDNA for canine (Eysselein, 1982), porcine (Mutt, 1971) and human proCCK (Takahashi, 1985) were previously described. Several canine and porcine substitutions occur in these antisera recognition areas. These include an isoleucine substitution for leucine of the I11H recognition area and extensive canine substitutions in the L8D antisera recognition area (Deschenes, 1984). Human substitutions include valine and asparagine substitutions at position 24 and 19 of I11H, a glutamate at position -7 for D10Y, and extensive substitutions in the L8D recognition area (Takahashi, 1985). Interestingly, the G-17 recognition area is highly conserved in most species.

The L8D antisera was produced with the rat sequence: [Leu-Arg-Ala-Val-Leu-Arg-Pro-Asp] and does not detect CCK-8, CCK-33 or CCK-39. Previously the L8D antiserum was shown to be specific for procholecystokinin and CCK-58 and the amino terminal portion of CCK-58 left when CCK-39/33 is cleaved (Beinfeld, 1985).

I11H antisera was generated against [Ile(Leu)-Lys-Asn-Leu-Gln-Ser(Gly)-Leu-Asp-Pro-Ser-His] of the porcine sequence (rat sequence) and does not detect CCK-8, CCK-4, gastrin, peptide YY, rat pancreatic polypeptide, vasoactive intestinal polypeptide, growth hormone releasing hormone, gastric inhibitory

peptide, secretin, or motilin tested to 1 ug/ml (Han, 1987). The porcine sequence substitutions include an aliphatic side chain switch of Ile instead of Leu at the position corresponding to amino acid 24 and an aliphatic hydroxyl group of Ser for glycine corresponding to amino acid 19. The I11H antisera displays a 50% molar crossreactivity with CCK-33, CCK-39, and CCK-(1-21) and may also recognize the amino terminal segments of CCK-39/33 without the CCK-8 sequence (Han, 1987).

The D10Y antiserum #1578 was generated against the following synthetic peptide: Asp-Phe-Gly-Arg-Arg-Ser-Ala-Glu-Asp-Tyr corresponding to the rat sequence (Beinfeld, 1985). The D10Y antisera does not detect CCK-8s, CCK-30, CCK-33, CCK-39, human gastrin I, or CCK-4. Major peaks of D10Y-LI immunoreactivity in rat brain extracts upon sephadex gel filtration were similar in size to CCK-33 and slightly larger than CCK-8. D10Y immunoreactive peptides contain a minimum of Gly-Arg-Arg on their C-terminal extension because treatment of synthetic D10Y or CCK-33 carboxyl-extended forms (from sephadex columns) with trypsin eliminated their ability to cross-react with the antiserum (Beinfeld, 1985). The D10Y antisera detects carboxy-extended CCK forms: those which require further processing by carboxypeptidase H and amidation by peptidyl-glycine-α-amidating monooxygenase to produce the bioactive CCK-8s peptide (Allard, 1985).

The R5 or G17 antiserum was generated against synthetic CCK-8s (Beinfeld, 1981). G17 antisera has weak crossreactivity with nonsulfated CCK-8 (2%) and

no crossreactivity towards oxytocin, vasopressin, α-MSH, Met-enkephalin, TRH, somatostatin, substance P or VIP (Beinfeld, 1981). In our laboratory, the G-17 antisera recognized only 40% of gastrin, 47% of CCK-33, 64% of CCK-(30-33) and 70% of CCK-8s at the 1000 pg/tube level of the standards tested.

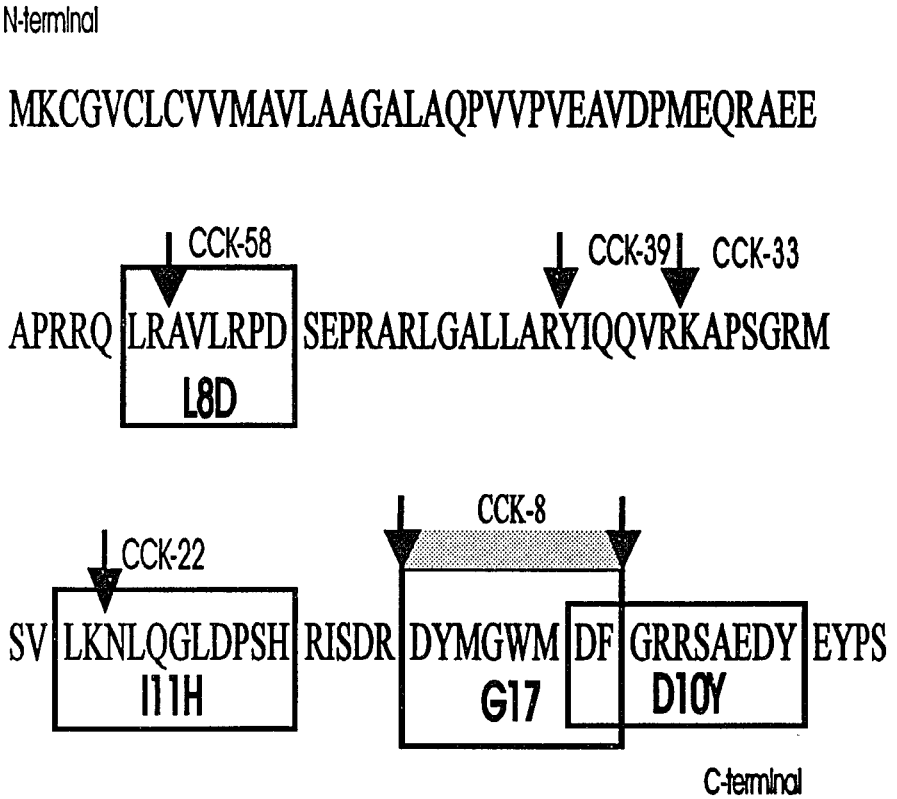


Figure 3. Antisera Recognition Sites on Cholecystokinin

Receptors

Cholecystokinin is capable of binding to two different types of receptors, the CCK-A receptor found predominantly in peripheral tissues and the CCK-B/gastrin receptor found predominantly in the brain.

Research has been undertaken to determine the specificity of CCK receptors with the use of new agonists and antagonists. Studies with conformationally constrained peptides have shown that when the CCK-A receptor interacts with a peptide the bound peptide is in a linear conformation. When CCK-B receptors interact with the peptide a bent or folded configuration of the bound peptide exists (Roy, 1990). In addition, the replacement of the aspartate with an N-methyl derivative in position 32 of CCK-33 has led to a more selective agonist for the CCK-A receptor. The agonist appears to have similar potency to that of CCK-8 (Lin, 1990) suggesting the ability to manipulate large forms of cholecystokinin to alter the specificity and possibly the response at particular receptors. Several classes of CCK receptor antagonists include: cyclic nucleotides, amino acid derivatives (e.g., proglumide), C-terminal analogues of cholecystokinin, and benzodiazepine derivatives (Miller, 1991).

The ability to isolate and clone these receptors has been complicated because no tissue is particularly rich in cholecystokinin receptors. This has previously led to the classification of cholecystokinin receptors based upon biological activity, binding activity and their cellular localization based upon morphology. The structural basis for varied specificity of agonists at these receptors has begun

since the cloning and sequencing of the receptors from cDNA. The CCK-A receptor has been cloned from the rat pancreas (Wank, 1992), the CCK-B receptor from the rat brain (Wank, 1992), human brain and stomach (Pisegna, 1992), and the gastrin receptor from a canine parietal cell library (Kopin, 1992). A comparison of the amino acid sequences for these receptors has presently shown that the rat CCK-B receptor is 90% homologous to the canine gastrin receptor. These isoforms and are now designated as a CCK-B/gastrin receptor.

CCK-A Receptor

CCK-A receptors have been found on the pancreatic acinar cell and islets, the gallbladder muscularis smooth muscle, pyloric smooth muscle, neurons in the lower esophageal sphincter and myenteric plexus. Along the gastrointestinal tract, CCK-A receptors have been found along the smooth muscle and endocrine cells. CCK-A receptors are also transported from the vagus nerve peripherally to the pyloric sphincter and vagal afferent mucosal fibers in the corpus, antrum or duodenum (Moran, 1990). Although thought of as a peripheral cholecystokinin receptor, the CCK-A receptor has been found in certain brain nuclei. Central CCK-A receptors were detected in the interpeduncular nucleus, the area postrema, the nucleus tractus solitarious (Miller, 1991) and in nuclei of the hypothalamus (Woodruff, 1991).

These CCK-A receptor "sites" have been characterized by the ability of the tissue to respond to different forms of cholecystokinin. In general, the CCK-A

receptors have relative affinities for CCK-8 which are 500 times that for CCK-8 nonsulfated and up to 10,000 times that for CCK-4 (Miller, 1991). Previous authors have shown that the minimum requirement for pancreatic acinar cell stimulation is the C-terminal heptapeptide including the sulfated tyrosine (Asp-Tyr-Met-Gly-Trp)(Jensen, 1989). Thus, the CCK-A receptor binding affinities appear to follow the following profile: CCK-8>CCK-8 non-sulfated>>CCK-4. CCK-A receptors in peripheral tissues may be positively coupled to phosphoinositide hydrolysis (Florholmen, 1989).

CCK-B /Gastrin Receptor

CCK-B receptors have primarily been found in the brain and predominate throughout the cerebral cortex, nucleus accumbens, amygdala, hippocampus and olfactory bulbs (Hill, 1990). In the periphery, the CCK-B receptor is suggested to be on gastric and enteric smooth muscle from some species (Menozzi, 1989).

These receptors have relative affinities for CCK-8 which are approximately ten times that for nonsulfated CCK-8, gastrin and CCK-4 making it less selective than the CCK-A type of receptor (Miller, 1991). The minimal requirement for the CCK-B receptor appears to be the C-terminal tetrapeptide (Jensen, 1989). The profile for agonists based upon relative affinities for the CCK-B receptor appear to be CCK-8 > CCK-8 nonsulfated = gastrin = CCK-4. CCK-B receptors may be coupled to adenylate cyclase via an inhibitory G protein (Studler, 1986). The CCK-B receptor isolated from the human brain has been shown to augment

phosphoinositide hydrolysis and cytosolic free calcium levels in chinese hamster ovary cells (Ito, 1993).

Carboxypeptidase H (EC 3.4.17.10)

Carboxypeptidase H, a neuropeptide processing enzyme, has been localized in the storage granules of peptide containing secretory vesicles (Lynch, 1990). CPH removes basic amino acids such as lysine or arginine after initial cleavage by a trypsin-like enzyme. Carboxypeptidase H processes propeptides within the granules to produce bioactive forms such as CCK-8s, that may be released into the synapse. Carboxypeptidase H is tightly associated with membranes at pH 5.5 (> 90%) (Fricker, 1990) and has highest activity at this pH corresponding to the environment inside storage granules. Thus, our carboxypeptidase H activity data is considered membrane bound (>90%) since samples were prepared and analyzed for activity in pH 5.5 buffer.

Distribution

Carboxypeptidase H purified from rat brain showed highest activity in thalamus/hypothalamus, lower activity in cerebral cortex and very low activity in the cerebellum when incubated with dansyl-Phe-Leu-Arg (Fricker, 1982). In the stomach and colon, binding with ^{3}H-GEMSA, a specific inhibitor of carboxypeptidase H has shown highest concentrations in the mucosa, lower levels in the muscle layer and low binding in the submucosa (Lynch, 1987).

Specificity

Carboxypeptidase H is also referred to as enkephalin convertase in the literature because of it's ability to process enkephalin precursors (Hook, 1982). Carboxypeptidase H is involved in the processing of prohormones such as proinsulin (Docherty, 1983), proopiomelanocortin, provasopressin and others (Gainer, 1985) into smaller biologically active forms.

Here, carboxypeptidase H activity was analyzed by the scintillation method of Stack, Fricker and Snyder (Stack, 1984) and is illustrated in figure 4. This radiometric assay uses [Benzoyl-2,5-^{3}H(N)]-Phe-Ala-Arg-OH as the substrate. The K_m of the substrate for this substrate is 0.06 mM (Rossier, 1989). Guanidinoethane mercaptosuccinic acid (GEMSA) is a specific inhibitor of carboxypeptidase H with a K_i of 8.8 nM for the purified enzyme (Fricker, 1983). The assay is based upon the differential solubility of ^{3}H-labeled substrate and product in chloroform. The substrate, ^{3}H-benzoyl-Phe-Ala-Arg-OH is poorly soluble in chloroform because of the charged arginine. The product ^{3}H-benzoyl-Phe-Ala is partitioned from the acidic aqueous phase into the chloroform layer to allow a rapid separation of product from substrate. This assay is 100 times more sensitive than the fluorometric assay utilizing dansyl-Phe-Ala-Arg as the substrate. As little as 25 pg of purified enkephalin convertase can be readily detected in the radiometric assay than the 100 times more required in the fluorometric assay. CPH is remarkably stimulated by Co^{2+} while other CPB-like enzymes are not.

GEMSA is a potent active site directed inhibitor, displaying a K_i value for purified enkephalin convertase of 5-10 nM while its influence on other carboxypeptidase B-like enzymes is several fold weaker. With maximally inhibiting concentrations of GEMSA the same level of activity in the presence and absence of cobalt is obtained.

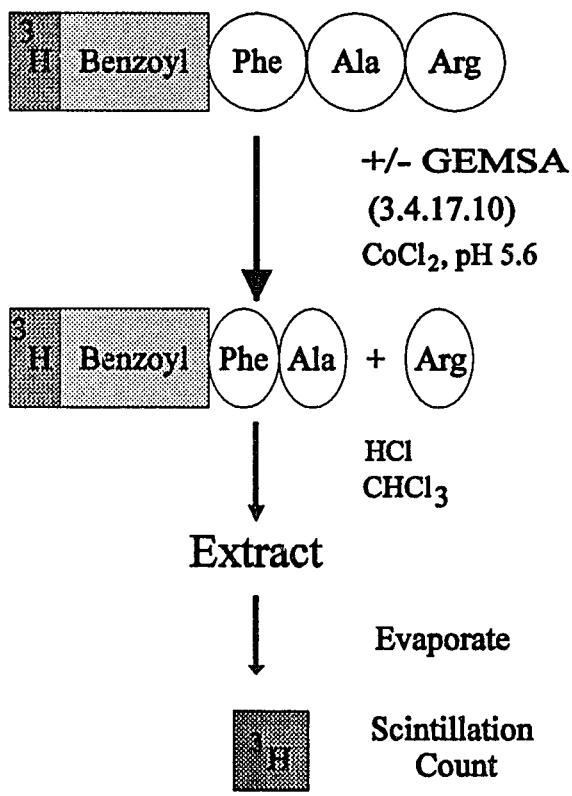


Figure 4. Carboxypeptidase H (EC 3.4.17.10) Assay

Neutral Endopeptidase (EC 3.4.24.11)

Neutral endopeptidase (NEP), also known as "enkephalinase" is a membrane-associated ectoenzyme that cleaves peptides on the amino side of hydrophobic amino acids. NEP is highly localized to the particulate fraction, namely synaptic membranes (Alstein, 1980). NEP has been shown to be an integral membrane protein containing a hydrophobic segment that may function as a transmembrane region with the bulk of the protein being found extracellularly (Letarte, 1988). For cholecystokinin, neutral endopeptidase cleaves at the Asp^{7}-Phe^{8} bond and also produces an active CCK-4 fragment by cleavage at the Gly^{4}-Trp^{5} bond (Bunnett, 1988; Zuzel, 1985).

Distribution

Binding studies with ^{3}H-HACBO-Gly, a selective inhibitor of NEP, showed moderate levels of binding in the cortex, the molecular layer of the cerebellum and the hypothalamus (Waksman, 1986). Fluorescent histochemical methods have shown high NEP activity in the cerebral cortex and other regions (Back, 1989). The distribution of NEP was determined by measuring fragments produced/hr/mg protein when incubated with CCK-8. This showed highest activity in the striatum followed by hypothalamus and low levels in the frontal cortex and cerebellum (Deschodt-Lankman, 1984). NEP mRNA was expressed by the epithelium and muscle layers of the gastrointestinal tract. Highest levels of expression are by the epithelial cells of the small intestinal brush border (Bunnett, 1991).

Specificity

Neutral endopeptidase cleaves a variety of peptides including the endorphins and enkephalins (Hersh, 1984), cholecystokinin (Deschodt-Lanckman, 1984), substance-P (Matsas, 1983), and atrial natriuretic factor (ANF) (Seymour, 1991). Neutral endopeptidase can cleave CCK-8 into the bioactive CCK-4 form with a K_m and V_{max} of 56 μM and 6 μmol/min/mg for CCK-8 in striatal membranes (Zuzel, 1985).

Neutral endopeptidase activity was analyzed using a modified version of the method of Hersh and Morihara (Hersh, 1986) as illustrated in figure 5. This assay utilizes glutaryl-Ala-Ala-Phe-4-methoxy-2-napthylamide as the specific substrate that is cleaved by NEP with a K_m of 69 μM and V_{max} was 3.5 μmol/min/mg (Bateman, 1987) similar to that for CCK-8 degradation in striatal membranes. The specific inhibitor, phosphoramidon, binds tightly to the active site of NEP at low concentrations with a K_i of 3 nM. In the presence of the substrate, NEP cleaves between the alanine and phenylalanine residues to release a phenylalanine bound chromophore. Aminopeptidase M is then added to the reaction and incubated. Aminopeptidase M cleaves the phenylalanine residue attached to the chromophore only after the alanine is cleaved by NEP. The absorbance of the free liberated product, 4-methoxy-2-napthylamine was read at 240 nm excitation and 425 nm emission.

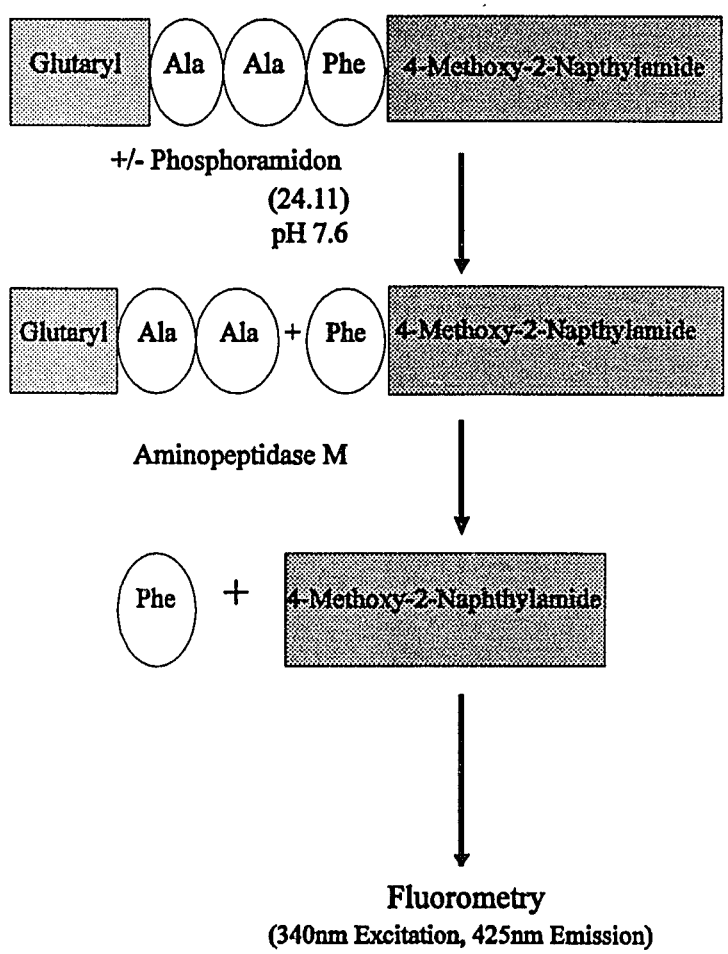


Figure 5. Neutral Endopeptidase (EC 3.4.24.11) Assay

Metallo Endopeptidase (EC 3.4.24.15)

The membrane-associated form of the metabolic enzyme, metallo endopeptidase represents approximately 20-25% of the total activity in brain homogenates and is associated with brain particulate fractions and synaptosomes (Acker, 1987). Metallo endopeptidase preferentially cleaves peptide bonds with high specificity when an aromatic amino acid such as phenylalanine is present to the right of the cleaved bond (Orlowski, 1983). The cleavage site leading to degradation of CCK includes cleavage of the Trp^5-Met^6 bond by metallo endopeptidase.

Distribution

The regional distribution of metallo endopeptidase in rat brain was determined in crude homogenates. Highest specific activity was found in cerebellum, followed by lower activity cortex and hypothalamus (Chu, 1985). To date, metallo endopeptidase levels have not been determined in the gastrointestinal tract.

Specificity

Metallo endopeptidase degrades several neuropeptides such as bradykinin (McDermott, 1987), leutenizing hormone releasing hormone (Molineaux, 1988), cholecystokinin (Malesci 1980), and others (Chu, 1985). CCK-8s was cleaved to produce the active fragment (i.e., CCK-4) and other inactive fragments by

peptidases present in synaptosomal fractions of cortex and hypothalamus (Deschodt-Lanckman, 1984).

The metallo endopeptidase assay employed the substrate Benzoyl-Gly-Ala-Ala-Phe-pAB for membrane samples based on the method of Orlowski, Michaud and Chu (Orlowski, 1983) and is depicted in Figure 6. The specific inhibitor N-[1-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB has a K_i of 27 nM for metallo endopeptidase (Orlowski, 1988). The substrate, although having a K_m of only 0.49 mM has good solubility and is not as difficult to synthesize as other substrates (Orlowski, 1983). Incubation of the substrate with regional samples containing metallo endopeptidase resulted in the cleavage between the glycine and arginine residues. The fragment Ala-Ala-Phe-pAB was further incubated with aminopeptidase M to cleave the alanine and phenylalanine residue from the fragment. The released p-amino benzoic acid and p-amino benzoic acid standards were diazotized with the addition of sodium nitrite, ammonium sulfamate and N-(1-napthyl)-ethylenediamine and read on a spectrophotometer at 555 nm. Sample values were calculated from the standard curve and metallo endopeptidase activity expressed as pmoles/mg protein/min.

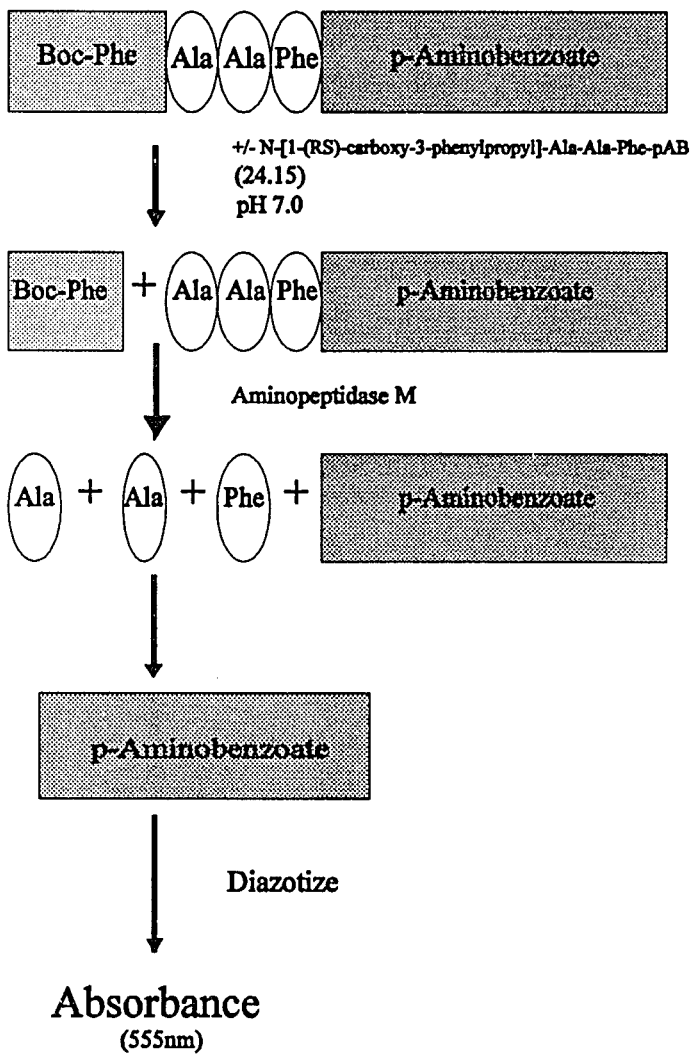


Figure 6. Metallo Endopeptidase (EC 3.4.24.15) Assay

MATERIALS AND METHODS

Chemicals and Reagents

Benzoyl-Gly-Ala-Ala-Phe-pAB and N-[1-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB were synthesized in the laboratory (Orlowski, 1983). Phosphoramidon, p-aminobenzoic acid, normal rabbit serum, goat antirabbit, polyethylene glycol (8000 mw), and CCK-8 (nonsulfated) were purchased from Sigma Chemical Co. (St. Louis, MO). Glutaryl-Ala-Ala-Phe-4-methoxy-2-napthylamide was purchased from Enzyme Systems Products (Livermore, CA). [Benzoyl-2,5-^{3}H(N)]-L-Phe-Ala-L-Arg-OH was purchased from Dupont Chemicals (Wilmington, DE). Guanidinoethane mercaptosuccinic acid (GEMSA) was purchased from Calbiochem (San Diego, CA). I11H (or CCK-PZ[10-20]), CCK-(1-21), CCK-(3-8), CCK-8s and Human Gastrin I were obtained from Bachem (Torrance, CA). CCK-33 (porcine) and CCK-tetrapeptide (30-33) were purchased from Peninsula (Belmont, CA). D10Y, L8D, Y9D, and antisera #1578, 67273, 1940, and R5 were generated in the lab of M.C. Beinfeld (St. Louis University Medical Center, St. Louis, MO).

Dissection Procedure

Young rats were obtained from the breeding colony of the Division of Animal Resources at the University of Arizona. Ninety day old male Sprague Dawley rats were purchased from Harlan (Indianapolis, IN) and allowed to acclimate for one

week prior to sacrifice. When necessary, litters were culled to ten pups on the second day after birth. Littermates were housed together in individual cages with a 12 hour light-dark cycle (6:00 a.m. - 6:00 p.m.). Ninety day old rats were housed four per cage, fed standard lab chow (Ralston Purina Inc., St. Louis, MO) and water ad libitum. Rats were sacrificed by decapitation at postnatal ages P0, P4, P7 and P30. Adult or P90 rats were anesthetized with ether before sacrifice. Decapitation is suggested to cause an abrupt release of neurotransmitters that may alter our findings. Our studies were not concerned with quantifying released forms but were mainly involved in the alteration of processing enzymes and levels of cholecystokinin forms found in tissue with age. Decapitation should therefore not present a problem. It is possible, however, that treatment of only the adult rats with ether prior to sacrifice may affect the levels of peptides in the brain. If this were the case, we would expect to find alterations of al peptidase activity and/or all peptide levels from P30-P90 that was not the case.

The brain was removed and immediately placed on ice. A 1-2 mm slice corresponding to plate #27 (Paxinos, 1986) was dissected. Hypothalamus and cortex were dissected from this slice and the cerebellum was removed from the rest of the brain. The samples were frozen on dry ice in preweighed tubes and pooled 10, 7, 5, 3, and 1 for ages P0, P4, P7, P30 and P90, respectively with an n of 5. Samples for enzyme activities consisted of 52, 46, 52, 47, and 100% males at ages P0, P4, P7, P30 and P90 respectively. Radioimmunoassays of cholecystokinin levels have previously shown sexual variation. Samples used for

radioimmunoassay of the brain regions consisted of 84, 92, 80, 80, and 100% male for ages P0, P4, P7, P30 and P90, respectively.

The abdomen was opened by a midline incision and the gastrointestinal tract removed. The stomach was separated and the entire small intestine from the ligament of Treitz to the cecum was dissected. Once the duodenum was removed, the small intestine was placed in an S-configuration and trisected. The more proximal region was designated as the jejunum, followed by the midjejunum and the more distal region as the ileum. The pancreas and mesenteric fat were removed from each of the tissues. Stomach samples were rinsed in 25 ml of iced normal saline prior to freezing. Remaining tissues were flushed with varying volumes of iced normal saline and equal volumes of air. Small intestinal tissues of day 0 animals were not flushed because of difficulty in handling. No contamination of our samples by CCK present in maternal milk occurred since pups were taken prior to suckling. P4, P7, P30 and P90 samples were flushed with 1, 3, 6, and 10 mls, respectively. The samples were frozen on dry ice in preweighed foil and pooled (3 for P0, P4, and P7). Gastrointestinal samples were not pooled for P30 or P90 since the amount of tissue per region was adequate for enzyme and radioimmunoassay analyses. Each age consisted of an n of 5. Samples for enzyme activities consisted of 67-100, 67, 67, 100 and 100 % male for gastrointestinal regions at ages P0, P4, P7, P30 and P90 respectively. Samples used for radioimmunoassay consisted of 100% male.

Sample Preparation for Enzyme Analysis

The frozen brain sections and gastrointestinal samples were thawed on ice and homogenized for 30 seconds or 1 minute with a Tekmar Tissumizer (50% output; Cincinnati, OH) in 1 mM Tris HCl, pH 7.4 buffer to produce a 4% weight/volume homogenate. Samples were sonified for 30 seconds on a Branson Sonifier 450 (90% duty cycle, 3.0 output; Branson Ultrasonics Corp., Danbury, CT). The homogenates were lysed at 0°C for 30 minutes. Half the volume of 50 mM Tris HCl, pH 7.4 was added to aid in pellet formation. Samples were homogenized and sonified for 10 seconds. For carboxypeptidase H activity analysis, the tissues were processed in 1 mM and 50 mM sodium acetate, pH 5.5 up to this point. Samples for neutral endopeptidase and metallo endopeptidase activity analysis were then centrifuged in a Sorvall Superspeed RC2-B (Newton, CT) at 49,000 G using an SM24 rotor for 45 minutes. The supernatant was decanted and saved. The pellet was resuspended to a 4% volume with 50 mM Tris HCl, pH 7.4 and sonified for 10 seconds followed by centrifugation as above. The supernatant was saved and the pellet was resuspended in 50 mM Tris HCl buffer, pH 7.4 to an approximately 1.0 mg/ml concentration. Aliquots of homogenate and resuspended membranes were analyzed for protein by the Lowry Method (Lowry, 1951).

Enzyme Analysis

Carboxypeptidase H Activity

Samples were diluted in sodium acetate, pH 5.6 such that 10 μg of homogenate protein/tube was analyzed for carboxypeptidase H activity for both brain and stomach, jejunum, and midjejunum samples. The midjejunum required analysis at 5 μg and the ileum at 20 μg protein/tube. Samples were assayed in triplicate with an inhibitor control. Carboxypeptidase H activity was expressed as pmoles/mg protein/min.

Neutral Endopeptidase Activity

Membrane protein was resuspended in Tris HCl, pH 7.4 to yield 20 μg protein/assay tube for brain samples. Gastrointestinal samples required differing amounts of protein/tube for analysis: 2 μg for duodenum, jejunum and midjejunum; 5 μg for ileum and 10 μg/tube for stomach. Absorbance of the free liberated product, 4-methoxy-2-napthylamine was read on an Aminco/Bowman Spectrofluorometer (American Instruments, Silver Spring, MD) at 340 nm excitation and 425 nm emission. Neutral endopeptidase activity was calculated in nanomoles product/mg protein/min from standard curve fluorescence response after subtracting phosphoramidon controls.

Metallo Endopeptidase Activity

Triplicate brain sample tubes contained 50 μg protein. Gastrointestinal samples ranging from 1-50 μg protein/tube were analyzed. A single inhibitor sample contained the specific inhibitor, N-[1-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB. Samples and p-amino benzoic acid standards were diazotized and read on a Beckman Model 25 spectrophotometer (555 nm). Sample values were calculated from the standard curve and metallo endopeptidase activity expressed as pmoles/mg protein/min.

All gastrointestinal samples resulted in very high activity with no difference between inhibited and uninhibited samples except for the stomach region. It was suggested that the small intestine may contain enzymes capable of cleaving our inhibitor or that trypsin/chymotrypsin may be responsible for cleavage of our substrate. The highest concentrations of inhibitors of trypsin/chymotrypsin were used to inhibit these enzymes. Aprotinin at 2 μg/ml and Bowman Burk's Inhibitor at 100 μg/ml were not capable of inhibiting the enzyme responsible for cleavage of our substrate in the small intestinal samples. To date, this enzyme responsible for cleavage of our substrate (or the inhibitor) is present in the small intestine, and is not inhibitable with either our specific metallo endopeptidase inhibitor or the trypsin/chymotrypsin inhibitors. Determination of the enzyme's identification requires further analysis. Thus, metallo endopeptidase activity in the enteric nervous system cannot be reported.

Sample Extraction for Radioimmunoassay

The method of extraction was based on the findings by Marley and Rehfeld (Marley, 1984) in which boiling and acetic acid extraction allowed high recovery of low and high molecular weight forms of CCK. Extraction of ^{125}I-I11H from various tissues allowed a 91% recovery from cortical, 66% from stomach (high loss found in the pellet), and 80% recover from duodenum and jejunum samples using the following extraction procedure. Brain samples and gastrointestinal were weighed and 10 ml/g brain tissue or 5 ml/g gastrointestinal tissue of distilled/deionized water was added. Samples were boiled immediately for 25 minutes and cooled on ice. Samples were homogenized for 30 seconds (brain) or 1 minute (gastrointestinal) on a Polytron Homogenizer (Brinkman Instruments, Westbury, NY, setting 5) and sonified for 30 seconds (duty cycle 50%, output 3). Samples were then brought to pH 2.5 with glacial acetic acid and centrifuged for 20 minutes at 15,000 x g. The supernatant was decanted and neutralized with 10 mM NaOH, sonified for 10 seconds and a 50 µl aliquot was set aside for protein analysis. Dilutions of the supernatant were made to produce 15, 50 and 75 µg in 200 µl for the RIA analysis of hypothalamic, cortical and cerebellar samples. For RIA analysis of gastrointestinal samples, samples were diluted to 15, 75, 10, 20 and 75 µg protein in 200 µl for stomach, duodenum, jejunum, midjejunum and ileum samples.

Samples were also pooled for HPLC/RIA analysis. Pooled samples were lyophilized, brought up in 1.0 ml 0.1 M acetic acid, and an aliquot saved for protein analysis. Cortical and cerebellar samples (approximately 0.5 mg/ml), hypothalamic samples (0.1 - 0.3 mg/ml), and gastrointestinal samples (0.5 - 1.0 mg/ml) were injected onto a Vydac (Hesperia, CA) analytical 5 mm C18 column at 37*C. Samples were separated on a reverse phase gradient using a Perkin Elmer (Norwalk, CT) series 4 chromatograph. Sample components were detected using a Perkin Elmer model LC-95 UV detector set near the isobestic point (215 nm) at 210 nm. Chromatograms were integrated with a Perkin Elmer model LCI-100 integrator. The samples were eluted on a linear gradient of 7-57% acetonitrile/water/TFA (70:29:1, v/v) vs 0.1% TFA for 70 minutes at a flow rate of 1 ml/min. TFA was added to increase solubility of the proteins and to minimize baseline drift while remaining transparent to the detector (Winkler, 1989). Two ml fractions were collected, lyophilized on a Savant Speed-Vac lyophilizer (Farmingdale, NY), brought up in 1 ml RIA buffer and 200 ml portions were analyzed by RIA. Standards were also prepared for HPLC/RIA and included: I11H, CCK(1-21), Human Gastrin I, CCK-33 (porcine), CCK-(26-29)s, CCK-tetrapeptide (30-33), CCK-(3-8), CCK-8s, CCK-8 nonsulfated, L8D and D10Y. Two to five mg of each standard were injected onto the HPLC separately and in combination. Blank samples were injected onto the column after every second brain sample and after each gastrointestinal sample to reduce alteration of

retention time. Typical recoveries for brain samples as determined by the addition of a known concentration of CCK-(26-29)s were greater than 90% for brain and small intestinal samples. Recovery for stomach samples ranged from 80% for younger rats to 50% for adult samples. A typical HPLC chromatogram of the combined standards is depicted in Figure 7 indicating the order of elution and retention time for relevant standards.

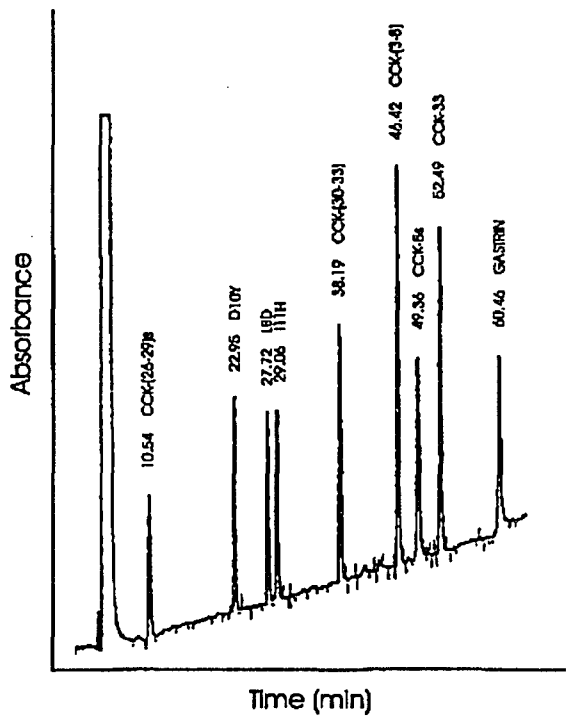


Figure 7. HPLC Chromatogram of Cholecystokinin Standards

Radioimmunoassay

The radioimmunassays were performed in triplicate with 10 mM phosphate buffered saline, pH 7.2 containing 0.25% bovine serum albumin (RIA buffer). Radioactive tracers were prepared using 1 μg peptide, and 1 mg/ml chloramine-T in a potassium phosphate buffer (50 mM, pH 7.4). After 0.5 - 1.0 minutes, buffer containing bovine serum albumin (BSA) was added. The radiolabeled peptide was purified on a C18 column (Beckman ODS column, 4.6 x 250 mm, 0.5 um) and separated from free peptide by reverse phase HPLC. The column was eluted with a 10-40% or 10-50% acetonitrile versus 0.1% TFA gradient in 30 or 40 minutes at a flow rate of 1 ml/minute depending upon the peptide being iodinated. One minute fractions were collected and tested for radioactivity on a Geiger counter. Tubes containing the radiolabeled peptide were dried under nitrogen to 0.5 ml and 0.5 ml of ethanol was added.

The L8D RIA, antiserum 1940 was used at a final dilution of 1: 40,000, ^{125}I-labeled Y9D as the tracer (L8D peptide containing an amino terminal tyrosine), and L8D as the standard. The I11H RIA used antiserum 67273 at a final dilution of 1: 1,200, ^{125}I-labeled I11H as the tracer and I11H as the standard. The G17 RIA used antiserum R5 at a final dilution of 1: 20,000, ^{125}I-labeled Gastrin 17 as the tracer, and CCK-8 as the standard. The D10Y RIA used antiserum 1578 at a final dilution of 1:1125 , ^{125}I-labeled D10Y as the tracer and D10Y as the standard.

Standard curves ranged from 20-1000 pg/tube by serial dilution and the G17 standard curve was extended down to 1 pg/tube. RIA tubes contained 200 µl sample, 100 µl of the appropriate dilution of antisera and 10 µl tracer corresponding to 20,000 cpm/tube. Total binding tubes consisted of 200 µl RIA buffer, 100 µl antisera and 10 µl tracer. Standard curve tubes consisted of 50 µl of the appropriate dilutions of standard, 150 µl RIA buffer, 100 µl antisera and 10 µl tracer. After incubation for 2 days at 4° C, the assay was terminated by addition of 0.1 ml normal rabbit serum, 0.1 ml goat antirabbit and 0.2 ml polyethylene glycol. Following vortex mixing, the samples were centrifuged at 2,000 x g for 20 minutes. The supernatant was aspirated and the pellet counted in a gamma counter for 1 minute.

Radioimmunoassay results on HPLC fractions for a combined standard sample are shown in figure 8 and are expressed as the percentage of total immunoreactivity seen by each specific antisera. CCK-(30-33) or CCK-4 was detected in fractions 20-23 and was immunoreactive with G17. CCK-(3-8) eluted in fractions 24-25 and was immunoreactive with G17. CCK-8s was eluted in fractions 26-29 and was immunoreactive to G17. CCK-33 corresponds to fractions 30-35 and was immunoreactive with G17 and I11H. Gastrin elutes in fractions 35-40 and is immunoreactive to G17 but is not shown on the RIA profile. Other fractions (fr) contained: D10Y [fr 15-20, D10Y-IR], the L8D

peptide [fr 17-23, L8D-IR], CCK-(10-20) also known as the I11H peptide [fr 21-23, I11H-IR] and CCK-(1-21) [fr 28-29, I11H-IR].

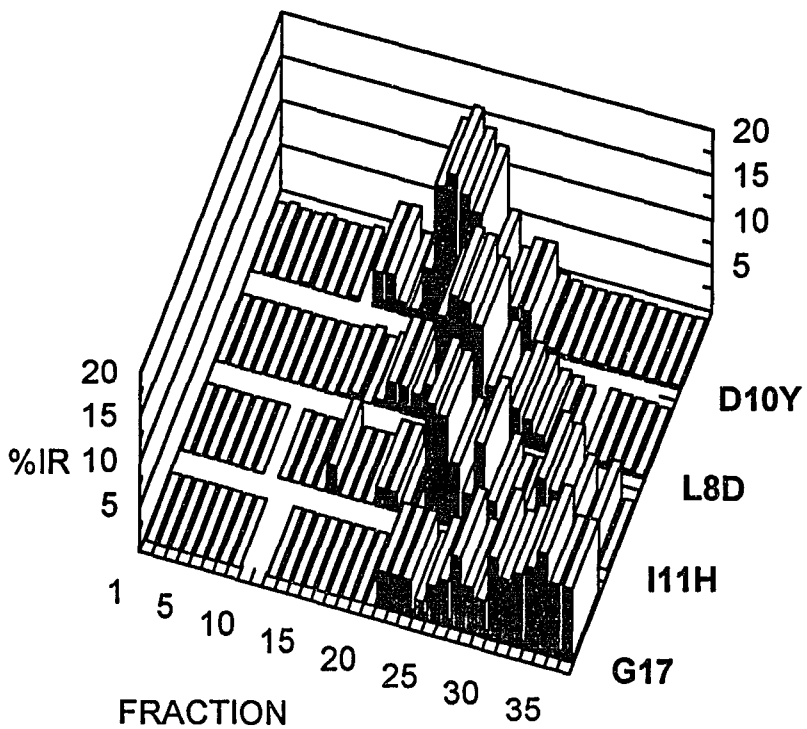


Figure 8. HPLC/RIA of Cholecystokinin Standards

For G17 immunoreactive forms determined in the fractions of pooled samples, fractions corresponding to standards for CCK-4, CCK-8, CCK-33 and gastrin were summed and a percent of immunoreactivity determined. This percent immunoreactivity was then multiplied by the pg/mg of samples analyzed by the G17 antisera that were not fractionated. This method illustrates how these four forms of cholecystokinin relate to the G17-IR seen of the samples not fractionated. Thus, the G-17 immunoreactivity of each fractionated sample is reported as CCK-4, CCK-8, CCK-33 and gastrin concentrations in pg peptide/mg protein.

Statistical Analysis

For enzyme activity and peptide levels the data was analyzed by a Newman-Keuls test at a 95% confidence interval. (The asterisks on the plots represent the significance level of values compared to birth levels. (** $p<0.01$, * $p<0.05$)

RESULTS

Central Nervous System

Enzyme Activity

Carboxypeptidase H and Neutral Endopeptidase activity during development are shown in figures 9, 10 and 11 for the hypothalmus, cortex and cerebellum, respectively. Carboxypeptidase activity is reported in pmoles/mg protein/min and neutral endopeptidase activity is reported as nmoles/mg protein/min.

Carboxypeptidase H activity in the hypothalamus (figure 9) increased 2-fold between postnatal ages P0 and P7, after which time activity levels remained constant through P90. Neutral endopeptidase activity increased 2-fold in the hypothalamus by P7, but decreased to birth levels by P90.

In cortex (figure 10), carboxypeptidase H activity also increased between P0 and P7 but decreased to birth levels by P90. Neutral endopeptidase activity decreased from P0 to P7 then showed a slight rise to adult levels.

Carboxypeptidase H activity in the cerebellum (figure 11) remained constant to P7, then decreased with age until P90. Neutral endopeptidase activity quickly decreased to half of birth levels at age P7 and continued to decrease to on-fifth of birth levels by P90.

Interestingly, CPH activity at birth was expressed at highest levels in the cortex followed by the cerebellum and hypothalamus (4.98, 4.29 and 3.48 pmoles/mg/min). By adult age, the pattern of CPH activity changed to where

hypothalamus contained the highest activity followed by cortex and cerebellum (7.65, 4.79 and 2.76). NEP activity was higher than CPH activity in hypothalamus, cortex, and cerebellum throughout development. Neutral endopeptidase activity was detectable at P0 with highest activity in the cortex followed by hypothalamus and cerebellum (2702, 1658 and 1410). By adult age, the NEP activity profile resembled that for CPH where hypothalamus>cortex>cerebellum (2604, 930 and 304).

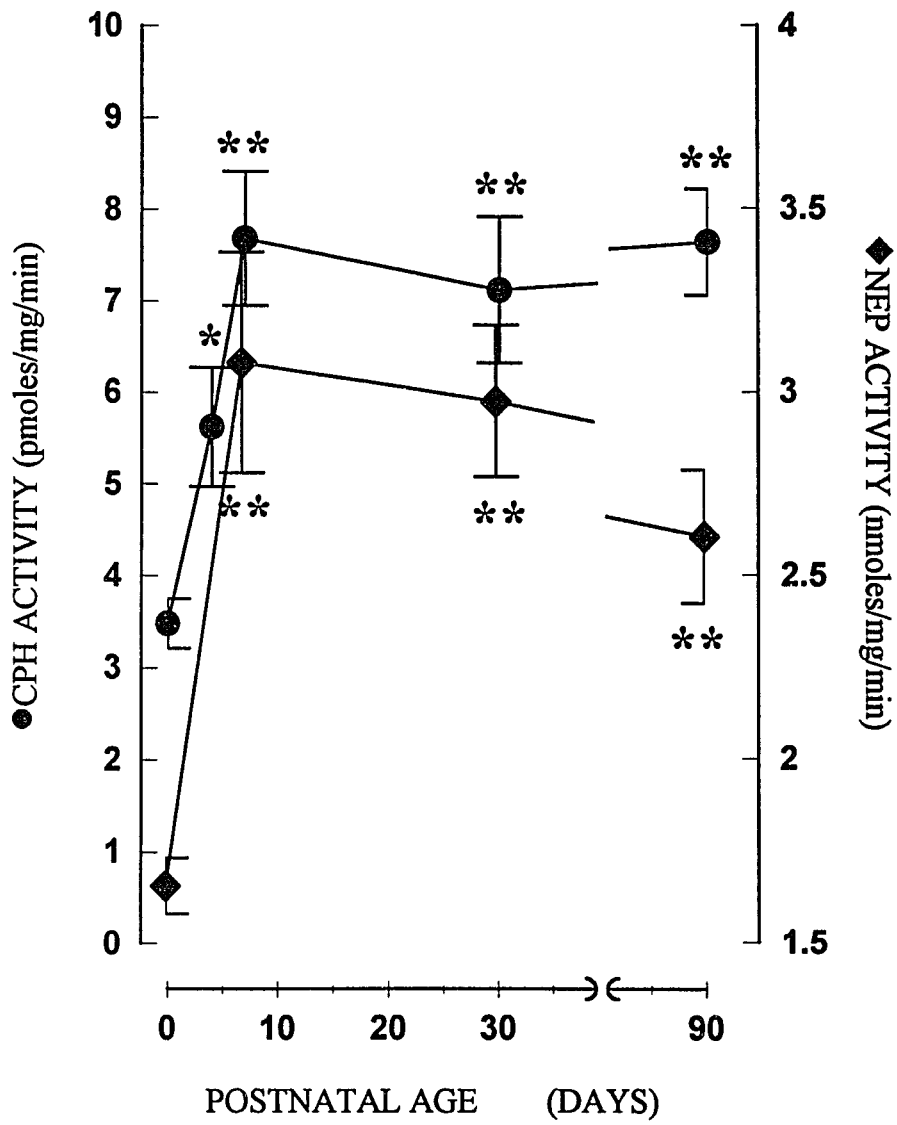


Figure 9. Carboxypeptidase H and Neutral Endopeptidase Activity in the Hypothalamus

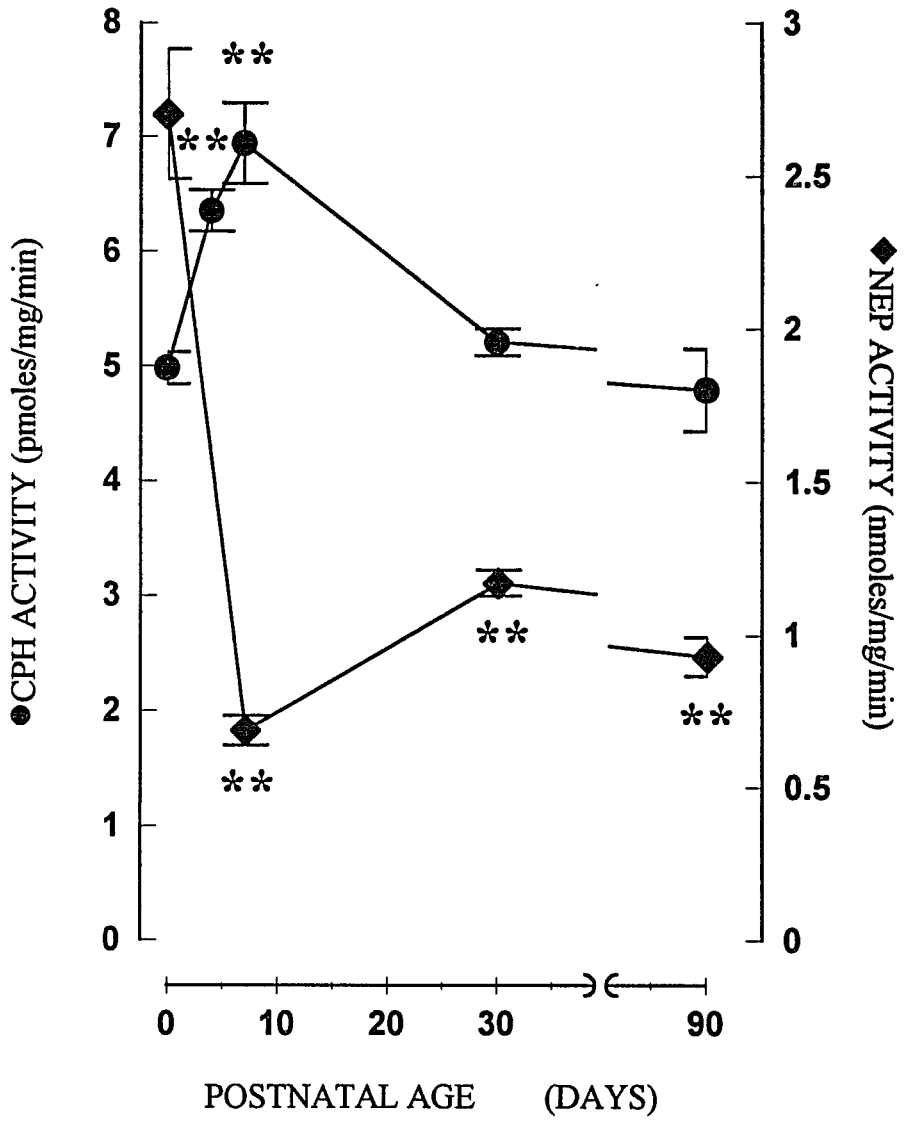


Figure 10. Carboxypeptidase H and Neutral Endopeptidase Activity in the Cortex

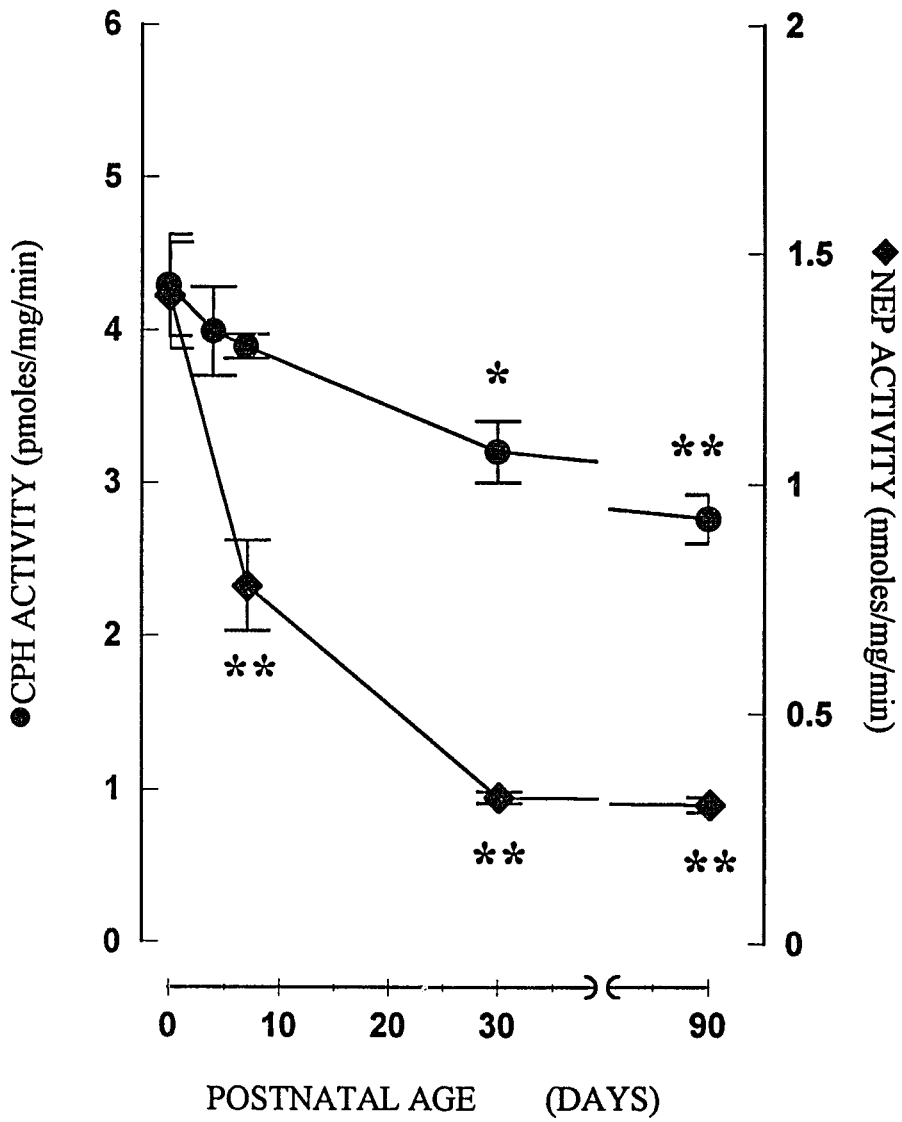


Figure 11. Carboxypeptidase H and Neutral Endopeptidase Activity in the Cerebellum

The developmental pattern for metallo endopeptidase activity is shown in figure 12 and is expressed as pmoles/mg/min. In hypothalamus, activity remained constant from P0 to P30 but significantly decreased by P90. In cortex, activity increased from P0 to P7, decreased to birth levels by P30 and then remained constant. Activity in the cerebellum also remained constant from P0 to P30 but decreased below birth levels at P90.

Metallo endopeptidase activity at birth was highest in the cerebellum followed by cortex and hypothalamus (1138, 946 and 756 pmoles/mg/min). By P90, metallo endopeptidase activity was highest in cortex and cerebellum and lowest in hypothalamus (915, 840 and 481). At adult age, metallo endopeptidase activity was six times lower than that of NEP in the hypothalamus (481 vs 2604 pmoles/mg/min). Metallo endopeptidase activity was similar to NEP activity in the cortex (915 versus 930), and almost three fold higher than NEP in the cerebellum (840 versus 304).

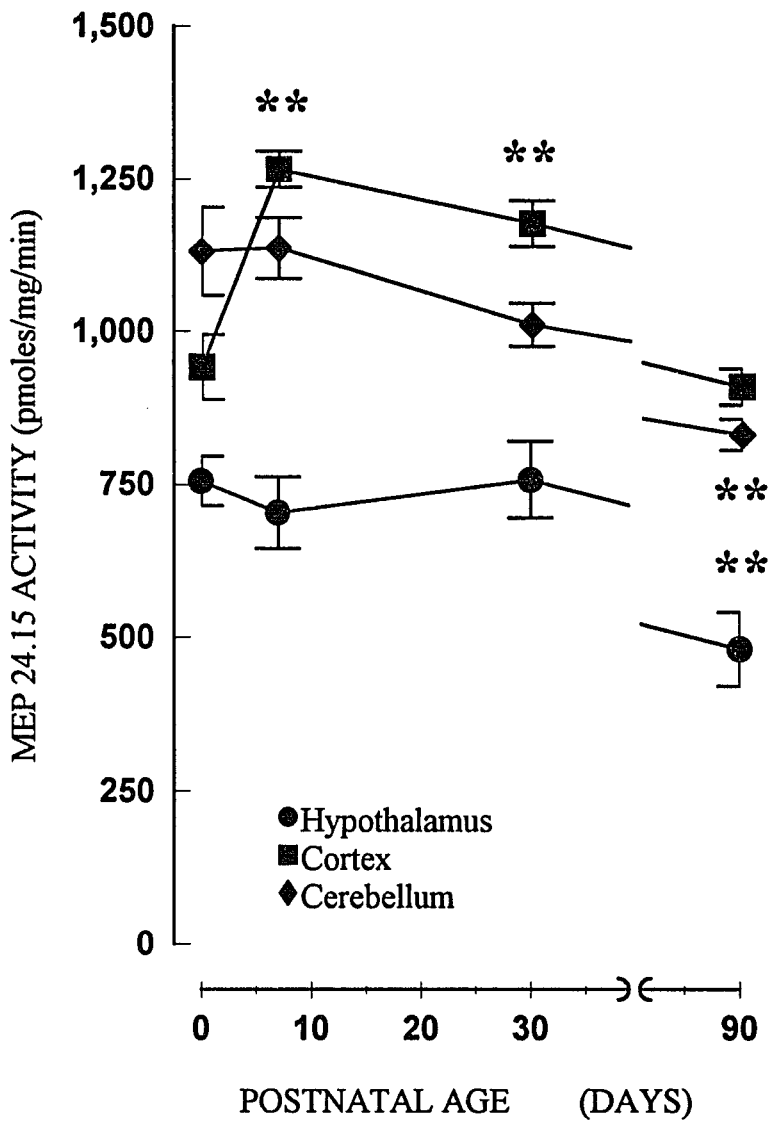


Figure 12. Metallo Endopeptidase Activity in Specific Regions of the Central Nervous System

Radioimmunoassay

CCK like peptides also illustrate regional patterns for levels and forms throughout development, as determined by radioimmunoassay (RIA). CCK-forms with development as detected by radioimmunoassay for hypothalamus, cortex and cerebellum are shown in figures 13, 14 and 15.

In hypothalamus (figure 13), I11H immunoreactivity (I11H-IR) is greater than L8D-IR, D10Y-IR and G17-IR throughout development. Here, L8D-IR and D10Y-IR did not significantly change throughout development whereas I11H-IR increased 3.5-fold above birth levels between P7 and P90 and G17-IR increased 5.5-fold above birth levels in this period. At P90, I11H-IR was higher than G17, D10Y, and L8D immunoreactivities.

In the cortex (figure 14) I11H was the highest immunoreactive peptide found in at all ages examined. L8D-IR remained constant throughout development as it had in the hypothalamus. D10Y-IR in this region increased 2-fold by P7 and then returned to initial levels by P90. I11H-IR remained constant throughout development. G17-IR levels increased after P4 with a 3.5-fold increase over birth levels by P90.

In cerebellum (figure 15), I11H also was the highest immunoreactive peptide throughout development. In this region, I11H-IR and G17-IR did not change with age. D10Y-IR at P0 was 2.8-fold and 5.4-fold higher in this region in comparison to hypothalamus and cortex, respectively. D10Y-IR and L8D-IR decreased between P4 and P90 to almost half the immunoreactivity found at P0.

In the central nervous system, L8D-IR does not change in the hypothalamus and cortex but decreases in the cerebellum with development. At adult age, L8D-IR peptides are present in highest concentrations in hypothalamus with a three times lower concentration in cortex and cerebellum. I11H-IR peptides appear to be produced in high quantities throughout development in all three regions. By adult age, I11H-IR is highest in the hypothalamus, twenty times lower in the cortex and only 10 times lower in the cerebellum. D10Y-IR does not change in the hypothalamus with development, a peak occurs on P7 in the cortex and D10Y-IR decreases in the cerebellum. By adult age D10Y-IR peptides are found in highest concentrations in the cerebellum and hypothalamus compared to cortex. Interestingly, G17-IR increases throughout development of the hypothalamus whereas an initial increase is seen in the cortex but no change in the cerebellum. The level of G17-IR in the adult rat is highest in hypothalamus, three times lower in cortex and almost non-detectable in cerebellum.

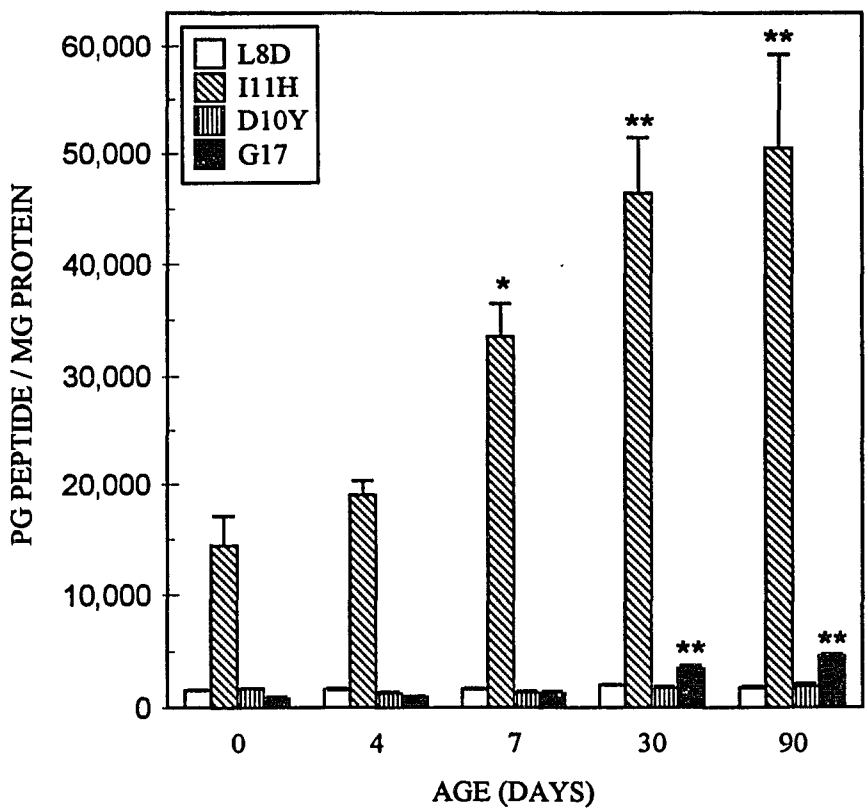


Figure 13. Cholecystokinin-like Immunoreactivity in the Hypothalamus

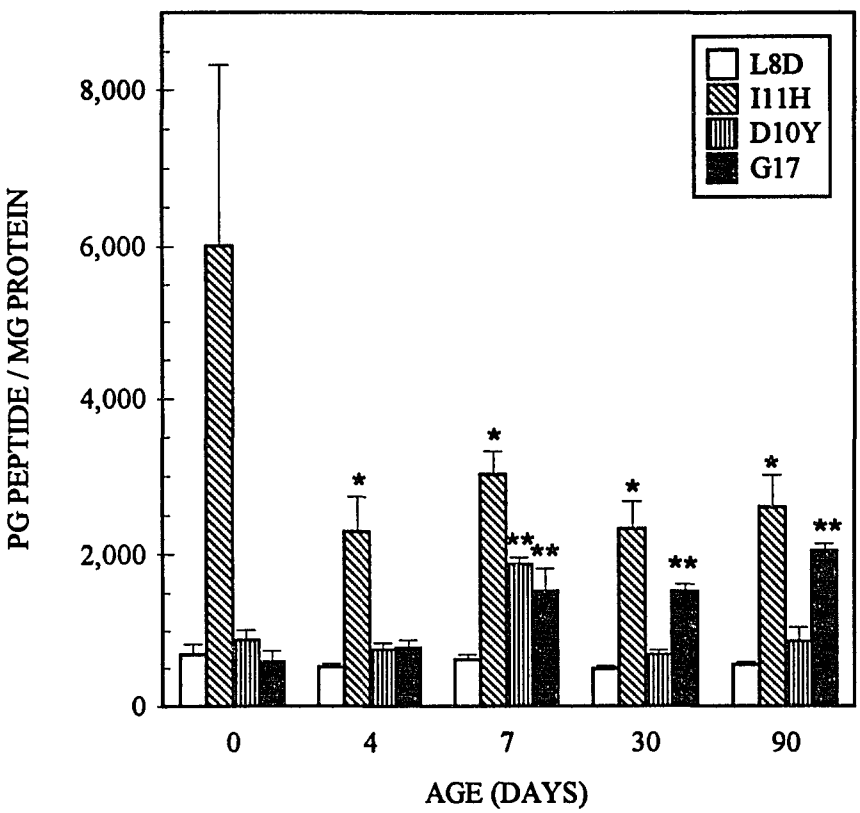


Figure 14. Cholecystokinin-like Immunoreactivity in the Cortex

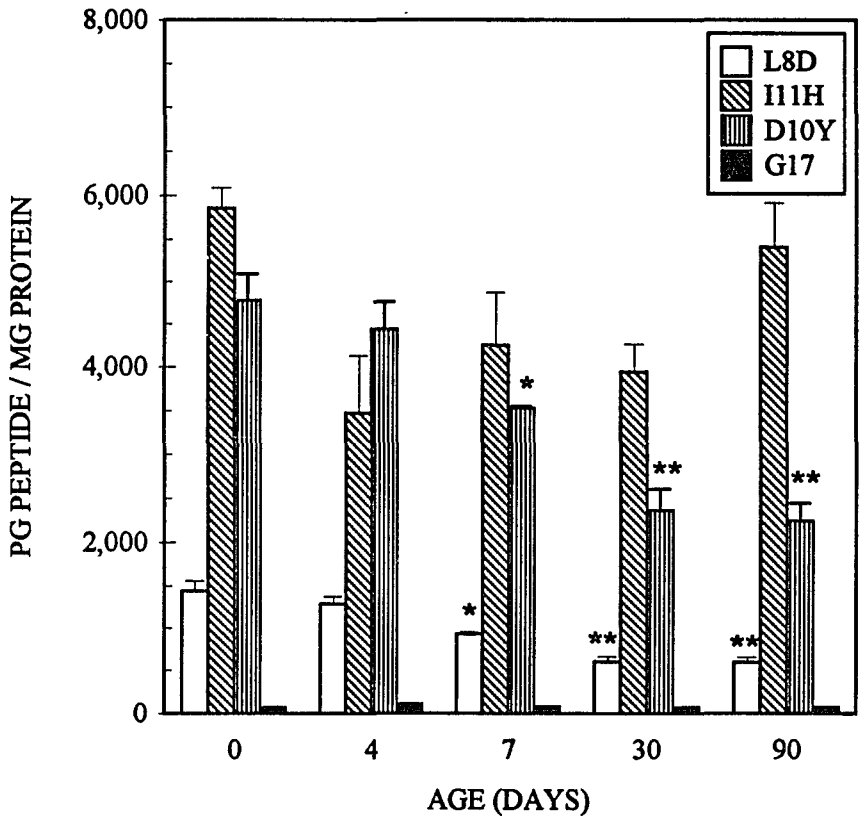


Figure 15. Cholecystokinin-like Immunoreactivity in the Cerebellum

Samples were also pooled and placed over the HPLC and fractions were also analyzed by radioimmunoassay with the G17 antisera. The HPLC/G17-IR analysis of pooled hypothalamic samples at different ages is shown in Figure 19. HPLC treatment of pooled samples followed by radioimmunoassay with the G17 antisera allows us to tease out the immunoreactive forms shown by the figures. No immunoreactive peaks were observed in the first 14 fractions. CCK-(30-33) or CCK-4 eluted in fractions 20-23, CCK-8s in fractions 28-29, and fractions 30-35 represent CCK-33 immunoreactivity. Results were expressed as pg/mg protein to aid in illustrating the role of enzyme activity in relation to peptide levels found.

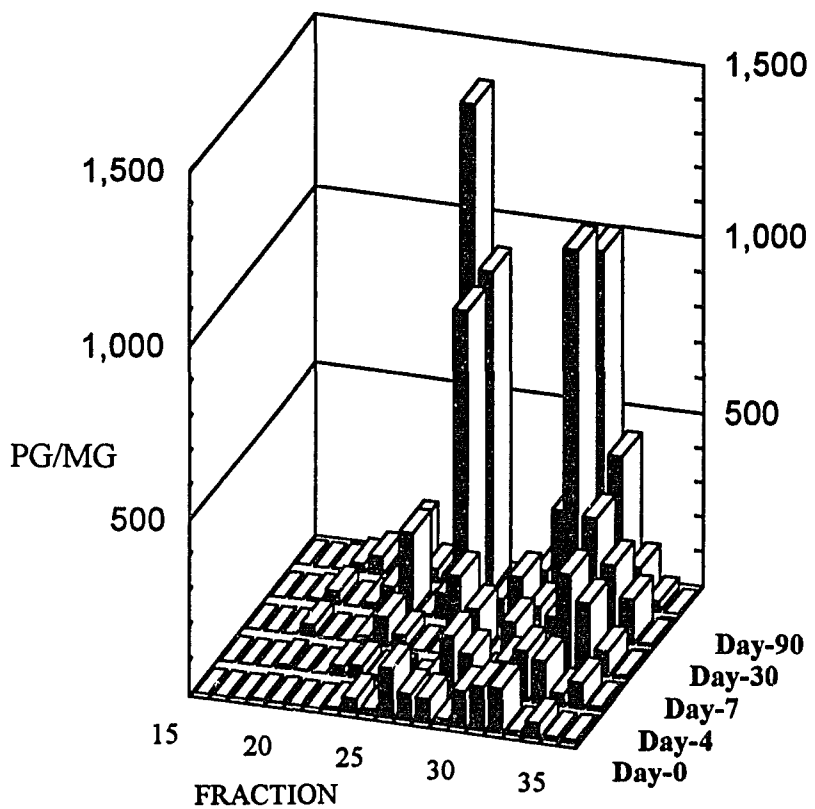


Figure 16. Pooled HPLC/G17-RIA Profile of the Hypothalamus
(G17 Immunoreactivity expressed as pg peptide / mg protein)

The peaks of immunoreactivity shown in Figure 16 were quantified and forms of cholecystokinin determined for each age. The data from HPLC/G17 peaks are shown in Table 1 and include all central nervous system regions studied. An increase of CCK-4, CCK-8 and CCK-33 levels occur in the hypothalamus from P0 to P30. Each peptide then decreases slightly from P30 to adult age. In cortex, CCK-4 levels decrease, CCK-8 levels increase to P30 and fluctuate to adult levels. CCK-33 in the cortex increases from P0-P90. From cerebellum data we may only conclude that levels of CCK-4, CCK-8 and CCK-33 are low.

Table 1. HPLC/G17-IR Forms in the Central Nervous System
(Levels expressed as pg peptide/mg protein)

POSTNATAL AGE	P0	P4	P7	P30	P90
HYPOTHALAMUS					
CCK-4	49	75	136	321	221
CCK-8	263	336	485	307	352
CCK-33	324	481	575	1846	1601
CORTEX					
CCK-4	49	27	26	83	21
CCK-8	89	388	593	119	394
CCK-33	292	316	804	906	982
CEREBELLUM					
CCK-4	0	0	0	2	0
CCK-8	5	54	3	13	8
CCK-33	20	49	8	24	59

Enteric Nervous System

Enzyme Activity

Carboxypeptidase H and neutral endopeptidase activities during development are shown in figures 17-21 for the stomach, duodenum, jejunum, midjejunum and ileum, respectively.

In stomach (figure 17), carboxypeptidase H activity peaked at P7 and then decreased to birth levels by P90 whereas neutral endopeptidase activity decreased throughout development.

Carboxypeptidase H activity in the duodenum (figure 18) remained constant throughout development with a decreased activity at P30 compared to birth. NEP activity in the duodenum decreased throughout development as it had in the stomach.

For jejunum, CPH activity (figure 19) remained constant but decreased from P7 to P90. NEP activity decreased in this region throughout development.

CPH activity in the midjejunum (figure 20) was similar to that found in the jejunum except for a slight increase of activity before P7 followed by a decrease afterwards. In midjejunum, a slight decrease of NEP activity occurred at P4 and P7 with activity approaching that seen at birth from P30 to P90.

The pattern of CPH activity in the ileum (figure 21) is similar to that found in the midjejunum. A more significant increase of CPH activity occurred in the ileum from P0 to P7 than in the midjejunum. Activity decreased below birth levels

by P90. Neutral endopeptidase activity is very low in the ileum at P0, P4 and P7. A 50- to 100-fold increase of NEP activity occurred by P90 in this region.

Carboxypeptidase H activity was highest in the duodenum, moderate in jejunum and midjejunum and lowest in stomach and ileum at P0. With development, all enteric regions to showed a peak of CPH activity at age P7. After P7 CPH activity decreased to adult levels. By adult age, the profile of CPH activity was highest in duodenum (15.17 pmoles/mg/min), moderate in stomach, jejunum and midjejunum (4.12, 3.40, 3.28) and lowest in ileum (1.16).

Neutral endopeptidase activity was higher than carboxypeptidase H activity in all enteric regions throughout development. At birth, NEP activity was highest in jejunum, followed by duodenum, midjejunum, ileum and stomach. By adult age the jejunum contained highest NEP activity (1285 pmoles/mg/min). Moderate NEP activity was found for midjejunum, duodenum and ileum (819, 375, 129). The lowest NEP activity at adult age was present in stomach (0.34).

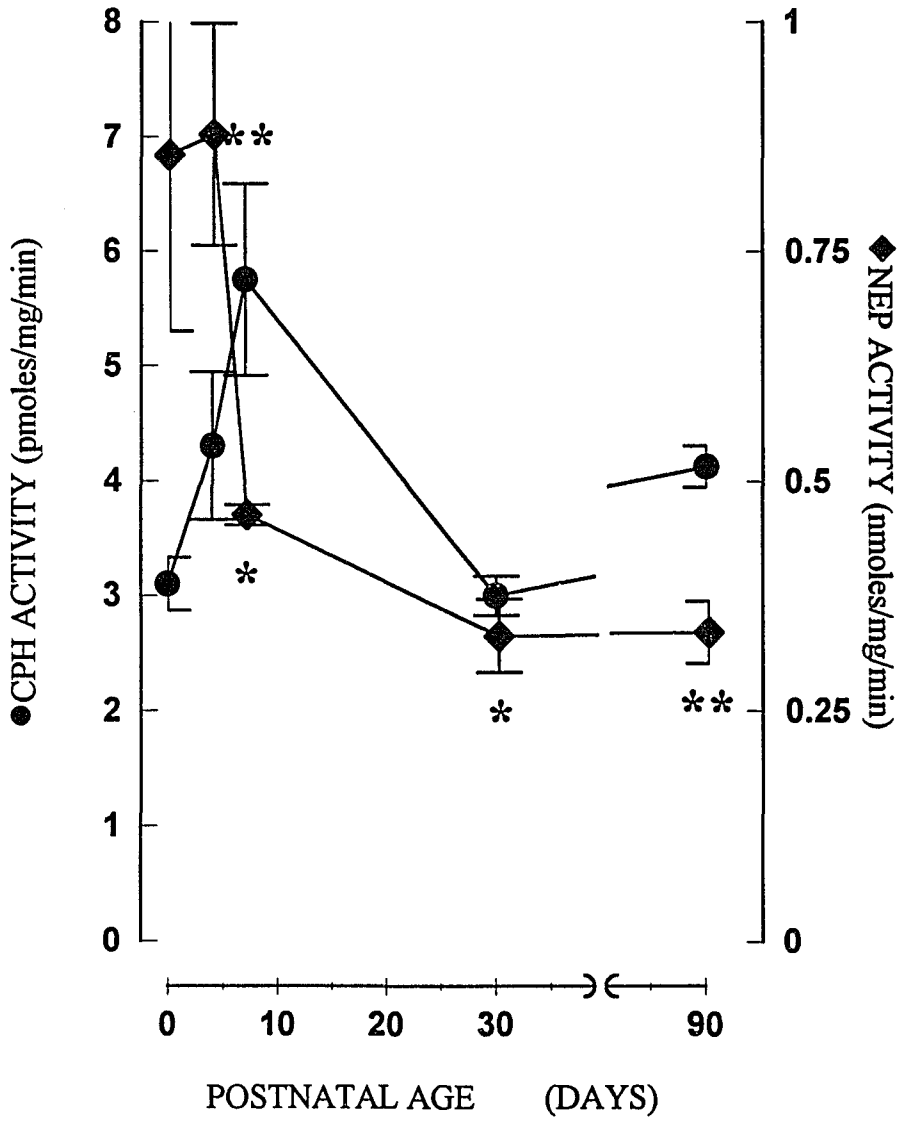


Figure 17. Carboxypeptidase H and Neutral Endopeptidase Activity in the Stomach

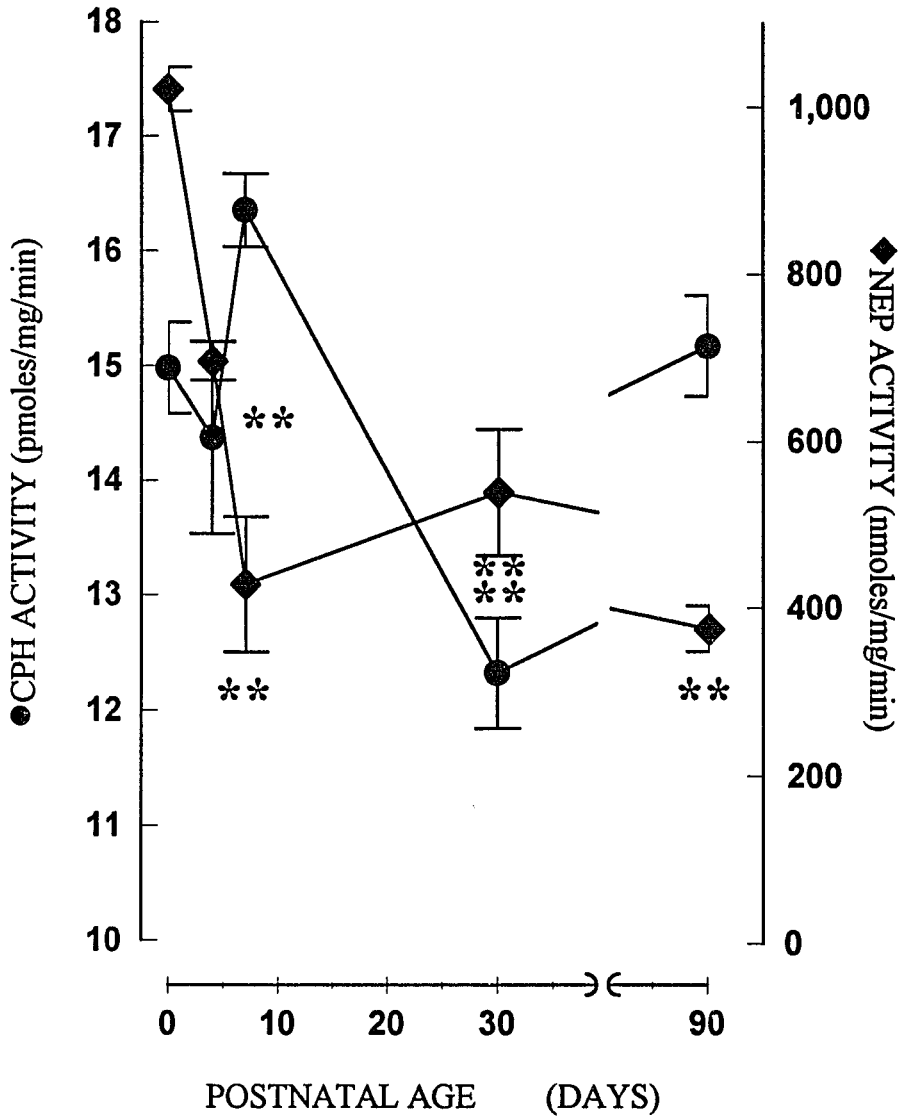


Figure 18. Carboxypeptidase H and Neutral Endopeptidase Activity in the Duodenum

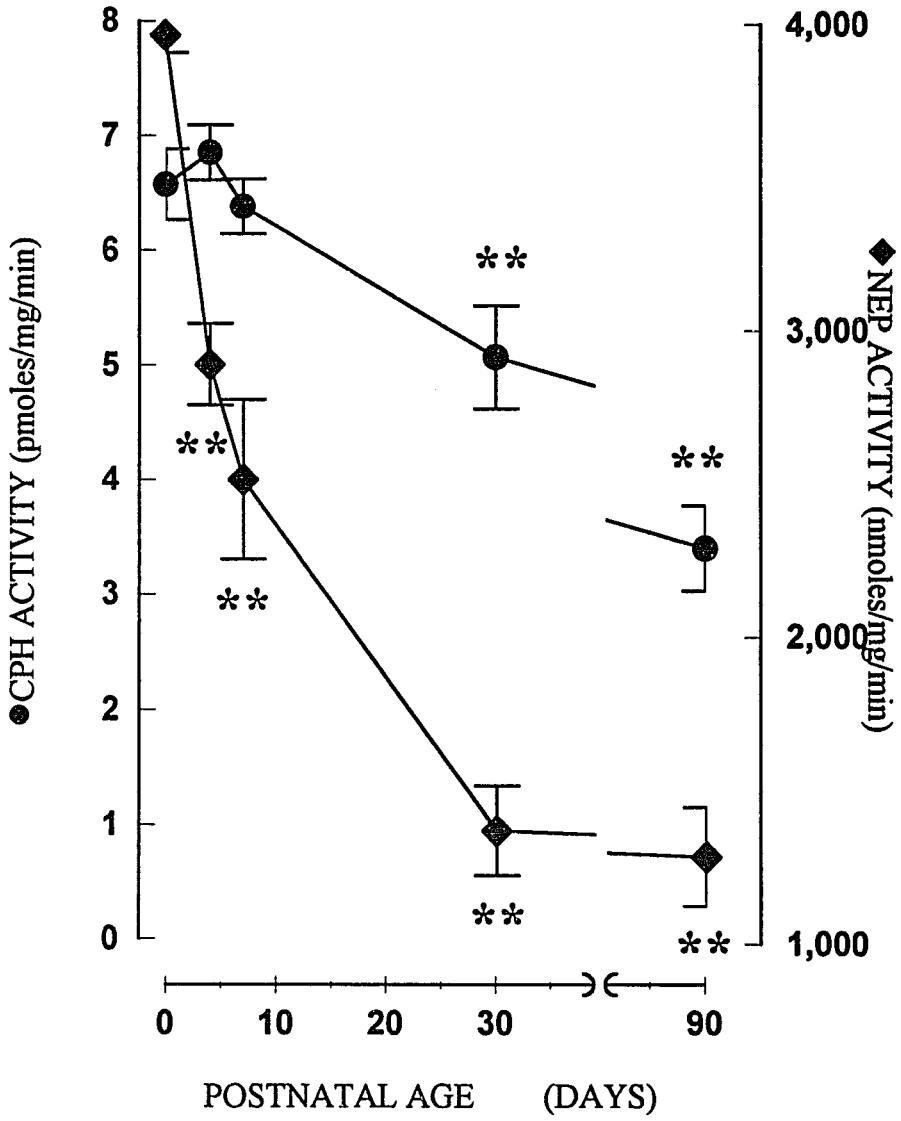


Figure 19. Carboxypeptidase H and Neutral Endopeptidase Activity in the Jejunum

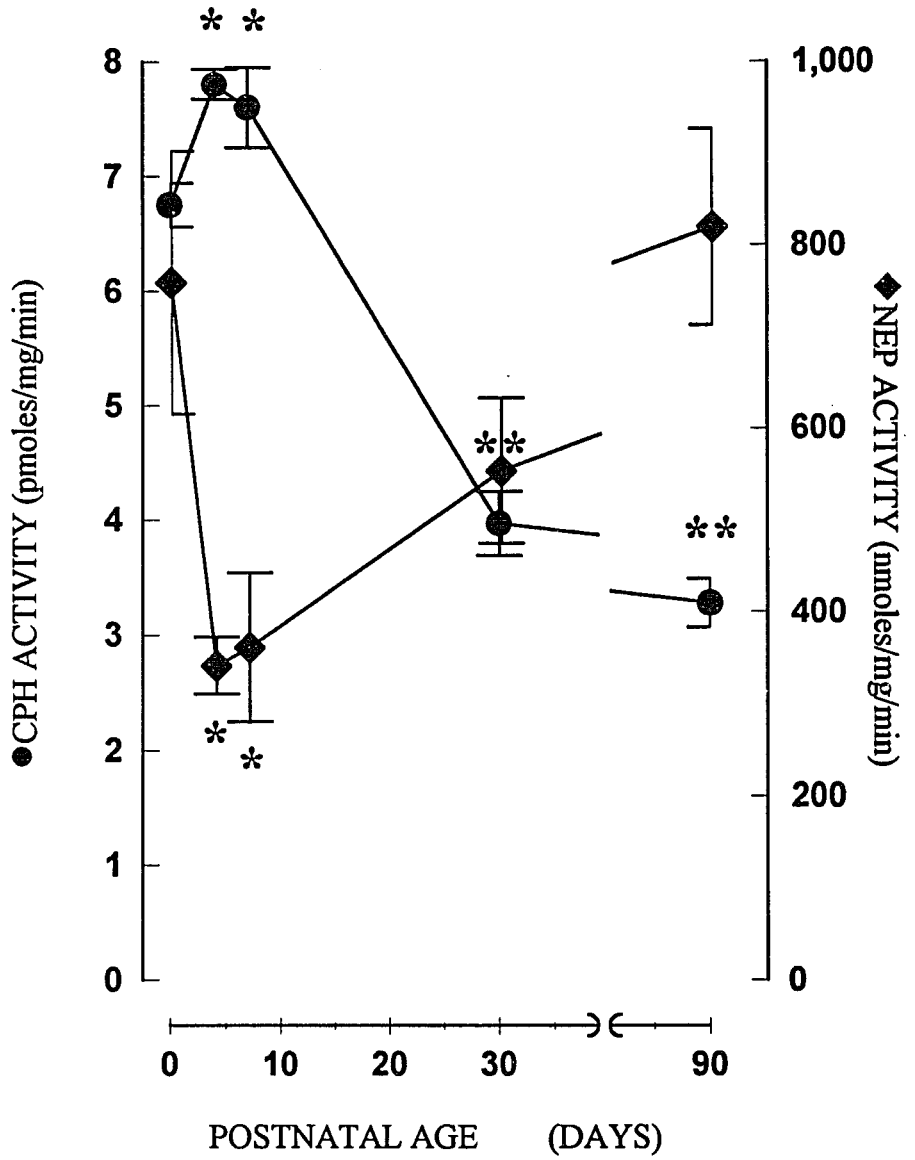


Figure 20. Carboxypeptidase H and Neutral Endopeptidase Activity in the Midjejunum

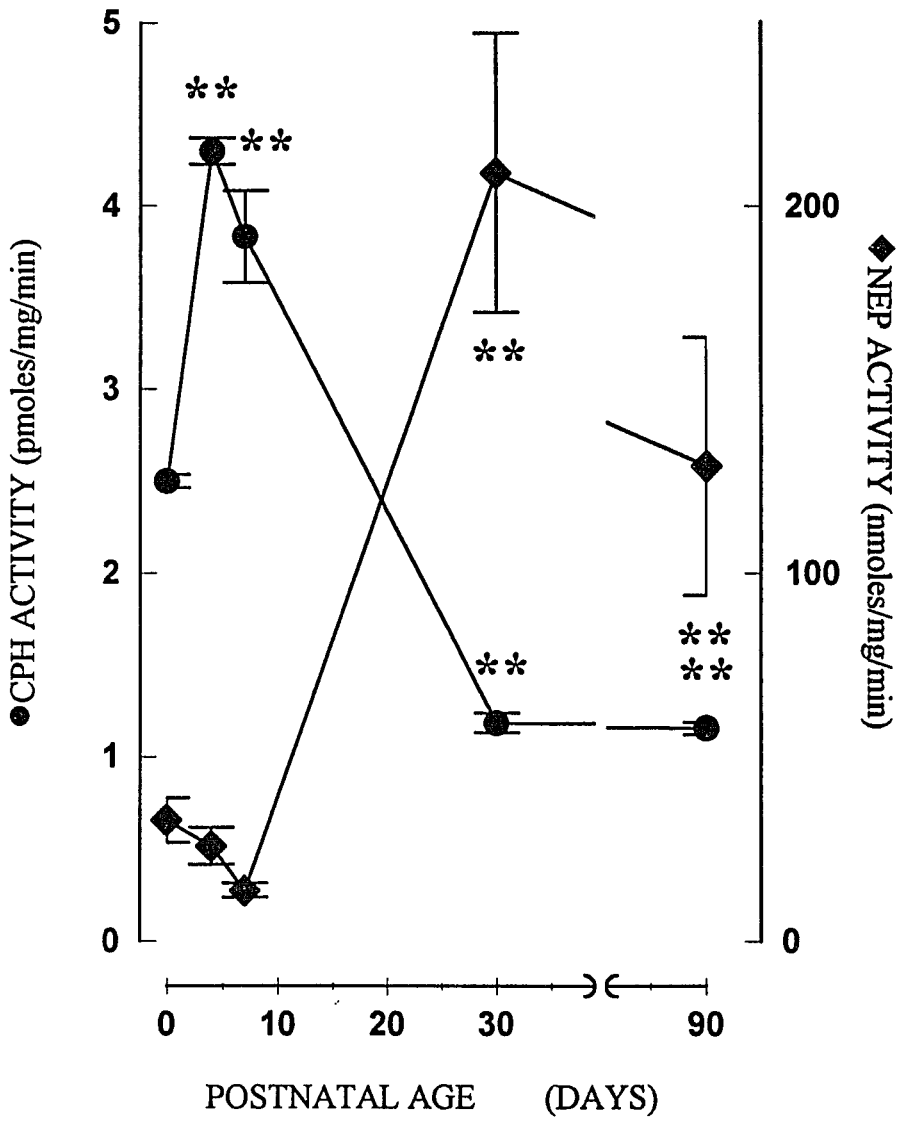


Figure 21. Carboxypeptidase H and Neutral Endopeptidase Activity in the Ileum

Radioimmunoassay

CCK-like peptides in the gastrointestinal tract also showed regional patterns of levels and forms throughout development, as determined by RIA. The levels of immunoreactive forms found for the stomach, duodenum, jejunum, midjejunum and ileum during development are shown in figures 22-26, respectively.

For stomach (figure 22), no appreciable change in L8D-IR or I11H-IR occurred with development. D10Y-IR increased at P4 and P7 and then decreased to adult levels by P30 however G17-IR increased throughout development of the stomach.

In duodenal samples (figure 23), a slight decrease of L8D-IR occurred at P90. I11H-IR increased at P30 but then decreased by P90. D10Y-IR forms in the duodenum remained constant throughout development with a slight decrease at P90. Similar to the stomach, G17-IR in the duodenum also increased throughout development.

L8D-IR, I11H-IR and D10Y-IR in the jejunum (figure 24) remained constant throughout development except for L8D-IR that increased by P90. G17-IR forms also increased throughout development to highest levels at P90.

The midjejunum (figure 25) presents a similar profile as the jejunum in that L8D-IR, I11H-IR and D10Y-IR forms remained constant throughout development. Only L8D-IR increased by P90 in the midjejunum. Interestingly, G17-IR forms also increased throughout development but peaked at P30.

Ileum (figure 26), unlike all other gastrointestinal regions showed a decrease of L8D-IR throughout development. I11H-IR forms continued to increase after P7 to very high levels by P90. D10Y-IR forms increased early in development, levels peaked by P7 and then decreased by P30. An increase of G17-IR forms occurred in the ileum at P7 followed by a decrease at P30 and an increase at P90.

Thus, L8D-IR remained constant from P0 to P30 in the stomach, duodenum, jejunum and midjejunum. By P90, L8D-IR did not change for stomach, increased in the jejunum and showed a slight decrease in duodenum and midjejunum. The ileum, unlike all other regions showed an initial decrease of L8D-IR that remained fairly constant throughout the rest of development. At adult age, L8D-IR was highest in jejunum (915 pg/mg protein) followed by moderate levels in stomach (729), and lower levels in midjejunum, duodenum, and ileum (378, 231, 126).

I11H-IR also showed no change during development for stomach, jejunum and midjejunum regions. In duodenum and ileum, I11H-IR remained constant to P7 after which time I11H-IR increased at P30 in the duodenum, decreased at P30 and increased at P90 in the ileum. At adult age the level of I11H-IR was highest in stomach and jejunum (6384, 6303 pg/mg protein) followed by moderate levels in duodenum (3957), and lower levels in the ileum and midjejunum (1714, 1222).

No substantial change of D10Y-IR occurred during development for duodenum, jejunum or midjejunum regions. In stomach, an increase of D10Y-IR occurred from P0 to P7 but then decreased back to birth levels by P30. D10Y-IR fluctuated in the ileum with a general decrease of immunoreactivity during

development. At adult age, D10Y-IR was highest in jejunum (5116 pg/mg protein) followed by moderate levels in stomach (3416) and lower levels in duodenum, midjejunum and ileum (1111, 811, 394).

Interestingly, G17-IR showed a substantial and significant increase of imunoreactivity at P90 compared to birth levels for all enteric regions. The increased G17-IR occurred in the stomach at P30, whereas it occurred in the duodenum, jejunum, midjejunum and ileum at P7. By P90, highest levels of G17-IR forms were found in stomach (15337 pg/mg protein) followed by jejunum (1943) and lower levels in duodenum, ileum, and midjejunum (593, 566, 536).

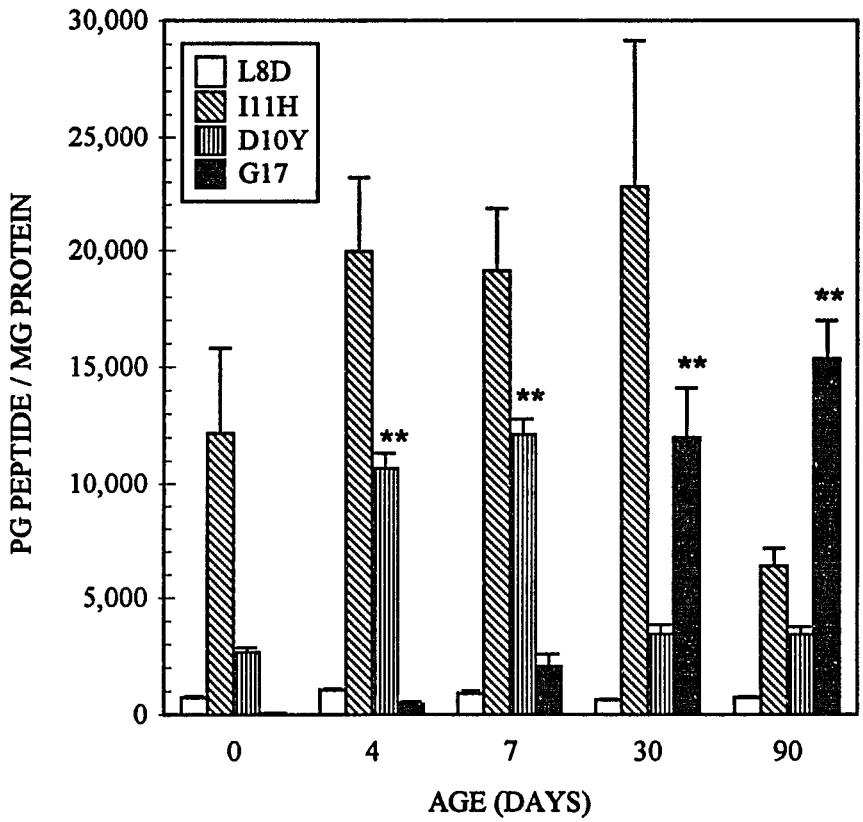


Figure 22. Cholecystokinin-like Immunoreactivity in the Stomach

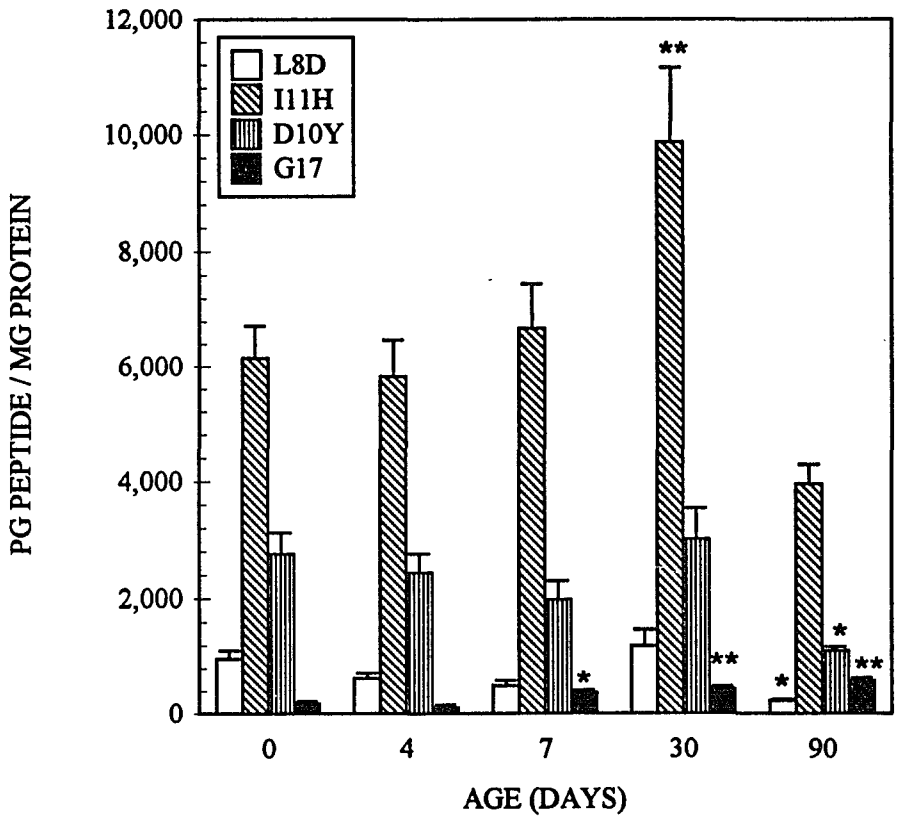


Figure 23. Cholecystokinin-like Immunoreactivity in the Duodenum

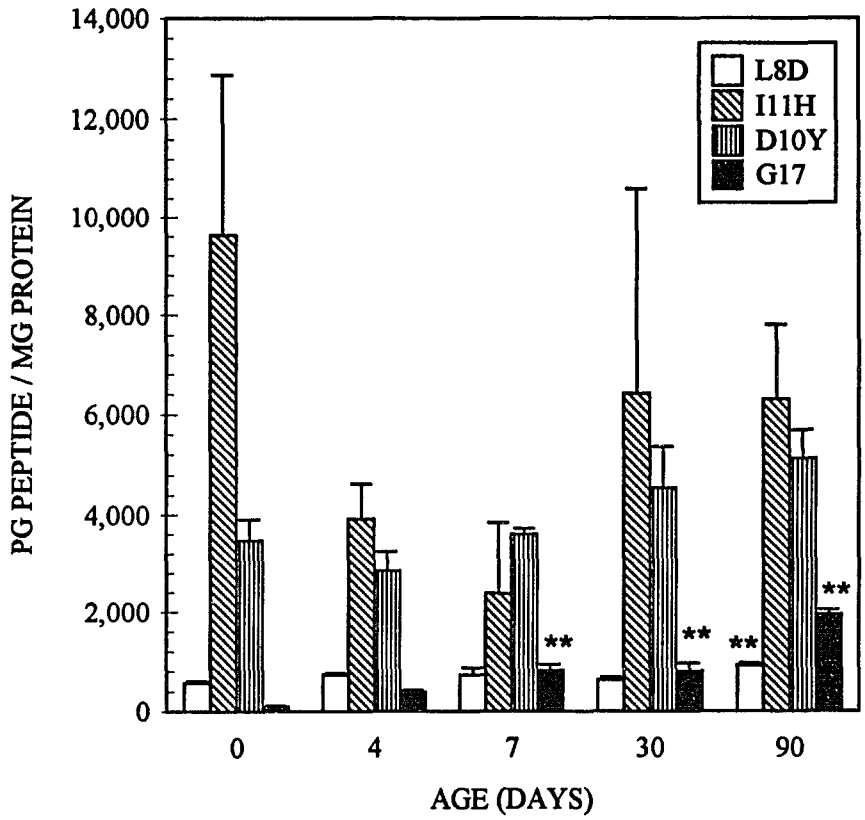


Figure 24. Cholecystokinin-like Immunoreactivity in the Jejunum

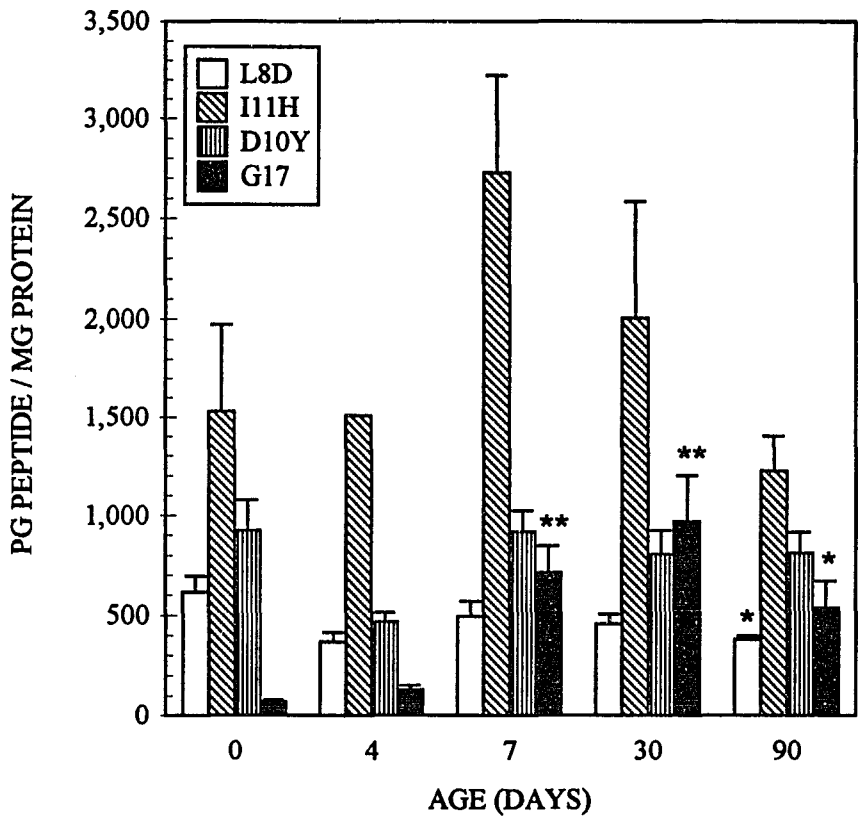


Figure 25. Cholecystokinin-like Immunoreactivity in the Midjejunum

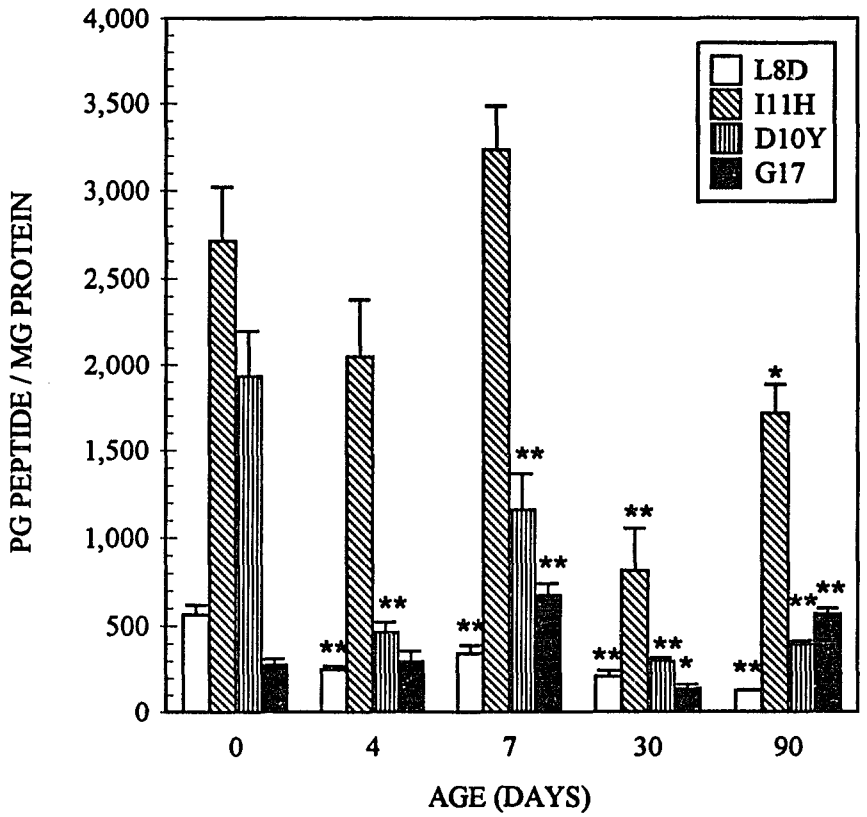


Figure 26. Cholecystokinin-like Immunoreactivity in the Ileum

Fractions of pooled samples separated by HPLC were also analyzed by G17 radioimmunoassay. Levels of CCK-4, CCK-8, CCK-33 and gastrin are reported in Table II. In stomach, these four peptides increased from P0 to P30. CCK-4, CCK-8, and CCK-33 decreased after P30 but gastrin increased at P90. By adult age, gastrin and CCK-33 were the predominant G17-IR forms found in the stomach. For duodenum, an increase of all four peptides occurred with development such that CCK-8 and CCK-33 were the predominant peptides found at adult age followed by CCK-4 and even lower levels of gastrin. In jejunum, G17 forms also increased with age with CCK-8 and CCK-33 in highest concentrations by adult age. In midjejunum and ileum we found CCK-8, CCK-33 and CCK-4 in highest quantities at adult age.

CCK-33 and gastrin appeared in highest (and similar) concentrations in the stomach compared to other regions of the adult enteric nervous system. CCK-4 and CCK-8 were also found in highest concentrations in the stomach but were about 25% lower than that of gastrin and CCK-33 in this region.

Table 2. HPLC/G17-IR Forms in the Enteric Nervous System
(Levels expressed as pg peptide/mg protein)

POSTNATAL AGE	P0	P4	P7	P30	P90
STOMACH					
CCK-4	0	61	305	2790	1523
CCK-8	0	59	1664	3233	1584
CCK-33	7	20	154	3371	2362
GASTRIN	2	1	5	871	2188
DUODENUM					
CCK-4	36	8	76	79	105
CCK-8	41	7	85	105	166
CCK-33	49	11	98	131	166
GASTRIN	17	11	42	49	55
JEJUNUM					
CCK-4	16	65	140	136	301
CCK-8	27	94	230	236	551
CCK-33	26	108	236	214	486
GASTRIN	8	41	72	63	320
MIDJEJUNUM					
CCK-4	15	26	98	58	121
CCK-8	16	37	189	332	153
CCK-33	17	39	222	341	139
GASTRIN	5	10	67	104	15
ILIUM					
CCK-4	34	75	139	35	124
CCK-8	81	68	141	31	122
CCK-33	112	79	170	36	158
GASTRIN	20	29	130	18	84

DISCUSSION

Comparison to Previous Adult Levels

Previous studies dealing with cholecystokinin levels and activities of carboxypeptidase H, neutral endopeptidase and metallo endopeptidase have been extensively researched in the adult rat brain but not in the gastrointestinal tract. Thus, a comparison of our data will with previously published data in the adult rat is limited to our particular regions of the rat central nervous system.

Enzyme Activity

Our data at P 90 showed highest carboxypeptidase H activity in the hypothalamus, intermediate activity in the cortex and lowest activity in the cerebellum. This adult data agrees with previous immunocytochemical localization studies (Lynch, 1990), with binding studies using the specific carboxypeptidase H inhibitor ^{3}H-GEMSA in rat brain slices (Lynch, 1984) and with activity studies of purified enzymes (Fricker, 1982).

Neutral endopeptidase activity was highest in the hypothalamus, intermediate in the cortex and lowest in the cerebellum at adult age. Localization of NEP with a fluorescent histochemical technique produced staining throughout the grey matter of the cortex, intense staining in the granular cell layer and scattered staining in the molecular cell layer of the cerebellum and in several hypothalamic nuclei (Back, 1989).

staining in the molecular cell layer of the cerebellum and in several hypothalamic nuclei (Back, 1989).

In the adult rat, high metallo endopeptidase activity was found in both the cortex and cerebellum with an approximately two-fold less activity for hypothalamus. Immunocytochemistry has shown the highest density of staining in the cerebellar Purkinje cells, some staining seen in the granule cells of the cerebellum as well as the preoptic region and arcuate nucleus of the hypothalamus (Healy, 1992).

Peptide Levels

Different molecular weight forms of cholecystokinin have been found in the rat brain. Our studies suggest that large cholecystokinin (L8D-IR) forms were found in highest concentrations in the hypothalamus followed by cortex and cerebellum. Previously, L8D-IR using the same antisera was most abundant in areas where CCK terminals predominate such as the septum, striatum, and olfactory tubercle/nucleus accumbens. These authors also found abundant levels in the hypothalamus, moderate levels in the midbrain, olfactory bulb, medulla and low levels in the cerebellum and cerebral cortex and pituitary of adult rats (Beinfeld, 1985).

Moderate molecular weight (I11H-LI) forms or CCK-33 like are reported to be 10- and 20- fold higher in the hypothalamus compared to cerebellum and cortex. Previously, high levels of I11H-LI were found in the adult rat frontal

cortex. Previously, high levels of I11H-LI were found in the adult rat frontal cortex, olfactory bulb and caudate putamen with only moderate levels of I11H-LI in the hypothalamus and low levels in the cerebellum (Han, 1987). The discrepancy between our cortical levels and the previous levels determined with the same antisera may result from differences in reporting of values or in the dissection of cortical, hypothalamic and cerebellar regions. The ng/g of immunoreactive peptide reported earlier would change slightly when reporting the values as pg/mg protein and may account for this discrepancy since cortical tissue has a higher protein concentration at adult age than the hypothalamus. We report our values in this manner to aid in illustrating the relationship between enzyme activities and peptide levels.

The carboxy-extended CCK (D10Y-IR) showed high concentrations in the hypothalamus and cerebellum at adult age with lower amounts for cortex. Carboxy-extended CCKs were previously found in highest amounts in the olfactory bulb, followed by the hippocampus. Lower amounts were detected in the cerebral cortex followed by cerebellum and hypothalamus of adult rats (Beinfeld, 1985) when reported as ng/g tissue.

In the adult rat bioactive CCK (G17-IR) concentrations were three-fold higher in the hypothalamus than in the cortex. The cerebellum contained very low and almost undetectable levels of G17-IR. Previous studies suggest that CCK-8 like forms show highest concentrations in the caudate nucleus, followed by the cerebral cortex and nucleus accumbens. G17-LI was previously found widely distributed in the hypothalamus with highest concentrations in the median eminence and ventromedial nucleus. Low G17 immunoreactivity was found in the pituitary. No substantial immunoreactivity was found in the cerebellum (Beinfeld, 1981).

To our knowledge, this is the only publication where enzyme activities have been reported with peptide levels that have been analyzed under similar conditions. Thus, a discussion of this relationship between post-translational enzymes and peptide levels follows for each of the regions of the central and enteric nervous system.

Central Nervous System

It is in general belief that the active form of cholecystokinin in the brain is CCK-8 and that the cortex produces the highest concentrations followed by hypothalamus. No appreciable production of cholecystokinin takes place in the cerebellum and gastrin has not been found in the rat brain. Interestingly, CCK-8 was produced in highest levels in both the hypothalamus and cortex whereas CCK-4 was detected in highest levels in the hypothalamus at adult age.

Interestingly, both CCK-A and CCK-B receptors were found in the hypothalamus, only CCK-B in the cortex and no receptors in the cerebellum.

A discussion for each region considering the forms of cholecystokinin detected, the activity of post-translational enzymes, and the receptor population follows to show how post-translational enzymes may be involved in regulating bioactive CCK-8 and therefore its actions at the receptor.

Hypothalamus

In hypothalamus, large pro-CCK (L8D-IR) forms did not change throughout development (figure 13) suggesting that the rate of pro-CCK transcription remained constant in this region. CCK mRNA levels in adult rat hypothalamus have previously been shown to be between levels found in cortex and cerebellum (Iadorola, 1989), however to date developmental levels have not been reported for this region. A conversion of these pro-CCK forms to shorter CCK-33 like (I11H-IR) forms occurred from P7 to P90 as shown by an increase of I11H-IR. These CCK-33 like forms may then be enzymatically cleaved by trypsin-like enzymes to the carboxy-extended (D10Y-IR) forms detected at constant levels throughout development.

Carboxypeptidase H activity doubled from P0 to P7 and remained at this high level throughout development (figure 9) suggesting that it is partly responsible for the increase of bioactive (G17-IR) forms seen with development. Further analysis of G17 immunoreactivity by HPLC/G17-RIA suggests that the levels of CCK-8

and CCK-4 increased slightly with age, however CCK-33 levels increased four fold by P90. Thus, the overall increase of bioactive (G17-IR) forms may be due to an increased production of CCK-33 as is also suggested by the increased CCK-33 like (I11H-IR) forms discussed previously.

The activity of the degradative enzyme, neutral endopeptidase, doubled between P0 and P7 (figure 9) and remained at that level to adult age. Metallo endopeptidase activity remained constant to P30 but decreased by P90 (figure 12). The increased neutral endopeptidase activity early in development results in the production of bioactive CCK-4 as shown in Table I where CCK-4 levels increased with age. It is interesting that metallo endopeptidase activity decreased only after P30 whereas neutral endopeptidase activity remained constant. This suggests that at least in the hypothalamus, the role for neutral endopeptidase is in producing CCK-4 as well as the degradation of CCK-8. On the other hand, metallo endopeptidase activity appeared to be mainly responsible for the degradation of CCK-8s after P7 as suggested by the lower CCK-8 levels detected in table I. Thus, the increased detection of CCK-4 and the constant level of CCK-8 in are capable of acting upon both CCK-A and CCK-B receptors found in this region (Woodruff, 1991).

This data is in close agreement to similar studies in the rat hypothalamus that showed high levels of products extended beyond glycine at the C-terminus, but low levels of processed bioactive CCK (carboxyamidated CCK) at birth (Mogensen, 1991).

Cortex

Pro-CCK (L8D-IR) forms remained constant in the cortex as they had in the hypothalamus with development (figure 14). This may suggest a constant level of transcription even though CCK mRNA levels have previously been shown to peak on days P10 and P20 and then to decrease by P100 (Yoshikawa, 1988). Here CCK-33 like (I11H-IR) forms decreased throughout development in the cortex. This may signify an increased conversion to carboxy-extended (D10Y-IR) forms by the trypsin-like enzymes of this region. In fact, the carboxy-extended forms increased 2-fold by P7. After P7 carboxy-extended forms decreased to previous birth levels by P30 indicating a higher conversion of these forms to bioactive (G17-IR) forms.

Carboxypeptidase activity, responsible for the conversion of carboxy-extended forms to bioactive forms increased from P0 to P7 (figure 10) whereas bioactive (G17-IR) forms increased 2-fold from P4 to P7 and then remained constant. Carboxypeptidase H activity decreased after P7 suggesting that the amount of G17-IR peptide detected should decrease as well since the enzyme responsible for producing it decreases. Further analysis of HPLC/G17-IR of fractions (table I) suggests that the initial increase of G17-IR (figure 14) was due to an increase of CCK-8 and CCK-33 from P0 to P7 with development. CCK-8 and CCK-33 levels increased after P7 even though carboxypeptidase H activity remained constant during this time frame. This indicates that metabolic peptidase activity may be regulating levels of peptide after P7.

The degradative enzyme, neutral endopeptidase, significantly decreased between P0 and P7 and then remained constant to P90. In contrast, metallo endopeptidase activity increased between P0 and P7 and then slowly decreased back to birth levels by P90. The decrease of NEP activity early in development should produce a higher level of bioactive CCK forms detected after birth that was shown by an increased G17-IR and the increased CCK-8 detected. A high CCK-B receptor population (Woodruff, 1991) and low CCK-8 peptide detected at adult age would suggest that in this region the amount of bioactive peptide present in the synapse is under stringent control. This control is provided by the high activity of metallo endopeptidase to degrade the peptide into fragments not detectable by our antisera. Neutral endopeptidase provides less control over peptide levels since there is low production of CCK-4.

Cerebellum

In cerebellum, L8D-IR (pro-CCK forms) decreased after P0 suggesting limited transcription of the message (figure 15). The low levels of CCK mRNA detected (Iadorola, 1989) combined with low levels of bioactive CCK-8 suggests limited functions for CCK-8 in this region. High levels of CCK-33 forms are present throughout development. These high levels may be the result from antisera binding characteristics due to the I11H antisera recognizing the porcine sequence. Nevertheless, I11H forms do not change with development. The carboxy-extended (D10Y-IR) forms decreased after P4 and continued to decrease

significantly until P90 whereas bioactive CCK (G17-IR) forms showed no change throughout development. Bioactive forms of CCK are very low in this region as shown by low G17-IR and low concentrations of CCK-4, CCK-8 and CCK-33 of table I.

Interestingly, carboxypeptidase H activity in this region also decreased throughout development suggesting a limited capacity of this tissue to produce bioactive CCK. The amount of ^{3}H-CCK-8 binding in this region was not appreciable in adult rats (Van Dijk, 1984) suggesting not only a low CCK receptor population but a low need for this peptide in this region. The degradative enzymes, neutral endopeptidase and metallo endopeptidase, were present at birth but also decreased with development. Although low levels of CCK were present in this tissue, these metabolic enzymes may degrade other peptides found in this region such as Met-enkephalin, somatostatin, corticotropin releasing factor, dynorphin or substance P.

Enteric Nervous System

It is in general belief that the active form of cholecystokinin produced and released into circulation is CCK-33 from the rat enteric nervous system. Cholecystokinin is believed to directly stimulate pancreatic secretion and gall bladder contraction and to inhibit gastric emptying, however the forms of cholecystokinin capable of these actions is still quite controversial. The CCK-A receptor has been found in the pancreas, gall bladder, pyloric smooth muscle and neurons of the myenteric plexus. CCK-B receptors, having a high affintity for CCK-4 and gastrin have also been found in gastric and enteric smooth muscle. Changes of peptide levels seen in the enteric nervous system may be dependent upon several factors. These include the absorption of cholecystokinin from maternal milk during suckling age and changes in diet composition during weanling. A description of cholecystokinin forms, post-translational enzyme activities, and their receptors follows for each region of the enteric nervous system studied.

Stomach

In stomach, pro-CCK (L8D-IR) forms remained constant throughout development suggesting constant translation of message (figure 22). CCK-33 like (I11H-IR) forms also remained constant however the carboxy-extended (D10Y-

IR) forms produced by trypsin-like enzymes showed a five fold increase at P4 and P7 but decreased to birth levels by P30.

Carboxypeptidase H activity increased above birth levels only at P7 (figure 17) and may be responsible for the decreased carboxy-extended (D10Y-IR) forms and the increased bioactive (G17-IR) forms detected after P7. The bioactive (G17-IR) forms increased to very high levels by adult age.

Neutral endopeptidase activity decreased throughout development and therefore allowed the detection of bioactive forms (figure 17). Neutral endopeptidase was 100 to 1000 times less active in the stomach as compared to other enteric nervous system regions. As a matter of fact, the stomach appears to be highly involved in the production of bioactive CCK since these forms were much higher in the stomach than other regions. HPLC analysis of the bioactive forms showed a high production of gastrin and CCK-33 and a slightly lower production of CCK-8 and CCK-4 in this region (table II). Thus, high levels of CCK-8, CCK-33 and gastrin may be released from the stomach at adult age to alter digestive activities.

Interestingly, CCK-4 concentrations increased with development. This suggests that neutral endopeptidase activity, even though at low levels, is still quite capable of degrading the peptide into non-detectable fragments as well as the CCK-4 bioactive fragment.

Duodenum

Pro-CCK (L8D-IR) forms remained constant from P0 to P30 (figure 23). By P90, pro-CCK forms were 3-fold less than that at birth. CCK-33 like (I11H-IR) forms peaked at P30 which then decreased to P90. Carboxy-extended (D10Y-IR) forms remained constant to P30 but decrease by P90. Here, carboxypeptidase H activity responsible for the carboxy-extended (D10Y-IR) form conversion to bioactive (G17-IR) forms only showed decreased activity at P30. This does not explain the increased detection of bioactive (G17-IR) forms found during development however carboxypeptidase H shows highest activity in this region compared to other enteric regions. A decreased neutral endopeptidase activity immediately after birth may explain the increased detection of bioactive forms found later in development. These bioactive forms at adult age consist mainly of CCK-8 and CCK-33 with less gastrin and even lower amounts of CCK-4 being detected (table II).

It is interesting that carboxypeptidase H activity is much higher in this region compared to all other enteric regions studied. It is possible that the turnover rate for producing and degrading cholecystokinin is very high in this region and/or that carboxypeptidase H may be involved in processing other peptides in this region.

Jejunum

In the jejunum constant levels of pro-CCK (L8D-IR) forms were produced up to P30 (figure 24) with an increase seen thereafter. No change occurred during development for CCK-33 like (I11H-IR) forms or carboxy-extended (D10Y-IR) forms suggesting a constant production of these forms with development.

Carboxypeptidase H activity decreased after P7 (figure 19) whereas bioactive (G17-IR) forms increased from P7 to P90. The increase of bioactive forms may not be attributed to carboxypeptidase H activity since CPH activity decreased during this period. Neutral endopeptidase activity decreased right after birth and therefore may allow the increased detection of the bioactive forms during this time frame. The HPLC/G17-IR data of table II showed a high production of CCK-8 and CCK-33 followed by gastrin and CCK-4. This data is similar to that previously noted in which we have an approximately 50/50 production and possible release of large and small forms when considering pmolar concentrations.

Midjejunum

The midjejunum is very similar to the jejunum in that pro-CCK (L8D-IR) forms and CCK-33 like (I11H-IR) forms did not change dramatically with development (figure 25). Carboxy-extended (D10Y-IR) forms also remained constant with development. Here, pro-CCK, CCK-33 like and carboxy-extended forms were lower than that found in the jejunum.

Bioactive (G17-IR) forms increased after P4 and remained at this level until adult age. The increased bioactive forms from P4 to P7 may be attributed to a slight increase of carboxypeptidase H activity (figure 20) after birth. The increase of bioactive forms after P7 may not be related to carboxypeptidase H since CPH activity decreased below birth levels. It is possible that CPH levels are still high enough to produce this level of peptide. In this region, neutral endopeptidase activity fluctuated with a decreased activity between P4 and P7 that increased by P90. If we examine the bioactive forms in this region (table II) we find a decreased levels of CCK-8 from P30 to P90 and an increased production of CCK-4 during this time frame. Thus, carboxypeptidase H appears to be responsible for the increased production of bioactive (G17-IR) forms throughout development. The increased activity of neutral endopeptidase later in development appears to be responsible for the decreased CCK-8 and increased CCK-4 detected.

Ileum

Unlike all of the other enteric regions described, the ileum showed a decreased production of pro-CCK (L8D-IR) forms throughout development as compared to birth levels (figure 26). This may suggest a lower transcription of the peptide from CCK mRNA however it may also suggest a higher turnover rate for the peptide undergoing post-translational processing. CCK-33 like (I11H-LI) forms increased after P7 suggesting that this region is highly active in turning over

bioactive forms. The carboxy-extended (D10Y-IR) forms fluctuated with an abrupt decrease from P0 to P4, an increase to P7 (still below birth levels) and a continued decrease to P90 levels.

Interestingly, carboxypeptidase H activity increased right after birth to P7 but decreased afterwards to below birth levels by P30 (figure 21). Carboxypeptidase H activity thus correlates with the changes of carboxy-extended peptides seen during development. Bioactive (G17-IR) forms also fluctuate with development as well. An increase of bioactive forms occurred from P4 to P7 and may be due to the increased carboxypeptidase H activity during this same time frame. After P7, bioactive forms decreased below birth levels at P30 that is consistent with the decreased carboxypeptidase H activity during this time frame. After P30, bioactive forms increased again even though carboxypeptidase H activity shows no change. The bioactive (G17-IR) forms for this region in table II show similar levels of CCK-4, CCK-8, and CCK-33 being produced with much lower levels of gastrin.

Interestingly, neutral endopeptidase activity remained low from P0 to P7 but then increased dramatically by P30 (figure 21). An increased production of CCK-4, CCK-8 and CCK-33 occurred from P0 to P7. This suggests that the early detection of CCK peptides was due to carboxypeptidase H activity since these peptides decreased at P30 and then increased to high levels detected at P90. The dramatic increase of neutral endopeptidase activity at P30 that continues to P90 would suggest a decrease of bioactive forms produced. CCK-4 levels increased

from P0 to P7 and decreased by P30. This is predictable according to the amount of bioactive (G17-IR) peptides produced by carboxypeptidase H that can be degraded by neutral endopeptidase present during the same period. From P30 to P90 we see an increased production of CCK-4 with may be explained by the increase of neutral endopeptidase activity seen at P30.

Central vs. Enteric Nervous System

Dramatic changes in CCK-like immunoreactivity occur in the central and enteric nervous systems and vary considerably by region. In general, large molecular weight forms of cholecystokinin (L8D-IR) did not change with development in either the central or enteric nervous system regions suggesting that translation of CCK mRNA does not change. CCK-33 like forms (I11H-IR) showed regional variations in the central nervous system whereas these forms decreased in the enteric nervous system suggesting a more rapid turnover of cholecystokinin in the enteric nervous system. Carboxy-extended (D10Y-IR) forms showed regional alterations in both the central and enteric nervous systems suggesting that the activity of carboxypeptidase H in each region is variable.

In general, carboxypeptidase H activity in the adult rat brain is highest in the hypothalamus, moderate in the cortex and low in the cerebellum. With development, carboxypeptidase H activity showed specific regional activity patterns, however most alterations of CPH activity in the central nervous system occurred early in gestation, at about P4. For enteric nervous system regions, CPH

activity showed no change in the more proximal tissues (stomach, duodenum and jejunum) but presented a decreased activity in distal tissues (midjejunum and ileum) by P4. CPH activity in the enteric nervous system was similar to that found in the central nervous system, however the duodenum had a two-fold higher activity than the hypothalamus that resulted in higher levels of bioactive cholecystokinin forms being produced in these two regions. Thus carboxypeptidase H activity shows a variety of regional activity patterns in the central nervous system however significant alterations occurred by P4. The enteric nervous system shows fewer changes of CPH activity with either no change in proximal tissues with development or a decreased activity by P4 in the most distal regions.

Neutral endopeptidase activities were altered early in development of the central nervous system by postnatal age 4 days (P4) and also showed varied regional patterns of activity with development. Neutral endopeptidase activity increased in the hypothalamus and decreased in the cortex and cerebellum with development. For enteric nervous system regions, neutral endopeptidase activity decreased with age around P30 in the proximal regions and P4 in more distal regions of the enteric nervous system. Thus, neutral endopeptidase activity was similar to CPH activity in the central nervous system because of the varied pattern of activity shown for each region. NEP activity in the enteric nervous system also showed fewer changes of activity as did CPH. Unlike CPH, neutral

endopeptidase activity decreased in the proximal tissues by P30 and decreased in the more distal tissues by P7.

Metallo endopeptidase has a more constant activity throughout development in the CNS than did CPH or NEP. Metallo endopeptidase activity slowly decreased in the hypothalamus, fluctuated in the cortex and slowly decreased in the cerebellum. The role of metallo endopeptidase in the degradation of cholecystokinin in the central nervous system remains unclear.

Bioactive cholecystokinin forms increased in the enteric nervous system but showed regional alterations in the central nervous system. Bioactive forms of cholecystokinin were found in highest concentrations in the stomach followed by hypothalamus. In the stomach, high bioactive forms are due to moderate activity of the processing enzyme, CPH, in producing the bioactive forms and the extremely low metabolic activity of NEP. In hypothalamus, bioactive forms arise from the high processing activity of carboxypeptidase H and by the low activity of both neutral endopeptidase and metallo endopeptidase. Regions where bioactive forms are very low have shown high neutral endopeptidase activity and were discussed previously.

In conclusion, bioactive forms of cholecystokinin are regulated by processing and metabolic enzymes throughout development and the regulation of these peptidases are relevant in the control of both central and peripheral actions of peptides.

SUMMARY

The ability to alter peptidase activity in specific central nervous system regions upon chronic drug administration sparked my interest in understanding the ontogeny of how peptidases alter peptide levels in both the central and enteric nervous systems. In order to determine if peptidases involved in post-translational processing were capable of modulating peptide levels we decided to study development in order to avoid pharmacological manipulation of animals. We found wide variations of activity for the processing enzyme, carboxypeptidase H and the two metabolic enzymes; neutral endopeptidase and metalloendopeptidase in both the central and enteric nervous systems. Carboxypeptidase H, involved in the production of bioactive peptides showed alterations of activity early in development that correlated with bioactive cholecystokinin-forms detected. Peptidases involved in the degradation of the bioactive cholecystokinin-forms such as neutral endopeptidase and metalloendopeptidase also showed regional variations of activity. Changes of degradative enzyme activity (e.g. neutral endopeptidase) correlated with bioactive peptide levels when a fragment produced upon cleavage of CCK-8 (e.g. CCK-4) was also detectable. In some cases, the data suggest that peptidase activity (even if low) may still be capable of processing cholecystokinin. Additionally, our data show that detectable bioactive cholecystokinin forms were regulated by processing and metabolic enzymes acting simultaneously.

These data show how enzymes involved in the post-translational processing of cholecytokinin are intimately involved in regulating levels of cholecystokinin-forms detected. Other biologically active peptides (e.g. β-endorphin, substance P, met-enkephalin, neurotensin) which are processed by these same peptidases can be studied in a similar manner to show how peptidases regulate other peptides detected in the central and enteric nervous systems. Once the relationship between peptidases and peptide levels is understood we may be able to pharmacologically exploit this relationship with the use of new drugs developed to alter peptidase activity. These alterations of peptidase activity could produce different peptide levels and therefore alter the physiological state of the central and enteric nervous systems. Manipulation of peptidase activity has already been shown to have clinical significance (e.g. captopril inhibition of angiotensin converting enzyme as a potent antihypertensive). Manipulation of peripheral cholecystokinin levels may aid in altering many digestive functions for anorexic and obese patients. Alteration of cholecystokinin levels in the central nervous system may have an impact on neurological diseases such as Alzheimers, Schizophrenia, Depression, and others. Understanding how peptidases control peptide levels in development may aid in the selective use of drugs during infancy, adolescence and adult years. A further extension of this work is needed to determine how peptidase activity regulates peptide levels in a geriatric age group.

REFERENCES

Acker GR, Molineaux C and Orlowski M. Synaptosomal membrane-bound form of endopeptidase-24.15 generates leu-enkephalin from dynorphin$_{1-8}$, α- and β-neoendorphin, and met-enkephalin from met-enkephalin-Arg^6-Gly^7-Leu^8. *J Neurochemistry* 48: 284-292, 1987.

Allard LR and Beinfeld MC. The subcellular distribution of peptides immunoreactive for the carboxyl-terminal extension of cholecystokinin in rat brain. *Neuropeptides* 6: 239-245, 1985.

Alstein M and Vogel Z. On the inactivation of enkephalin by enkephalinase. In *Neurotransmitters and their receptors.* Littauer UZ, Dudai Y, Silman I, Teichberg VI and Vogel Z (eds). John Wiley and Sons, Chichester, 497-507, 1980.

Amer MS. Studies with cholecystokinin. II: Cholecystokinetic potency of porcine gastrins I and II and related peptides in three systems. *Endocrinology* 84: 1277-1281, 1969.

Bacarese-Hamilton A, Adrian T, Chohan P, Antony T and Bloom S. Oxidation/reduction of methionine residues in CCK: A study by radioimmunoassay and isocratic reverse phase high pressure liquid chromatography. *Peptides* 6: 17-22, 1985.

Back SA and Gorenstein C. Histochemical visualization of neutral endopeptidase-24.11 (enkephalinase) activity in rat brain: cellular localization and codistribution with enkephalins in the globus pallidus. *J Neuroscience* 9: 4439-4455, 1989.

Barr PJ. Mammalian subtilisins: the long-sought dibasic processing endoproteases. *Cell* 66: 1-3, 1991.

Bateman Jr RC and Hersh LB. Evidence for an essential histidine in neutral endopeptidase 24.11. *Biochemistry* 26: 4237-4242, 1987.

Bayon A, Shoemaker WJ, Bloom FE, Mauss A and Guillemin R. Perinatal development of the endorphin- and enkephalin-containing systems in the rat brain. *Brain Research* 179: 93-101, 1979.

Beglinger C, Fried M, Whitehouse I, Jansen JB, Lamers CB and Gyr K. Pancreatic enzyme response to a liquid meal and to hormonal stimulation. Correlation with plasma secretin and cholecystokinin levels. *J Clinical Investigation* 75: 1471-1476, 1985.

Beinfeld MC, Meyer DK, Eskay RL, Jensen RT and Brownstein MJ. The distribution of cholecystokinin immunoreactivity in the central nervous system of the rat as determined by radioimmunoassay. *Brain Research* 212: 51-57, 1981.

Beinfeld MC. Peptides in rat brain immunoreactive for the carboxyl terminal extension of cholecystokinin: Distribution and chromatography. *Peptides* 6: 857-860, 1985.

Beinfeld MC. The distribution and chromatographic characterization of an amino-terminal fragment of cholecystokinin (CCK) 58 in rat brain. *Biochem Biophys Res Commun* 127: 720-725, 1985.

Beinfeld MC. Cholecystokinin (CCK) gene-related peptides: distribution and characterization of immunoreactive pro-CCK and an amino-terminal proCCK fragment in rat brain. *Brain Research* 344: 351-355, 1985.

Bradbury AF, Finnie MDA and Smyth DG. Mechanism of C-terminal amide formation by pituitary enzymes. *Nature* 298: 686-688, 1982.

Brand SJ. The postnatal development of cholecystokinin-like activity in the brain and small intestine of the rat. *J Physiology* 326: 425-433, 1982.

Buchan AMJ, Polak JM, Solcia E, Capella C, Hudson D and Pearse AGE. Electron immunohistochemical evidence for the human intestinal I cell as the source of CCK. *Gut* 19: 403-407, 1978.

Bunnett NW, Debas HT, Turner AJ, Kobayashi R and Walsh JH. Metabolism of gastrin and cholecystokinin by endopeptidase 24.11 from the pig stomach. *Am J Physiology* 255 (Gastrointest Liver Physiol 18): G676-G684, 1988.

Bunnett NW, Kobayashi R, Wu V and Sternini C. Cellular localization and abundance of mRNA encoding for neutral endopeptidase in the alimentary tract (Abstract). *J Neurochemistry* 57: Suppl.:S82C, 1991.

Burks TF, Hirning LD, Galligan JJ and Davis TP. Motility effects of opioid peptides in dog intestine. *Life Sciences* 31: 2237-2240, 1982.

Butera PC, Bradway D.N and Cataldo NJ. Modulation of the satiety effect of cholecystokinin by estradiol. *Physiology and Behavior* 53:1235-1238, 1993.

Calam J, Ellis A and Dockray G. Identification and measurement of molecular variants of cholecystokinin in duodenal mucosa and plasma. *J Clinical Investigation* 69: 218-225, 1982.

Calderon P, Furnelle J and Christophe J. Phosphatidylinositol turnover and calcium movemnt in the rat pancreas. *Am J Physiology* 238: G247-G254, 1980.

Checler F, Vincent JP and Kitabgi P. Inactivation of neurotensin by rat brain synaptic membranes partly occurs through cleavage at the Arg^8-Arg^9 peptide bond by a metalloendopeptidase. *J Neurochemistry* 45: 1509-1513, 1985.

Chou C, Hsieh C and Dabney J. Comparison of vascular effects of gastrointestinal hormones on various organs. *Am J Physiology* 232: H103-H109, 1977.

Chowdhury JR, Berkowitz JM, Praissman M and Fara JW. Interaction between octapeptide-cholecystokinin, gastrin, and secretin on cat gallbladder in vitro. *Am J Physiology* 229: 1311-1315, 1975.

Chu TG and Orlowski M. Soluble metalloendopeptidase from rat brain: action on enkephalin-containing peptides and other bioactive peptides. *Endocrinology* 116: 1418-1425, 1985.

Crawley JN, Rojas-Ramirez JA and Mendelson WB. The role of central and peripheral cholecystokinin in mediating appetitive behaviors. *Peptides* 3: 535-538, 1982.

Crawley J and Beinfeld M. Rapid development of tolerance to the behavioural actions of cholecystokinin. *Nature* 302: 703-706, 1983.

Crawley JN. Comparative distribution of cholecystokinin and other neuropeptides. Why is this peptide different from all other peptides? *In Neuronal Cholecystokinin*. Vanderhaeghen J-J and Crawley JN, editors. Annals of the N.Y. Academy of Sciences. The N.Y. Academy of Sciences, New York, NY, pp. 1-8, 1985.

Dale WE, Turkelson CM and Solomon TE. Role of cholecystokinin in intestinal phase and meal-induced pancreatic secretioin. *Am J Physiology* 257: G782-G790, 1989.

Davis TP, Schoemaker H and Culling AJ. Centrally acting drugs alter *in vitro* β-endorphin processing in the rat. *Eur J Pharmacology* 100: 249-251, 1984.

Davis TP, Culling-Berglund AJ and Schoemaker H. Specific regional differences of *in vitro* β-endorphin metabolism in schizophrenics. *Life Sciences* 39: 2601-2609, 1986.

Davis TP and Culling-Berglund A. Neuroleptic drug treatment alters *in vitro* central neurotensin metabolism. *Psychoneuroendocrinology* 12: 253-260, 1987.

Deschenes RJ, Lorenz LJ, Haun RS, Roos BA, Collier KJ and Dixon JE. Cloning and sequence analysis of a cDNA encoding rat preprocholecystokinin. *Proc Natl Acad Sci* 81: 726-730, 1984.

Deschenes RJ, Haun RS, Funckes CL and Dixon JE. A gene encoding rat cholecystokinin. Isolation, nucleotide sequence, and promotor activity. *J Biological Chemistry* 260: 1280-1286, 1985.

Deschodt-Lanckman M, Bui N, Noyer M and Christophe J. Degradation of cholecystokinin-like peptides by a crude rat brain synaptosomal fraction: A study by high pressure liquid chromatography. *Regulatory Peptides* 2: 15-30, 1981.

Deschodt-Lanckman M, Bui N, Koulischer D, Paroutaud P and Strosberg A. Cholecystokinin octa- and tetrapeptide degradation by synaptic membranes. II. Solubilization and separation of membrane-bound CCK-8 cleaving enzymes. *Peptides* 4: 71-78, 1983.

Deschodt-Lanckman M, Koulischer D, Przedborski S and Lauwereys M. Cholecystokinin octa- and tetrapeptide degradation by synaptic membranes. III. Inactivation of CCK-8 by a phosphoramidon-sensitive endopeptidase. *Peptides* 5: 649-651, 1984.

De Wied D, Kovacs GL, Bohus B, Van Ree JM and Greven HM. Neuroleptic activity of the neuropeptide β-LPH_{62-77} ([Des-Tyr^1]γ-endorphin; DTγE). *Eur J Pharmacology* 49: 427-436, 1978.

Docherty K and Hutton JC. Carboxypeptidase activity in the insulin secretory granule. *FEBS* 162:137-141, 1983.

Dockray GJ. Immunochemical evidence of cholecystokinin-like peptides in brain. *Nature* 264: 568-570, 1976.

Dockray G. Immunoreactive component resembling cholecystokinin octapeptide in intestine. *Nature* 270: 359-361, 1977.

Dockray GJ, Gregory RA and Hutchinson JB. Cholecystokinin. In *Gut Hormones*. Bloom SR and Polak JM (eds). Livingstone, Edinburgh, pp 229-239, 1980.

Dockray GJ. Cholecystokinin in rat cerebral cortex: identification, purification, and characterization by immunochemical methods. *Brain Research* 188: 155-165, 1980.

Dourish CT, Rycroft W and Iversen SD. Postponement of satiety by blockade of brain cholecystokinin (CCK-B) receptors. *Science* 245: 1509-1511, 1989.

Eberlein GA, Eysselein VE, Hesse WH, Goebell H, Schaefer M and Reeve Jr. JR Detection of cholecystokinin-58 in human blood by inhibition of degradation. *Am J Physiology* 253: G477-G482, 1987.

Emson PC, Checler F, Camargo AC, Pittaway K, Williams B, Kitabgi P and Vincent JP. Neurotensin degradation by soluble and membrane-associated enzymes from the brain. *Biochem Soc Trans* 13: 53-54, 1985.

Eng J, Du BH, Pan YC, Chang M, Hulmes J and Yalow R. Purification and sequencing of a rat intestinal 22 amino acid C-terminal CCK fragment. *Peptides* 5: 1203-1206, 1984.

Eng J, Gubler U, Raufman JP, Chang M, Hulmes JD, Pan YCE and Yalow RS. Cholecystokinin-associated COOH-terminal peptides are fully sulfated in pig brain. *Proc Natl Acad Sci* 83: 2832-2835, 1986.

Eysselein VE, Reeve Jr. JR, Shively JE, Hawke D and Walsh JH. Partial structure of a large canine cholecystokinin (CCK58): Amino acid sequence. *Peptides* 3: 687-691, 1982.

Eysselein VE, Deveney C, Sankaran H, Reeve Jr. JR and Walsh J. Biological activity of canine intestinal cholecystokinin-58. *Am J Physiology* 245: G313-G320, 1983.

Eysselein VE, Eberlein GA, Hesse WH, Singer MV, Goebell H and Reeve Jr. JR. Cholecystokinin-58 is the major circulating form of cholecystokinin in canine blood. *J Biological Chemistry* 262: 214-217, 1987.

Eysselein VE, Eberlein GA, Schaeffer M, Grandt D, Goebell H, Niebel W, Rosenquist GL, Meyer HE and Reeve Jr. JR. Characterization of the major form of cholecystokinin in human intestine: CCK-58. *Am J Physiology* 258 (Gastrointest Liver Physiol 21): G253-G260, 1990.

Florholmen J, Malm D, Vonen B and Burhol PG. Effect of cholecystokinin on the accumulation of inositol phosphates in isolated pancreatic islets. *Am J Physiology* 257: G865-870, 1989.

Fricker LD, Supattapone S and Snyder SH. Enkephalin convertase: a specific enkephalin synthesizing carboxypeptidase in adrenal chromaffin granules, brain, and pituitary gland. *Life Sciences* 31: 1841-1844, 1982.

Fricker LD, Plummer Jr. TH and Snyder SH. Enkephalin convertase: potent, selective, and irreversible inhibitors. *Biochem Biophys Res Commun* 111: 994-1000, 1983.

Fricker LD, Das B and Angeletti RH. Identification of the pH-dependent membrane anchor of carboxypeptidase E (EC 2.4.17.10). *J Biological Chemistry* 265: 2476-2482, 1990.

Fujimura M, Sakamoto T, Lluis F, Beauchamp RD, Townsend CM Jr, Greeley GH Jr and Thompson JC. Effect of a cholecystokinin antagonist, proglumide, on cholecystokinin-8-induced gallbladder contraction in conscious dogs. *Biological Signals* 1: 94-100, 1992.

Furuta S, Kisara K, Sakurada S, Sakurada T, Sasaki Y and Suzuki K. Structure-antinociceptive activity studies with neurotensin. *Br J Pharmacology* 83: 43-48, 1984.

Gainer H, Russell JT and Loh YP. The enzymology and intracellular organization of peptide precursor processing: the secretory vesicle hypothesis. *Neuroendocrinology* 40: 171-184, 1985.

Garlicki J, Konturek PK, Majka J, Kwiecien N and Konturek SJ. Cholecystokinin receptors and vagal nerves in control of food intake in rats. *Am J Physiology 258* (Endocrinol. Metab. 21): E40-E45, 1990.

Gibbs J and Smith G. Cholecystokinin and satiety in rats and rhesus monkeys. *Am J Clinical Nutrition* 30: 758-761, 1977.

Grignaschi G, Mantelli B, Fracasso C, Anelli M, Caccia S and Samanin R. Reciprocal interaction of 5-hydroxytryptamine and cholecystokinin in the control of feeding patterns in rats. *Br J Pharmacology* 109: 491-494, 1993.

Han SP, Allard LR and Beinfeld MC. Peptides in rat brain immunoreactive for the amino terminus of cholecystokinin 33: Distribution and chromatography. *Biochem Biophys Res Commun* 149: 902-907, 1987.

Harper AA and Raper HS. Pancreozymin, a stimulant of the secretion of pancreatic enzymes in extracts of the small intestine. *J Physiology* 102: 115-125, 1943.

Healy DP and Orlowski M. Immunocytochemical localization of endopeptidase 24.15 in rat brain. *Brain Research* 571:121-128, 1992.

Henning SJ, Chang S-SP and Gisel EG. Ontogeny of feeding controls in suckling and weanling rats. *Am J Physiology* 237: R187-R191, 1979.

Henning SJ. Functional development of the gastrointestinal tract. *In Physiology of the Gastrointestinal Tract*, Second Edition. Johnson LR, editor. Raven Press, New York, pp 285-300, 1987.

Hersh LB. Reaction of opioid peptides with neutral endopeptidase ("enkephalinase"). *J Neurochemistry* 43: 487-493, 1984.

Hersh LB and Morihara K. Comparison of the subsite specificity of the mammalian neutral endopeptidase 24.11 (enkephalinase) to the bacterial neutral endopeptidase thermolysin. *J Biological Chemistry 261*: 6433-6437, 1986.

Hildebrand P, Beglinger C, Gyr K, Jansen JBMJ, Rovati LC, Zuercher M, Lamers CBHW, Setnikar I and Stalder GA. Effects of a cholecystokinin receptor antagonist on intestinal phase of pancreatic and biliary responses in man. *J Clinical Investigation* 85: 640-646, 1990.

Hill, DR and Woodruff GN. Differentiation of central cholecystokinin receptor binding sites using the non-peptide antagonists MK-329 and L-365,260. *Brain Research* 526: 276-283, 1990.

Hocker M, Schmidt WE, Wilms HM, Lehnhoff F, Nustede R, Schafmayer A and Folsch UR. Measurement of tissue cholecystoki8nin (CCK) concentrations by bioassay and specific radioimmunoassay: characterization of the bioactivity of CCK-58 before and after tryptic cleavage. *Eur J Clinical Investigation* 20: S45-S50, 1990.

Hokfelt T, Rehfeld JF, Skirboll L, Ivemark B, Goldstein M and Markey K. Evidence for coexistence of dopamine and CCK in meso-limbic neurons. *Nature* 285: 476-478, 1980.

Hook VYH, Eiden LEE and Brownstein MJ. A carboxypeptidase processing enzyme for enkephalin precursors. *Nature* 295: 341-342, 1982.

Hsiao S and Wang C. Continuous infusion of cholecystokinin and meal pattern in the rat. *Peptides* 4: 15-17, 1983.

Hynes MR, Culling AJ, Galligan JJ, Burks TF, Schoemaker H, Yamamura HI and Davis TP. Processing of b-endorphin in the dog intestine: regional specificity. *Proc West Pharmacol Soc* 26: 95-99, 1983.

Iadorola MJ, Naranjo JR, Duchemin AM and Quach TT. Expression of cholecystokinin and enkephalin mRNA in discrete brain regions. *Peptides* 10: 687-692, 1989.

Ito M, Matsui T, Taniguchi T, Tsukamoto T, Murayama T, Arima N, Nakata H, Chiba T and Chihara K. Functional characterization of a human brain cholecystokinin-B receptor. A trophic effect of cholecystokinin and gastrin. *J Biological Chemistry* 268: 18300-18305, 1993.

Ivy AC and Oldberg E. A hormone mechanism for gallbladder contraction and evacuation. *Am J Physiology* 86: 599-613, 1928.

Jensen S, Holst J, Nielsen O and Rehfeld J. Effect of sulfation of CCK-8 on its stimulation of the endocrine and exocrine secretion from the isolated perfused porcine pancreas. *Digestion* 22: 305-309, 1981.

Jensen RT, Wank SA, Rowley WH, Sato S and Gardner JD. Interaction of CCK with pancreatic acinar cells. *Trends in Pharmacological Sciences* 10: 418-423, 1989.

Jorpes JE, Mutt V Toczko K. Further purification of cholecystokinin and pancreozymin. *Acta Che Scand* 18: 2408-2410, 1964.

Jorpes JE and Mutt V. Cholecystokinin and pancreozymin, one single hormone? *Acta Physiol Scand* 66: 196-202, 1966.

Kerstens PJ, Lamers CB, Jansen JB, de Jong AJ, Hessels M and Hafkenscheid JC. Physiological plasma concentrations of cholecystokinin stimulate pancreatic enzyme secretion and gall bladder contraction in man. *Life Sciences* 36: 565-569, 1985.

Konkoy CS, Oakes MG and Davis TP. Chronic treatment with neuroleptics alters neutral endopeptidase 24.11 activity in rat brain regions. *Peptides* 14: 1017-1020, 1993.

Kopin AS, Lee Y-M, McBride EW, Miller LJ, Lu M, Lin HY, Kolakowski Jr. LF and Beinborn M. Expression, cloning and characterization of the canine parietal cell gastrin receptor. *Proc Natl Acad Sci* 89: 3605-3609, 1992.

Koulischer D, Moroder L and Deschodt-Lanckman M. Degradation of cholecystokinin octapeptide, related fragments and analogs by human and rat plasma in vitro. *Regulatory Peptides* 4: 127-139, 1982.

Larson BA and Vigna SR. Species and tissue distribution of cholecystokinin/gastrin-like substances in some invertebrates. *Gen Compar Endocrinology* 50: 469-475, 1983.

Larson BA and Vigna SR. Gastrin/cholecystokinin-like immunoreactive peptides in the Dungeness crab, Cancer Magister (DANA): immunochemical and biological characterization. *Regulatory Peptides* 7: 155-170, 1983.

Le Meuth V, Philouze-Rome V, Le Huerou-Luron I, Formal M, Vaysse N, Gespach C, Guilloteau P and Fourmy D. Differential expression of A- and B-subtypes of cholecystokinin/gastrin receptors in the developing calf pancreas. *Endocrinology* 133: 1182-1191, 1993.

Letarte M, Vera S, Tran R, Addis JBL, Onizuka RJ, Quackenbush EJ, Jongeneel CV and McInnes RR. Common acute lymphocytic leukemia antigen is identical to neutral endopeptidase. *J Exp Med* 168: 1247-1253, 1988.

Li ZW, Brendel K, Culling-Berglund AJ and Davis TP. Regional specificity of β-endorphin metabolism in brain slices of the rat. *Proc West Pharmacol Soc* 31: 67-70, 1988.

Lin CW, Holladay MW, Witte DG, Miller TR, Wolfram CAW, Bianchi BR, Bennett MJ and Nadzan AM. A71378: CCK agonist with high potency and selectivity for CCK-A receptors. *Am J Physiology* 258: G648-651, 1990.

Lundberg JM, Anggard A, Hokfelt T and Fahrenkrag J. Coexistence of peptides and chemical neurotransmitters in autonomic regulation of blood flow and exocrine secretion. In: *Proceedings of IUPHAR 9th International Congress of Pharmacology*, Vol. 2. Paton W, Mitchell J and Turner P, editors. Macmillan Press, London pp. 169-175, 1994.

Lynch DR, Strittmatter SM and Snyder SH. Enkephalin convertase localization by [^{3}H] guanidinoethylmercaptosuccinic acid autoradiography: selective association with enkephalin-containing neurons. *Proc Natl Acad Sci* 81: 6543-6547, 1984.

Lynch DR, Strittmatter SM, Venable JC and Snyder SH. Enkephalin Convertase in the gastrointestinal tract and associated organs characterized and localized with [^{3}H]guanidinoethylmercaptosuccinic acid. *Endocrinology* 121: 116-126, 1987.

Lynch DR, Braas KM, Hutton JC and Snyder SH. Carboxypeptidase E (CPE): immunocytochemical localization in the rat central nervous system and pituitary gland. *J Neuroscience* 10: 1592-1599, 1990.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. Protein measurement with the folin-phenol reagent. *J Biological Chemistry* 193: 265-275, 1951.

Mack A.J and Todd JK. A study of human gall bladder muscle *in vitro*. *Gut* 9: 546-549, 1968.

Malesci A, Straus E and Yalow RS. Cholecystokinin converting enzymes in the brain. *Proc Natl Acad Sci* 77: 597-599, 1980.

Malesci A, de Fazio C, Festorazzi S, Bonato C, Valentini A, Tacconi M, Rovati L and Setnikar I. Effect of loxiglumide on gallbladder contractile response to cerulein and food in humans. *Gastroenterology* 98: 1307-1310, 1990.

Marley PD and Rehfeld JF. Extraction techniques for gastrins and cholecystokinins in the rat central nervous system. *J Neurochemistry* 42: 1515-1522, 1984.

Matsas R, Fulcher IS, Kenny AJ and Turner AJ. Substance P and [Leu]enkephalin are hydrolyzed by an enzyme in pig caudate synaptic membranes that is identical with the endopeptidase of kidney microvilli. *Proc Natl Acad Sci* 80: 3111-3115, 1983.

McDermott JR, Dodd PR, Edwardson JA, Hardy JA and Smith AI. Pathway of inactivation of cholecystokinin octapeptide (CCK-8) by synaptosomal fractions. *Neurochemistry International* 5: 641-647, 1983.

McDermott JR, Gibson AM and Turner JD. Involvement of endopeptidase 24.15 in the inactivation of bradykinin by rat brain slices. *Biochem Biophys Res Commun* 146: 154-158, 1987.

McGregor GP, Woodhams PL, O'Shaughnessy DJ, Ghatei MA, Polak JM and Bloom SR. Developmental changes in bombesin, substance P, somatostatin and vasoactive intestinal polypeptide in the rat brain. *Neuroscience Letters* 28: 21-27, 1982.

McLaughlin CL, Baile CA and Buonomo FC. Effect of CCK antibodies on food intake and weight gain in Zucker rats. *Physiology and Behavior* 34: 277-282, 1985.

Melville LD, Smith GP and Gibbs J. Comparison of the satiating potencies of cholecystokinin-33 and cholecystokinin-8. *Pharmacol Biochem Behavior* 45: 85-87, 1993.

Menozzi D, Gardner JD, Jensen RT and Maton PN. Properties of receptors for gastrin and CCK on gastric smooth muscle cells. *Am J Physiology* 257: G73-G79, 1989.

Miller LJ. Heterogeneity of CCK receptors: classification and characterization. In *Cholecystokinin Antagonists in Gastroenterology*. Alder G and Beglinger C (Eds.). Springer-Verlag, Berlin Heidelberg, pp 27-34, 1991.

Mogensen NW, Hilsted L, Bardram L and Rehfeld JF. Ontogeny of procholecystokinin processing in rat hypothalamus. *Developmental Neuroscience* 13: 104-109, 1991.

Molineaux CJ, Lasdun A, Michaud C and Orlowski M. Endopeptidase-24.15 is the primary enzyme that degrades leutenizing hormone releasing hormone both in vitro and in vivo. *J Neurochemistry* 51: 624-633, 1988.

Moran TH, Smith GP, Hostetler AM and McHugh PR. Transport of cholecystokinin (CCK) binding sites in subdiaphragmatic vagal branches. *Brain Research* 415: 149-152, 1987.

Moran TH, Norgren R, Crosby RJ and McHugh PR. Central and peripheral vagal transport of cholecystokinin binding sites occurs in afferent fibres. *Brain Research* 526: 95-102, 1990.

Mutt V and Jorpes JE. Structure of porcine cholecystokinin-pancreozymin. *Eur J Biochemistry* 6: 156-162, 1968.

Mutt V and Jorpes E. Hormonal polypeptides of the upper intestine. *Proc Biochem Soc* 125: 57-58, 1971.

Myers RD. Neuroactive peptides: unique phases in research on mammalian brain over three decades. *Peptides* 15: 367-381, 1994.

Orlowski M, Michaud C and Chu TG. A soluble metalloendopeptidase from rat brain: purification of the enzyme and determination of specificity with synthetic and natural peptides. *Eur J Biochemistry* 135: 81-88, 1983.

Orlowski M, Michaud C and Molineaux CJ. Substrate-related potent inhibitors of brain metalloendopeptidase. *Biochemistry* 27: 597-602, 1988.

Paxinos G and Watson C (Eds.) *The Rat Brain in Stereotaxic Coordinates*, 2nd ed., Academic Press, Australia, 1986.

Peters A, Miller M and Kimerer LM. Cholecystokinin-like immunoreactive neurons in rat cerebral cortex. *Neuroscience* 8: 431-448, 1983.

Philipp E, Pirke KM, Kellner MB and JC Krieg. Disturbed cholecystokinin secretion in patients with eating disorders. *Life Sciences* 48: 2443-2450, 1991.

Philipp E, Wilckens T, Friess E, Platte P and KM Pirke. Cholecystokinin, gastrin and stress hormone responses in marathon runners. *Peptides* 13: 125-128, 1992.

Pisegna JR, De Weerth A, Huppi K and Wank SA. Molecular cloning of the human brain and gastric cholecystokinin receptor, structure, functional expression and chromosomal localization. *Biochem Biophys Res Commun* 189: 296-303, 1992.

Rajjo IM, Vigna SR and Crim JW. Cholecystokinin immunoreactivity in the digestive tract of Bowfin (*Amia calva*), Bluegill (*Lepomis macrochirus*), and Bullfrog (*Rana catesbeiana*). *Gen Comp Endocrinology* 70: 133-144, 1988.

Reeve Jr. J, Eysselein VE, Walsh JH, Sankaran JH, Deveney CW, Tourtellotte WW, Miller C and Shively JE. Isolation and characterization of biologically active and inactive cholecystokinin-octapeptides from human brain. *Peptides* 5: 959-966, 1984.

Rehfeld JF. Processing of preprocholecystokinin in the brain. *J Neurochemistry* 44: S11A, 1985.

Rehfeld JF and Hansen HF. Characterization of preprocholecystokinin products in the porcine cerebral cortex: evidence of different processing pathways. *J Biological Chemistry* 261: 5832-5840, 1986.

Richardson PD and Withrington PG. The effects of glucagon, secretin, pancreozymin and pentagastrin on the hepatic arterial vascular bed of the dog. *Br J Pharmacology* 59: 147-156, 1977.

Rossier J, Barres E, Hutton JC and Bicknell RJ. Radiomentric assay for carboxypeptidase H (EC 3.4.17.10) and other carboxypeptidase B-like enzymes. *Analytical Biochemistry* 178: 27-31, 1989.

Roy P, Charpentier B, Durieux C, Dor A and Roques BP. Conformational behavior of cyclic CCK-related peptides determined by 400-MHz ^{1}H-NMR: relationships with affinity and selectivity for brain receptors. *Biopolymers* 28: 69-79, 1990.

Rubin B, Engel SL, Drungis AM, Dzelzkalns M, Grigas EO, Waugh MH and Yiacas E. Cholecystokinin-like activities in guinea pigs and in dogs of the C-terminal octapeptide (SQ 19,844) of cholecystokinin. *J Pharmacological Sciences* 58: 955-959, 1969.

Schick RR, Reilly WM, Roddy DR, Yaksh TL and Go VLW. Neuronal cholecystokinin-like immunoreactivity is postprandially released from primate hypothalamus. *Brain Research* 418: 20-26, 1987.

Schick RR, Harty GJ, Yaksh TL and Go VLW. Sites in the brain at which cholecystokinin octapeptide (CCK-8) acts to suppress feeding in rats: a mapping study. *Neuropharmacology* 29: 109-118, 1990.

Schneider BS, Monahan JW and Hirsch J. Brain cholecystokinin and nutritional status in rats and mice. *J Clinical Investigation* 64: 1348-1356, 1979.

Schultzberg M, Hokfelt T, Nilsson G, Terenius L, Rehfeld JF, Brown M, Elde R, Goldstein M and Said S. Distribution of peptide- and catecholamine- containing neurons in the gastro-intestinal tract of rat and guinea-pig: immunohistochemical studies with antisera to substance P, vasoactive intestinal polypeptide, enkephalins, somatostatin, gastrin/cholecystokinin, neurotensin and dopamine-β-hydroxylase. *Neuroscience* 5: 689-744, 1980.

Seymour AA, Norman JA, Asaad MM, Fennell SA, Abboa-Offei B, Little DK, Kratunis VJ, Delaney NG, Hunt JT and Di Donato G. Possible regulation of atrial natriuretic factor by neutral endopeptidase 24.11 and clearance receptors. *J Pharmacol Exp Ther* 256: 1002-1009, 1991.

Shiratori K, Watanabe S, and Takeuchi T. Inhibitory effect of intraduodenal administration of somatostatin analogue SDZ CO 611 on rat pancreatic exocrine secretion. *Pancreas* 8: 471-475, 1993.

Skirboll L, Hokfelt T, Rehfeld J, Cuello AC and Dockray G. Coexistence of substance P- and cholecystokinin-like immmunoreactivity in neurons of the mesencephalic periaqueductal central gray. *Neuroscience Letters* 28: 35-39, 1982.

Solomon T, Yamada T, Elashoff J, Wood J and Beglinger C. Bioactivity of cholecystokinin analogues: CCK-8 is not more potent than CCK-33. *Am J Physiology* 247: G105-G111, 1984.

Stacher G, Steinringer H, Schmierer G, Schneider C and Winklehner S. Cholecystokinin octapeptide decreases intake of solid food in man. *Peptides* 1: 133-136, 1982.

Stacher G. Effects of cholecystokinin and caerulein on human eating behavior and pain sensation: a review. *Psychoneuroendocrinology* 11: 39-48, 1986.

Stack G, Fricker LD and Snyder SH. A sensitive radiometric assay for enkephalin convertase and other carboxypeptidase B-like enzymes. *Life Sciences* 34: 113-121, 1984.

Strittmatter SM, Lynch DR and Snyder SH. Differential ontogeny of rat brain peptidases: prenatal expression of enkephalin convertase and postnatal development of angiotensin-converting enzyme. *Developmental Brain Research* 29: 207-215, 1986.

Studler JM, Reibaud M, Herve D, Blanc J, Glowinski J and Tassin JP. Opposite effects of sulfated cholecystokinin on DA-sensitive adenylate cyclase in two areas of the rat nucleus accumbens. *Eur J Pharmacology* 126: 125-128, 1986.

Takahashi Y, Kato K, Hayashizaki Y, Wakabayashi T, Ohtsuka E, Matsuki S, Ikehara M and Matsubara K. Molecular cloning of the human cholecystokinin gene by use of a synthetic probe containing deoxyinosine. *Proc Natl Acad Sci* 82: 1931-1935, 1985.

Turkelson CM, Solomon TE, Bussjaeger L, Turkelson J, Ronk M, Shively JE, Ho FJ and Reeve Jr. JR. Chemical characterization of rat cholecystokinin-58. *Peptides* 9: 1255-1260, 1989.

Turner AJ, Hooper NM and Kenny AJ. Metabolism of neuropeptides. In *Mammalian ectoenzymes,* AJ Kenny and AJ Turner (Eds). Elsevier Science Publishers B.V. Biomedical Division. Amsterdam pp. 211-248, 1987.

Van Dijk A, Richards JG, Trzeciak A, Gillessen D and Mohler H. Cholecystokinin receptors: biochemical demonstration and autoradiographical localization in rat brain and pancreas using [^{3}H] cholecystokining as radioligand. *J Neuroscience* 4: 1021-1033, 1984.

Vanderhaeghen JJ, Signeau JC and Gepts W. New peptide in the vertebrate CNS reacting with gastrin antibodies. *Nature*, Lond 257: 604-605, 1975.

Vanderhaeghen JJ, Lotstra F, Vandesande F and Dierickx K. Coexistence of cholecystokinin and oxytocin-neurophysin in some magnocellular hypothalamo-hypophyseal neurons. *Cell Tissue Research* 221: 227-231, 1981.

Vargas F, Frerot O, Tuong MDT, Zuzel K, Rose C and Schwartz JC. Sulfation and desulfation of cerebral cholecystokinin. *Ann NY Acad Sci* 448: 110-120, 1985.

Vigna SR and Gorbman A. Effects of cholecystokinin, gastrin, and related peptides on coho salmon gallbladder contraction *in vitro*. *Am J Physiology* 232: E485-E491, 1977.

Vigna SR. Distinction between cholecystokinin-like and gastrin-like biological activities extracted from gastrointestinal tissues of some lower vertebrates. *Gen Compar Endocrinology* 39, 512-520, 1979.

Vigna SR. Radioreceptor and biological characterization of cholecystokinin and gastrin in the chicken. *Am J Physiology* 246 (*Gastrointest Liver Physiol* 9): G296-G304, 1984.

Vigna SR, Morgan JL and Thomas TM. Localization and characterization of gastrin/cholecystokinin-like immunoreactivity in the central nervous system of *Aplysia Californica*. *J Neuroscience* 4: 1370-1377, 1984.

Vigna SR, Fischer BL, Morgan JL and Rosenquist GL. Distribution and molecular heterogeneity of cholecystokinin-like immunoreactive peptides in the brain and gut of the Rainbow Trout, *Salmo Gairdneri*. *Comp Biochem Physiology* 82C: 143-146, 1985.

Villaneuva M, Collins S, Jensen R, and Gardner J. Structural requirements for action of cholecystokinin on enzyme secretion from pancreatic acini. *Am J Physiology* 242: G416-G422, 1982.

Waksman G, Hamel E, Fournie-Zaluski MC and Roques BP. Autoradiographic comparison of the distribution of the neutral endopeptidase "enkephalinase" and of μ and δ opioid receptors in rat brain. *Proc Natl Acad Sci* 83: 1523-1527, 1986.

Wank SA, Harkins R, Jensen RT, Shapira H, De Weerth A and Slattery T. Purification, molecular cloning, and functional expression of the cholecystokinin receptor from rat pancreas. *Proc Natl Acad Sci* 89: 3125-3129, 1992.

Wank SA, Pisegna JR and De Weerth A. Brain and gastrointestinal cholecystokinin receptor family: structure and functional expression. *Proc Natl Acad Sci* 89: 8691-8695, 1992.

Weatherford SC, Figlewicz DP, Park CR and Woods SC. Chronic alcohol consumption increases sensitivity to the anorexic effect of cholecystokinin. *Am J Physiology* 265: R211-5, 1993.

Weller A, Smith GP and Gibbs J. Endogenous cholecystokinin reduces feeding in young rats. *Science* 247: 1589-1591, 1990.

Winkler G. Increasing the sensitivity of UV detection in protein and peptide separations when using TFA-acetonitrile gradients. *LC-GC* 5: 1044-1045, 1989.

Woodruff GN, Hill DR, Boden P, Pinnock R, Singh L and Hughes J. Functional role of brain CCK receptors. *Neuropeptides* 19 (Suppl.) 45-56, 1991.

Yabu M, Himeno S, Kariya Y, Fujita H and Tarui S. Identification of gastrin- and cholecystokinin-cells in human gastric antrum and duodenum using in situ hybridization technique. *Acta Histochem Cytochem* 22: 309-314, 1989.

Yoshikawa K and Aizawa T. Transient increases and individual variations in preprocholecystokinin gene expression in rat telencephalic regions during postnatal development: a quantitative comparison between preprocholecystokinin mRNA and preproenkephalin mRNA. *Molecular Brain Research* 4: 87-96, 1988.

Zuzel KA, Rose C and Schwartz JC. Assessment of the role of "enkephalinase" in cholecystokinin inactivation. *Neuroscience* 15: 149-158, 1985.