NEUROTENSIN : A STUDY OF ITS DISTRIBUTION, RELEASE AND METABOLISM

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ABSTRACT

The development and modification of a radioimmunoassay for the measurement of neurotensin in human plasma and in tissue extracts is described. The distribution of neurotensin in extracts of rat peripheral tissues is compared with that of neuromedin N, a peptide recently isolated from the porcine spinal cord and structurally similar to neurotensin. In the gastrointestinal tract, the distribution of neurotensin is closely comparable to neuromedin N. However, a considerable amount of neuromedin N is found in the kidney whereas no neurotensin has been detected. The regulation of neurotensin release is investigated by using an isolated rat ileum preparation. Carbachol and the amphibian peptide, bombesin, are found to affect the neurotensin release in the isolated rat ileum. The peptidergic interaction is demonstrated: when a somatostain analogue (SMS 201-995) is infused in man, it suppresses the basal and abolishes the meal-stimulated release of neurotensin. Dexamethasone, nerve growth factor and forskolin are found to influence synergistically the release and synthesis of neurotensin in the PC 12 phaeochromocytoma cell line, which may be cAMP mediated. The pharmacokinetics and metabolism of

neurotensin are studied in man with region specific antisera. The metabolism of both exogenous infused and meal-stimulated neurotensin is demonstrated. The half life of exogenous infused neurotensin is calculated to be 1.4 minutes and 6.0 minutes when plasma immunoreactive neurotensin is measured with C- and N-terminally directed antisera respectively. Consistent with the pharmacokinetic data and the metabolic fate of exogenous infused neurotensin in man, meal-stimulated neurotensin is found to be exceedingly unstable in human circulation, being rapidly processed to its stable N-terminal fragments , in particular, neurotensin 1-8. The molecular forms of circulating human immunoreactive neurotensin are characterised by gel filtration and high pressure liquid chromatography. In vitro study demonstrates that EDTA may be of use in the preservation of neurotensin in human plasma.

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MATERIALS AND APPARATUS

Materials

Acetic acid (glacial):	BDH Chemicals Ltd Poole, Dorset
Citric acid:	
L-Cystein hydrochloride:	
Ethylenediaminetetra acidic acid (EDTA), disodium salt:	
Formic acid:	
Gelatin:	
Hydrochloric acid:	
Lactose:	
Potassium dihydrogen orthophosphate:	
Potassium hydroxide:	
Potassium iodide:	
Sodium azide:	
Sodium chloride:	
Sodium hydrogen carbonate:	
di-Sodium hydrogen orthophosphate dihydrate:	
Sodium hydroxide:	
Trifluoroacetic acid (TFA):	
Tris(hydroxymethyl) methylamine:	

All the above are Analar grade and from BDH Ltd

Arlacel A:	Sigma Chemical Co, St Louis, Mo, USA
Hexadecane:	

Benzidine dihydrochloride:	Sigma Chemical Co Ltd Poole, Dorset
Bovine serum albumin (Fraction V):	
Chloramine T:	
Cytochrome C (horse heart):	
Dextran (Clinical grade):	
Dextran Blue:	
Pepstatin A:	
Phenylmethylsulphonyl-fluoride (PMSF):	

All the above are from Sigma Ltd

Acetonitrile (HPLC grade):	Rathburn Chemicals, Peebleshire, Scotland
Acid tartrate nor-adrenaline:	Wintrop, London
Carbachol BP:	MacCarthy's, London

Charcoal (Norit GSX):	Hopkin & Williams Ltd Chadwell Heath, Essex
Heparin:	CP Pharmaceuticals Clwyd
Human serum albumin:	Lister Blood Products Lab, Elstree, Herts
Intralipid (10 %): (fractionated soya oil, 100 g; glycerol, 22.5 g)	KabiVitrum, London
Oxygen (95% O_2, 5% CO_2):	BOC, London
Phosphate buffered saline: pH 7.4	Oxoid Ltd, Basingstoke, Hants
Sephadex G 50 superfine: QAE Sephadex A25:	Pharmacia Fine Chemicals, Uppsala Sweden
M Tuberculosis (attenuated, heat-killed):	Ministry of Agriculture, Fisheries & Food, Central Veterinary Laboratory, Weybridge, Surrey

Equiments

Balance:-

Cahn Electrbalance (Model M-10):	Cahn Instrument Co, Paramount, Calif, USA
Oertling (Model 1201):	Orpington, Kent
Sartorious:	London

Centrifuges:-

Bench-top (EBA 35):	A R Howell, London
MSE Coolspin (GF8):	MSE Scientific Instruments, Sussex
IEC 6000B:	Damen/IEC (UK) Ltd, Dunstable, Beds

Chromatography:-

Pharmacia columns: Frac-100 fraction collector:	Pharmacia Fine Chemicals, Uppsala, Sweden
HPLC system: u-Bondapak C18 column: Sep-pak C18 cartridge:	Waters Associates, Milford, Mass, USA

Equipment	Supplier
HPLC system: Techsil C18 column:	HPLC Technology, Cheshire
Dispensers:-	
Hamilton PB 600 dispenser:	V A Howe & Co, London
Hamilton gas-tight syringes, £1725, £1750 £1002:	V A Howe & Co, London
Eppendorf dispenser and Combitips (10 - 250 ul):	Eppendorf, Hamburg West Germany
Microcaps (1 - 100 ul):	Volupette Pipets, DADE Division, Miami, Fl, USA and Volupettes, Medikent, London
Gamma-counter:-	
NE 1600:	Nuclear Enterprises, Edinburgh, Scotland
Wilj:	Electronics Ltd, Ashford, Kent
Homogeniser:-	
Ultra-Turrax:	Scientific Instruments, London
Lyophiliser:-	
Edwards High Vacuum:	Edwards, Crawley, Sussex
SpeedVac concentrator:	Savant, Mass, USA
Peristaltic Pump:-	
Pharmacia (Model P-3):	Pharmacia Fine Chemicals, Uppsala, Sweden
Autoanalyser pump:	Technicon, Tarrytown New York , USA
pH meter:-	
Corning pH meter 125:	London
Sample and Assay tubes:-	
GW3 polystyrene tubes:	Seward Labs, London
GW4 Polystyrene tubes:	Seward Labs, London

Glass vials (freeze drying):	Anchor Glass, London
Lithium heparin tubes:	Seward Labs, London
LP3 polystyrene tubes (10.5 x 63.5 mm):	Luckham Ltd, Burgess Hill, Sussex
Nunc cryoyubes (1.8 - 4.5 ml):	Nunc InterMed, DK-4000 Roskilde, Denmark
Polystyrene tubes (75 x 12 mm)	Sarstedt Ltd, Leicester, Leic
Universal containers	Sterilin, London
Z35 plastic containers	Seward Labs, London

Appendix and abbreviations

<u>Appendix A</u>: Formulae for calculations

<u>Appendix B</u>: Buffers and solutions

<u>Appendix C</u>: Cross-reactivity of neurotensin antisera

<u>Appendix D</u>: Binding curves of NT 58 with neurotensin and its fragments

Abbreviations

pmol/l	picomols per litre
mmol/l	millimoles per litre
Kg	kilogram
KJ	Kilojoule
Ci	Curie
Bq	Becquerel
HSA	Human serum albumin
BSA	Bovine serum albumin
KIU	Kallikrein inactivator unit
Ab	antibody
Ag	antigen
HPLC	high pressure liquid chromatography
RIA	radioimmunoassay
N/cm	newton per centemiter
NT	neurotensin
NTLI	neurotensin-like immunoreactivity
Min	minute
cpm	counts per minute

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This thesis is dedicated to the memory of my mother, Mrs Pui-Yue Yeung Lee.

Chapter 1

GENERAL INTRODUCTION

Discovery and isolation

In the course of the purification and isolation of substance P from bovine hypothalamus, Carraway and Leeman (1973) discovered a bioactive material which was chromatographically different from substance P in their column eluates. When this material was injected into the rat intravenously, it caused vasodilation of the exposed cutaneous region and hypotension. Because of its neural origin and its hypotensive effect, Carraway and Leeman (1973) named it neurotensin. Subsequently, peptide sequence analysis demonstrated that neurotensin consists of thirteen amino acid residues with pyroglutamate at its amino-terminal and a free carboxy group at its carboxy-terminal (Carraway & Leeman 1975a). In addition to its isolation and purification from bovine hypothalamic extracts, neurotensin was also isolated from calf and human gastrointestinal tract (Kitabgi et al 1976, Carraway et al 1978, Hammer et al 1980).

Structural homology

The amino acid sequence of neurotensin (Figure 1:1) isolated from the hypothalamus and gastrointestinal tract has been shown to be identical for several mammalian species. These species include bovine, rodent, porcine and man. Several peptides structurally related to neurotensin have also been isolated and sequenced from mammalian and non-mammalian vertebrates. For example, there is structural homology between neurotensin and Xenopsin, a peptide isolated from the skin of Xenopus laevis (Araki et al 1973). Carraway and Bhatnagar (1980a) reported the isolation of a thirteen amino acid peptide from chicken tissues (Figure 1:1). This thirteen amino acid peptide from chicken may represent the avian equivalent of mammalian neurotensin. This was followed by the isolation of a hexapeptide (Lant-6) from chicken intestine by Carraway and Ferris (1983a). Lant-6 immunoreactivity has been reported to be present in the rat central nervous system and gastrointestinal tract (Carraway et al 1983b). However, Goedert and co-workers reported that immunoreactive lant-6 was present in chicken but absent in rat tissues, and they proposed that the previously reported lant-6 immunoreactivity in rat tissues may be an

<u>Neurotensin</u>

pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-
Arg-Arg-Pro-Tyr-Ile-Leu-OH

<u>Neuromedin N</u> Lys-Ile-Pro-Tyr-Ile-Leu-OH

<u>Xenopsin</u> pGlu-Lys-Arg-Pro-Trp-Ile-Leu-OH

<u>Chicken neurotensin</u>

pGlu-Leu-His-Val-Asn-Lys-Ala-

Arg-Arg-Pro-Tyr-Ile-Leu-OH

<u>Lant-6</u> Lys-Asn-Pro-Tyr-Ile-Leu-OH

Figure 1:1. Amino acid sequences of neurotensin and peptides structurally related to neurotensin.

extraction artefact (Goedert et al 1985). More recently another structurally related peptide, namely neuromedin N, a six amino acid peptide, has been isolated from porcine spinal cord (Minamino et al 1984). It is noteworthy that a high degree of sequence homology is observed between all these recently isolated peptides and the carboxy-terminal of mammalian neurotensin. In fact the last four amino acid residues of the carboxy-terminal of all these peptides are identical to those of mammalian neurotensin (Figure 1:1). This suggests that the carboxy terminal of neurotensin is highly conserved during evolution.

Structure-activity

Structure-activity studies have indicated that the amino acids likely to be responsible for the biological activity of neurotensin reside at the C-terminal region of the molecule (Carraway et al 1975b). However the potency of these C-terminal fragments or analogues constitutes less than 30 % of the activity of neurotensin itself (Kitabgi 1982). Neurotensin 1-13 amide and amino-terminal partial sequences of neurotensin including neurotensin 1-8 and neurotensin 1-10 are biologically inactive (Leeman and Carraway 1982). Consistent with structure-activity studies of

neurotensin, the peptides structurally related to neurotensin, whether from chicken tissues, or porcine spinal cord, exhibit similar pharmacological actions to those of neurotensin (Carraway et al 1980a, Minamino et al 1984, Kalivas et al 1986). These peptides are structurally similar to the C-terminal region of the neurotensin molecule.

Distribution and cellular localization

With the development of radioimmunoassay for the measurement of neurotensin together with immunocytochemistry, the distribution of neurotensin has been described in various mammalian species (Uhl & Snyder 1976, Kobayashi et al 1977, Cooper et al 1981, Holzer et al 1982, Emson et al 1982a, Goedert & Emson 1983a).

In the central nervous system (CNS) highest concentrations of immunoreactive neurotensin were found in the hypothalamus, moderate amounts in the thalamus and amygdala, and lowest concentrations in the cortical areas (Carraway and Leeman 1976a, Kobayashi et al 1977). Within the hypothalamus, immunocytochemical analysis demonstrated the presence of neurotensin in multiple clusters of neuronal cell bodies and terminals within the

arcuate and paraventricular nuclei (Uhl et al 1977a, Kahn et al 1980,1982). Neurotensin-containing fibres have also been found projecting to the hypophysial portal system in the median eminence (Uhl and Snyder 1979). The presence of neurotensin has also been reported in the anterior lobe of the pituitary (Goedert et al 1982). In the spinal cord neurotensin was mainly found in the substantia gelatinosa (Gibson et al 1981, Hunt et al 1981). This distribution pattern in the CNS suggests that neurotensin may have possible roles in the regulation of neuroendocrine function.

In the peripheral organs, neurotensin has been shown mainly present in the gastrointestinal tract (Orci et al 1976, Sundler et al 1977) where it had a discrete distribution (Carraway and Leeman 1976b). In man radioimmunoassay and immunocytochemical studies demonstrated that neurotensin was mainly localised to the mucosal epithelium of the ileum within endocrine-like cells, namely the "N" cells (Polak et al 1977, Ferri et al 1983).

Immunoreactive neurotensin has been reported in the cat and rat adrenal glands (Lee et al 1981, Terenghi et al 1983, Rokeaus et al 1984), and in the heart of the guinea pig (Reinecke et al 1982).

However the presence of neurotensin-like immunoreactivity in these organs appeared not to be universal in all mammalian species investigated.

Release of neurotensin

Subcellular fractionation of rat hypothalamic homogenates demonstrated that highest concentrations of immunoreactive neurotensin were in the synaptosomal fraction (Uhl and Synder 1977b). The affinity of neurotensin to brain membranes was reported to be proportional to the distinctive distribution of immunoreactive neurotensin in the different regions of the brain (Uhl and Synder 1977b). Highest affinity was demonstrated in the hypothalamus and lowest in the cerebellar cortex. Iversen et al (1978) observed that in rat hypothalamic slice superfusion experiments there was a calcium-dependent release of neurotensin in the presence of potassium. Furthermore, rapid inactivation and degradation of neurotensin by as yet unidentified endopeptidases has been documented (Macdermott et al 1982, Checler et al 1983, Emson et al 1985b). These data suggest that neurotensin may play a neurotransmitter and/or neuromodulator role.

In response to food ingestion, neurotensin is

released into the general circulation both in animal and man (Mashford et al 1978a, 1978b; Blackburn and Bloom 1979). The most potent stimulus is fat (Rosell and Rokaeus 1979). Because of the marked increase of plasma immunoreactive neurotensin following fat ingestion, and its effects on gastric acid secretion and gastric motility, neurotensin has been postulated to be an enterogastrone (Rosell 1982, Fletcher et al 1985).

Biological action

Both in vitro and in vivo studies have demonstrated that neurotensin has potent and diverse pharmacological effects in the central nervous system and the periphery.

Central effects

In the central nervous system the effects of exogenous neurotensin include hypothermia, antinociception and alteration in locomotor activity (Martin et al 1980, Prange and Nemeroff 1982). In rat a significant reduction in core temperature was observed when neurotensin was injected into the anterior and dorsomedial hypothalamus (Nemeroff et al 1979, Martin et al 1980). Chandra et al (1981) postulated that this

effect was primarily caused by neurotensin reducing the rate of metabolism and hence of heat production and core temperature. The exact role and action of neurotensin in temperature control remains to be clarified. Nemeroff et al (1979) reported that in rat the antinociceptive potency of neurotensin was exceedingly high, much greater than that of morphine or enkephalin and was only exceeded by that of beta-endorphin. Ervin et al (1981) found that neurotensin inhibited amphetamine-induced locomotion when neurotensin was injected into the nucleus accumbens, a mesolimbic dopamine terminal area; while Kalivas et al (1984) reported that intra-nucleus accumbens neurotensin injection suppressed the behavioural hyperactivity seen when neurotensin was injected into the ventral tegmental area (VTA), the site of origin of the mesolimbic dopamine system. The bilateral intra-VTA injection of neurotensin produced a dose-dependent increase in locomotion in the rat (Kalivas et al 1983). The reason for these conflicting findings is not clear. However it has been postulated that the action of neurotensin on locomotor activity is site dependent as well as site selective (Prange and Nemeroff 1982). In addition, it appeared that intact dopaminergic transmission may be required for the behavioural effects of neurotensin (Nemeroff and Cains 1985), as it has been

demonstrated that destruction of mesolimbic dopamine by hypothalamic injection of 6-hydroxydopamine or pre-treatment with haloperidol abolishes the locomotion stimulation seen by intra-VTA injection of neurotensin (Kalivas et al 1983). It is well established that the mechanisms of dopamine synthesis in all dopamine-containing neurons in the CNS are identical, however, these neurons do not function identically.

Whether neurotensin is co-localised with dopamine is not clear. However, neurotensin receptors have been reported within the dopamine neurons in the rat mid brain (Palacios and Kuhar 1981). Experiments using isolated rat hypothalamic-pituitary axis preparations have shown that neurotensin enhanced the release of dopamine evoked by electrical and pharmacological stimulation (Davis and Kilt 1987).

Sheppard et al (1979) showed that neurotensin increased the release of somatostatin from isolated hypothalamic slices, while a dose-dependent release of somatostatin into the hypophysial portal blood was produced by the central adminstration of neurotensin (Abe et al 1981). Neurotensin may thus have a direct action on the hypothalamus. Neurotensin is present in the anterior pituitary

(Goedert et al 1982) and has been reported to affect the secretion of growth hormone, prolactin and luteinising hormone (Rivier et al 1977, Vijayan and McCann 1979, 1980). The release of these neuroendocrine hormones by neurotensin depends on whether neurotensin is injected centrally or peripherally. Abe et al (1981) reported that there was a reduction of growth hormone secretion from the anterior pituitary when neurotensin was administered via the cerebral ventricles in the rat. However, when injected peripherally a marked increase of growth hormone release was observed (Rivier et al 1977, Maeda and Froham 1978). Similarly neurotensin suppressed the release of prolactin and luteinising hormone when it was introduced centrally (Maeda and Froham 1978) whereas an increase of plasma prolactin and lutenising hormone was reported when neurotensin was administered systemically (Maeda and Froham 1978). The reason for these opposing effects of neurotensin between central and peripheral administration in animals is unclear.

The exact roles of neurotensin on pituitary function are complex. In view of the possible interaction of neurotensin and dopamine which is thought to have a general inhibitory effect on pituitary hormone secretion, the effect of

neurotensin discussed above with regard to pituitary function may be an indirect one involving multiple factors and neurotensin may act merely in a paracrine fashion.

Peripheral effects

Although neurotensin was first isolated and purified from the hypothalamus, relatively high concentrations of neurotensin are present in the gastrointestinal tract (Carraway and Leeman 1976b). It is well established that neurotensin is released into the circulation in response to physiological stimuli such as nutrient ingestion (Rosell and Rokaeus 1979, Blackburn and Bloom 1979, Go and Demol 1981). Because of its presence in plasma after food ingestion, much interest has been focused on its possible gastrointestinal effects. These possible gastrointestinal actions include reduction of gastric acid secretion (Andersson et al 1976, Kihl et al 1981), delaying of gastric emptying (Blackburn et al 1980a), inhibition of gut motility (Thor et al 1980) and the stimulation of exocrine and endocrine pancreatic secretion (Blackburn et al 1981a, Fletcher et al 1981, Baca et al 1982). Neurotensin was also reported to induce hypercholesterolaemia (Peric-Golia et al 1979) and to influence intestinal transport

(Mitchenere et al 1981).

In man, Blackburn et al (1980b) reported that intravenously infused neurotensin had no effect on pulse rate, blood pressure or the release of gastrointestinal peptides such as gastrin, motilin, enteroglucagon, glucagon or vasoactive intestinal polypeptide. However, the plasma pancreatic polypeptide concentration was significantly elevated when neurotensin was infused peripherally (Blackburn et al 1980b, Fletcher et al 1981, Shulkes et al 1984). Fletcher et al (1981) also reported that neurotensin stimulated pancreatic bicarbonate output and caused a small reduction of pancreatic trypsin. These effects may have resulted from the elevated plasma pancreatic polypeptide.

Discussion and Purpose of study

Nucleotide sequence analysis of cloned cDNA that encodes neurotensin has revealed the primary structure of a precursor protein (Dobner et al 1987) which encodes not only neurotensin but also neuromedin N, one of the mammalian peptides recently isolated from porcine spinal cord (Minamino et al 1984). Neurotensin and neuromedin N may thus be derived from the same precursor. The comparative distribution of neurotensin and

neuromedin N is not known. Neurotensin is released into the general circulation after a physiological stimulus such as nutrient ingestion. This response has been reported to be biphasic with an early peak within 30 minutes followed by a delayed peak (Rosell and Rokeaus 1979). This suggests that there may be a neural influence on its release. Neurotensin has a very short half life in plasma as was demonstrated when neurotensin was infused intravenously in the rat (Aronin et al 1982). However, the metabolic fates of either exogenous or endogenous neurotensin in man is not known. In addition the possible effects of neurotensin fragment, neurotensin 1-8, have not been investigated in man. Although neurotensin was discovered almost fifteen years ago, its exact physiological actions remain unclear. That neurotensin functions as a circulating hormonal factor is still open to discussion. One possibility is that neurotensin mediates the physiological response to stimuli in concert with other regulatory substances. Alternatively, the neurotensin released may act in a paracrine fashion.

This thesis begins by describing a radioimmunoassay for the measurement of plasma and tissue concentrations of immunoreactive neurotensin

(chapter 2). The distribution of immunoreactive neurotensin in extracts of rat peripheral tissues is described and compared with that of neuromedin N (chapter 3). In addition, the distribution of immunoreactive neurotensin in the central nervous system of rat and man is reported. The regulation of release and synthesis of neurotensin is investigated using a rat isolated ileal preparation (chapter 4) and a rat phaeochromocytoma (PC 12) tissue culture cell line (chapter 5). The possible influence of brain-gut peptide in the meal-stimulated release of neurotensin is examined using a synthetic somatostatin analogue (SMS 201-995) (chapter 4). The pharmacokinetics of neurotensin and its N-terminal fragment, neurotensin 1-8, in the human circulation are studied and compared (chapter 6). The metabolism of infused and endogenously released neurotensin is investigated in man (chapter 6,7). Possible means of the preservation of neurotensin in human plasma is addressed (chapter 8).

Chapter 2

RADIOIMMUNOASSAY OF NEUROTENSIN

Introduction

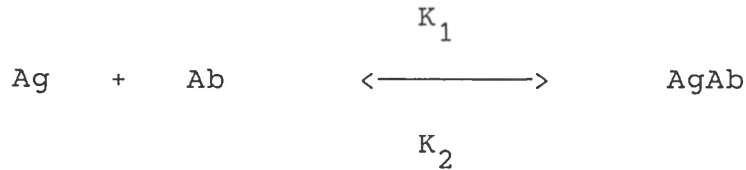
Historical Background

In the 1950s, Berson and Yalow, while studying the behaviour of ^{131}I labelled insulin in insulin-dependent diabetes, showed that the tracer could be displaced from the insulin-binding protein, i.e. insulin antibody, by the addition of a large quantity of unlabelled insulin. They recognized that the degree of binding of ^{131}I insulin was quantitatively related to the total amount of insulin present (Berson et al 1956). This subsequently formed the basis of the first report of the measurement of plasma insulin in man by radioimmunoassay (Yalow & Berson 1959). In the meantime, Ekins developed the method of measurement of thyroxin in human plasma with thyroxin-binding globulin (Ekins 1960).

Principle of radioimmunoassay

The principle of radioimmunoassay (RIA) may be

considered through the antigen(Ag)-antibody(Ab) reaction:



$$Ag + Ab \underset{K_2}{\overset{K_1}{\longleftrightarrow}} AgAb$$

where K_1 and K_2 are the rate constants for the forward and reverse reaction respectively. The absolute rate, i.e. the number of molecules which react in unit time, is dependent on the concentration of the molecules. At equilibrium, the formation of the antigen-antibody complex is equal to the dissociation of this complex to free antigen (Ag) and antibody (Ab) molecules.

This reversible reaction between antigen and antibody can be described in thermodynamic terms by the Law of Mass Action which states that at equilibrium the ratio of the products of the concentrations of the two sides of the equation will be constant:

$$K_a = \frac{[Ag\ Ab]}{[Ag]\ [Ab]}$$

where K_a is the affinity constant which provides a measure of the energy of the reaction between the

antigen and antibody. In binding assays, the affinity constant is often used to describe the avidity of the antiserum. At equilibrium, the affinity constant governs the quantitative relation between the concentration of free antigen, free antibody, and the antibody bound antigen. In most radioimmunoassays, iodinated antigen is used together with the biological samples in which endogenous antigen (Ag) may or may not be present. Assuming that the iodinated antigen and the endogenous antigen behave identically, the ability of the antigen present to inhibit the binding of the labelled antigen to its specific antibody forms the basis of the principle of radioimmunoassay. In the assay, labelled antigen and antigen (samples or standards) are incubated together with a limited amount of antibody. After reaching or approaching equilibrium, free labelled antigen and antibody bound antigen are separated, and the radioactivity of one, or the other, or both fractions is determined. When increasing concentrations of endogenous or added antigen are present, the ratio of the bound to free labelled antigen will decrease quantitatively with respect to the concentration of the unlabelled antigen present.

In this study, a RIA with an antiserum suitable for the measurement of tissue and unextracted plasma

neurotensin-like immunoreactivity (NTLI) was developed and validated.

Materials and Methods

Preparation of antigenic conjugate of neurotensin:

Neurotensin (1.2 mg, 800 nmol) was dissolved in freshly prepared bis-diazotised benzidine solution (250 ul, 1 umol) containing 80 umol (20.5 mg) benzidine dihydrochloride dissolved in 10 ml of 0.18 M HCL and mixed gently overnight at $4^{0}C$ with 1 ml of 0.16 M $NaNO_2$ (11 mg). 0.25 ml $NaHCO_3$ (40 umol, 3.4 mg) was added and followed immediately by the addition of 0.5 ml bovine serum albumin (14 mg, 100 nmol). The pH was adjusted to 9.8 with 0.5 M NaOH. The conjugate was lyophilized and stored at $-20^{0}C$ until use.

Immunisation:

The conjugate was dissolved in water (6 ml) and emulsified with an equal volume of Freund's complete adjuvant. The mixture was administered to 12 rabbits (1 ml each), each of which received approximately 100 ug neurotensin. Each rabbit received four subcutaneous injections, one into each groin and axilla. The animals were boosted

two months later with 50 ug neurotensin coupled to BSA in Freund's incomplete adjuvant and bled from the ear vein seven days and ten days after the boost. Subsequent boosts were carried out at 5 - 8 week intervals.

Antiserum titre:

The antisera harvested after each bleed were tested for their titre and displacement with labelled neurotensin. The titre was determined as the reciprocal of the dilution of antiserum which binds 45 - 55 % of the labelled neurotensin.

Five serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) of each antiserum were prepared in 0.06 M phosphate buffer containing EDTA and 0.3 % gelatin, pH 7.2 (assay buffer). Sample tubes contained 0.1 ml labelled neurotensin (2000 - 2500 cpm, equivalent to 1.0 - 2.0 fmol labelled neurotensin), 0.1 ml of an antiserum dilution and 0.8 ml of assay buffer. Thus the final dilution of the antiserum was 1/10 of the initial dilution. The incubation period of the assay was five days at 2^0 - 8^0C. At the end of the incubation the free labelled neurotensin was separated from the bound by the antiserum with the addition of 0.25 ml of dextran-coated activated charcoal containing 8.0 mg

activated charcoal and 0.8 mg dextran. The assay was performed in duplicate. To investigate the displacement with labelled neurotensin, synthetic neurotensin (Peninsular Laboratories, Merseyside, England), 0.02 ml (1.0 pmol/ml), was added to another series of assay tubes identically prepared and assayed as above.

The radioactivities of supernatant containing the antibody bound fraction and precipitate containing the free labelled fraction adsorbed to the charcoal were counted in a 16 well gamma counter (Nuclear Enterprise Ltd, Scotland) to obtain over 4000 counts or for 3 minutes . This allows results to be obtained with less than a 1.6% error due to counting.

Antiserum specificity:

The regional specificity of the antiserum chosen was investigated with neurotensin, and synthetic fragments of neurotensin including neurotensin 1-8, 1-9, 1-11 and 6-13, 7-13, up to 1×10^5 fmol/tube. The cross-reactivity of the neurotensin antiserum with structurally related substances, for example, lant 6 and neuromedin N, and with other brain-gut peptides icluding substance P, vasoactive intestinal peptide, cholecystokinin, gastrins,

pancreatic polypeptide, somatostatin, enkephalins, endorphins and bombesin was also studied.

Iodination of neurotensin:

[^{125}I]-neurotensin was prepared by the chloramine T method (Hunter and Greenwood 1962).

Neurotensin, 10-20 ug, was dissolved in 50 ul of 0.4 M phosphate buffer (pH 7.4) in a small conical vial. Na^{125}I (0.5 mCi, 5 ul) (Amersham International plc, Amersham, England or New England Nuclear Inc, Mass, USA) was added to the peptide solution and mixed. This was followed by the addition of 5 ul 0.4 % (wt/vol) chloramine T solution freshly prepared in 0.04 M phosphate buffer. The reaction time was 10-15 seconds and the reaction was stopped by the addition of 20 ul 0.24 % (wt/vol) sodium metabisulphite in 0.04 M phosphate buffer. This was followed by the addition of 50 ul 1 % (wt/vol) potassium iodide solution in 0.04 M phosphate buffer.

The iodination product was purified by gel-filtration or reverse phase high pressure liquid chromatography (HPLC). For purification by gel-filtration (Jorgensen and Larsen 1972), the reaction mixture was loaded on a QAE-Sephadex A25

column (Pharmacia, Uppsala, Sweden) previously equilibrated with Tris buffer containing 0.3 % BSA as column eluent. Fractions of 1 ml were collected every 15 minutes. For purification by reverse phase HPLC, the reaction mixture was loaded onto a Techsil C18 column (HPLC Technology, Cheshire, U K) eluted with a stepwise gradient of acetonitrile from 20 % to 80 % (vol/vol) containing 0.05 % aqueous trifluoroacetic acid (TFA) in water. The flow rate was 1.0 ml/min.

Immunological characterisation of the labelled neurotensin:

The peak fraction and two fractions on either side of the radioactive neurotensin peak were initially tested by RIA with the chosen antiserum (see result section below, p43) for non-specific (blank) and excess antibody binding. The fraction of the [^{125}I]-neurotensin preparation which gave the lowest blank (in the absence of antiserum), less than 5 % bound, and highest excess binding (1 ul neat antiserum), greater than 90 % bound, was further investigated for its immunoreactive characteristics in comparison with the unlabelled neurotensin.

The immunological identity of [^{125}I]-neurotensin

was characterised in two ways:- (1). Two dose response curves were constructed in duplicate; one with synthetic neurotensin containing 1, 2, 3, 5, 10, 15, 20 and 25 fmol neurotensin per tube, another with increasing concentrations of labelled neurotensin: 1, 2, 3, 5, 10, 15, 20, 25 fmol labelled neurotensin per tube. (2). Three antiserum dilution curves were constructed with the antiserum dilution ranging from 10^{-2} to 10^{-7}. The first dilution curve contained approximately 10.0 fmol (100 ul) labelled neurotensin per tube. The second and third dilution curves contained 1.0 fmol (10 ul) of labelled neurotensin per tube. No synthetic neurotensin was added to the first dilution curve. However, synthetic neurotensin was added to all tubes in the second (5.0 fmol per tube) and third (10.0 fmol per tube) dilution curves.

All assay tubes in the three antiserum dilution curves contained a total assay volume of 1.0 ml.

The assay was incubated at 2^{0} - 8^{0}C for five days and bound and free fractions separated by dextran-coated charcoal (8 mg per tube).

Results

Immunisation response and affinity constant of antiserum NT 58:

After the third boost, one of the rabbits produced an antibody against neurotensin, which was designated NT 58. At the final antibody titre of 1 in 80,000, a percentage bound (% bound) ratio of 50 % was obtained in the absence of neurotensin, and showed a dose response curve with synthetic neurotensin (Figure 2:1). The sensitivity of the assay was calculated to be 0.4 fmol/tube (see appendix A for calculation) with an assay incubation period of five days. The intra- and inter-assay coefficients of variation were 4 % and 11 % respectively (see appendix A). The affinity constant of antiserum NT 58 was determined by Scatchard analysis and calculated to be 4.4×10^{11} l/mol (Figure 2:2 and appendix A).

Specificity of antiserum NT 58:

The crossreactivity of antiserum NT 58 was calculated, with respect to neurotensin which was taken as 100%, to be 68 % with neurotensin 7-13, 58 % with neurotensin 6-13, and less than 0.1 % with neurotensin 1-9 and neurotensin 1-8 (Table

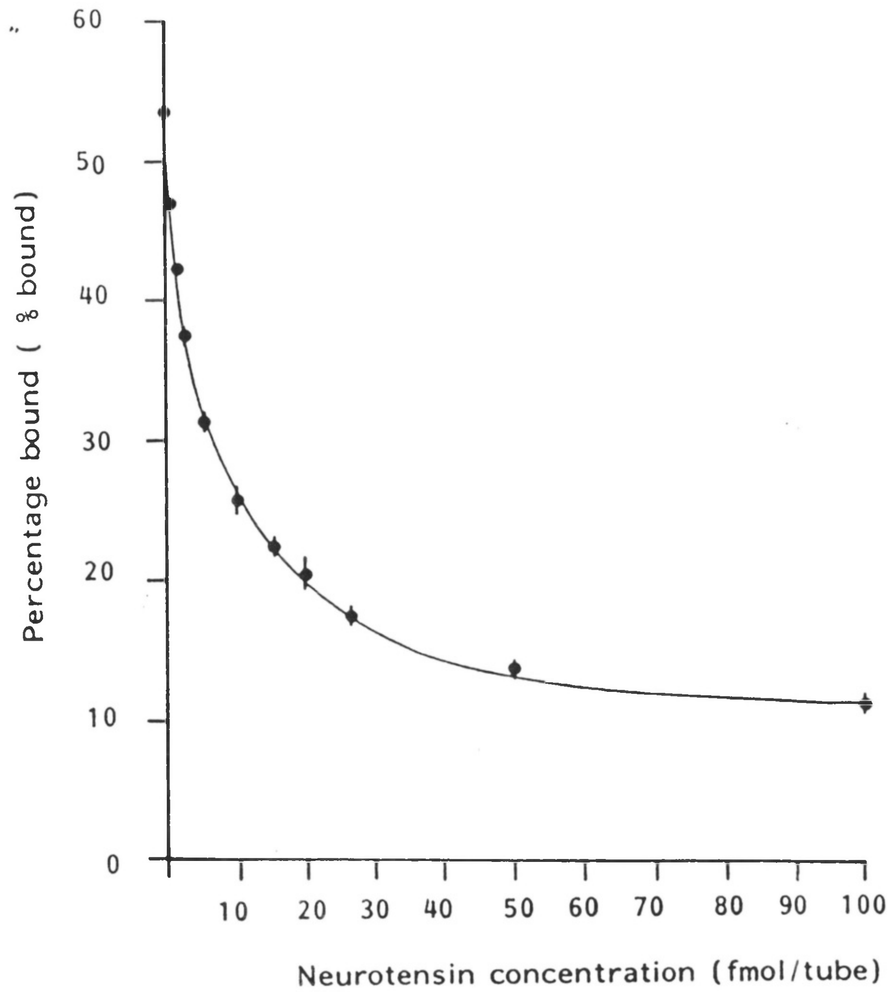


Figure 2: 1. Neurotensin standard curve (n = 4), each point is the mean and the SEM of the duplicate readings. Four standard curves were set up in the same assay.

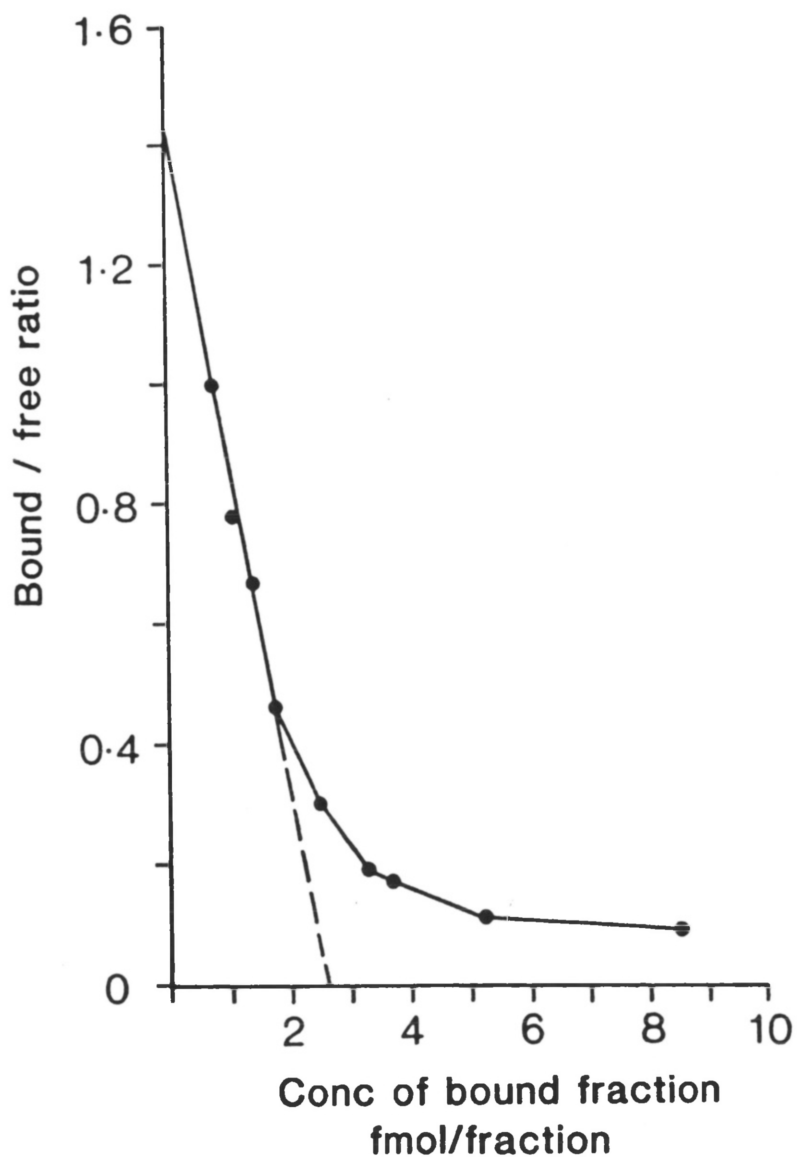


Figure 2:2. Scatchard plot. The intercept on the vertical axis (Y) is K (affinity constant) x N (concentration of antibody binding site in mole) and the intercept on the horizontal axis is N (calculation, see appendix A).

Table 2:1. Binding of antiserum NT 58 to neurotensin and its fragments.

Peptides or fragments	Cross-reactivity
Neurotensin	100 %
Neurotensin 6-13	58 %
Neurotensin 7-13	68 %
Neurotensin 1-8	< 0.1 %
Neurotensin 1-9	< 0.1 %
Neurotensin 1-11	< 0.1 %

The crossreactivity was relative to neurotensin to which the binding was taken as 100 %.

2: 1). It was thus concluded that the antiserum NT 58 is directed toward the C-terminal region of the neurotensin molecule. No cross-reactivity (less than 0.01 %) was found with the peptides structurally related to neurotensin: lant-6 or neuromedin N. There was no cross-reactivity (less than 0.01 %) of NT 58 with the other brain-gut peptides: substance P, vasoactive intestinal polypeptide, cholecystokinin, gastrins, pancreatic polypeptide, somatostatin, enkephalins, endorphins and bombesin.

Characterisation of labelled neurotensin:

The purification profiles of neurotensin iodination product by gel filtration or reverse phase HPLC are shown in Figure 2. 3. Labelled neurotensin was shown to be immunologically similar to unlabelled neurotensin both in the assay and in the antiserum dilution curves (Figure 2: 4 and 2: 5). The specific activity of the [^{125}I]-neurotensin was calculated to be over 60 Bq/fmol by the displacement method (Sarson 1982)

Radioimmunoassay of neurotensin

The assay for the measurement of plasma immunoreactive neurotensin is a modification of

(a)

(b)

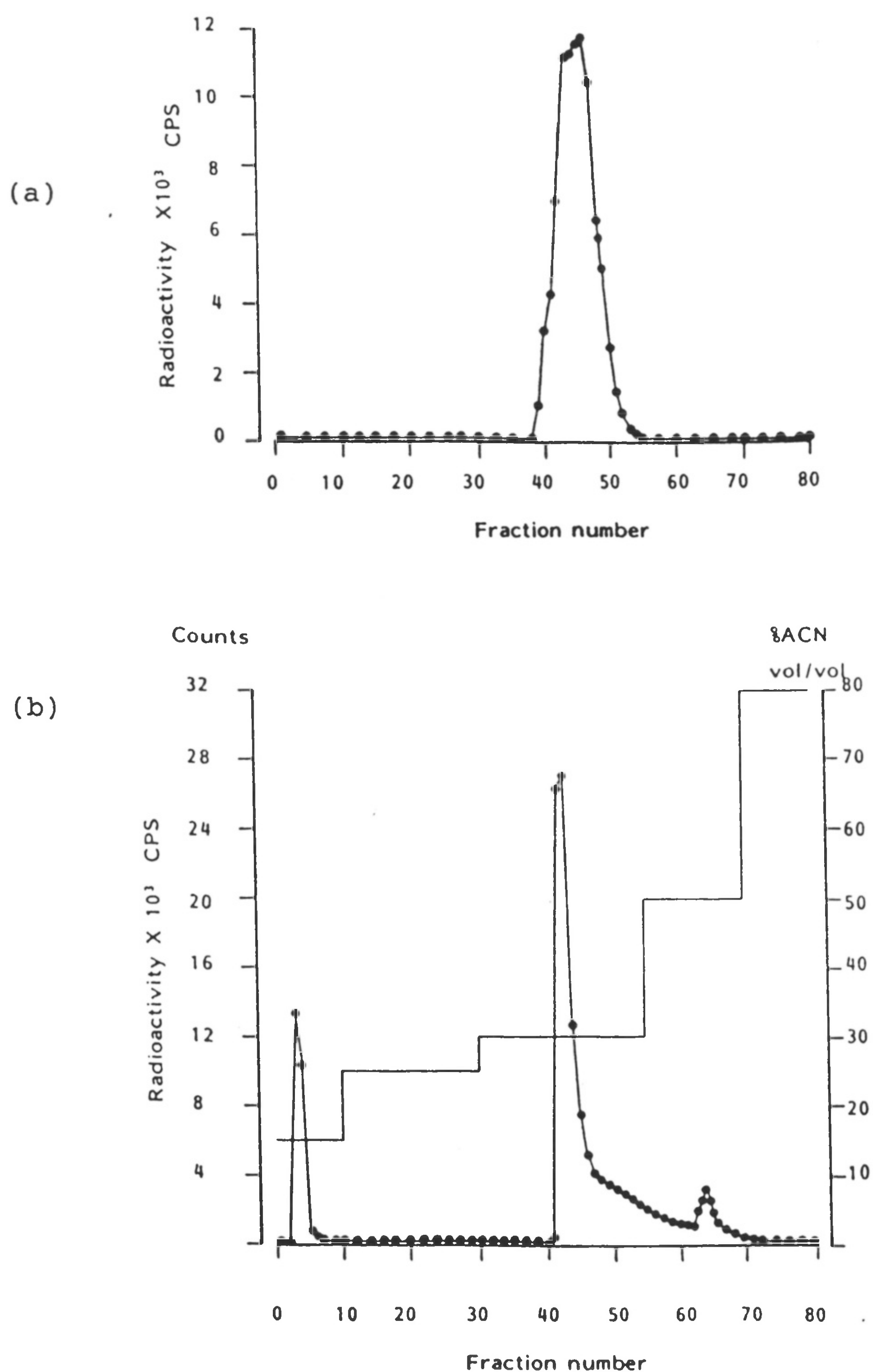


Figure 2:3. Neurotensin iodination profiles, the iodination product when purified by (a) gel filtration (upper) or (b) HPLC. Gel filtration was performed with a QAE A25 column eluted with Tris buffer containing 0.3 % BSA. HPLC purification was with a stepwise gradient of acetonitrile (vol/vol) with water in 0.05 % aqueous trifluoroacetic acid from 20 - 80 % over a period of 80 min

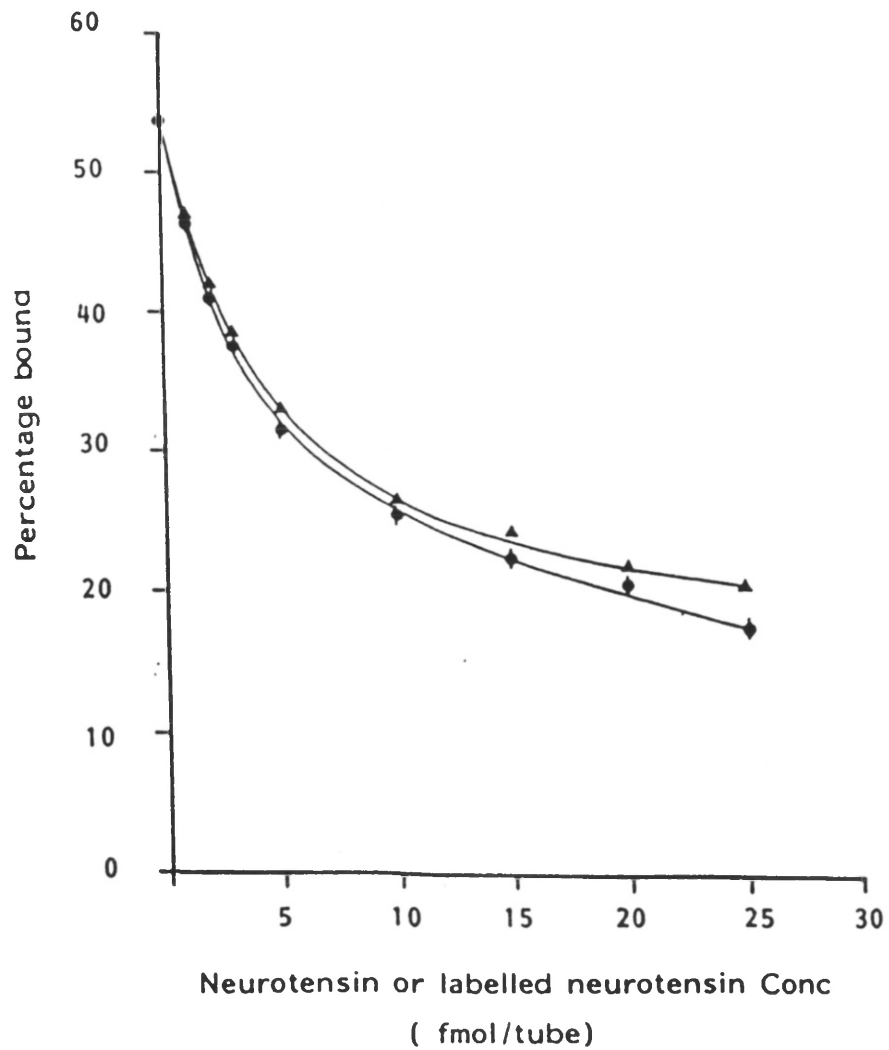


Figure 2:4. Neurotensin binding curves, (●) with increasing amount of synthetic neurotensin; (▲) with increasing amount of labelled neurotensin. To both curves, a fixed amount (1.0 fmol labelled NT per tube) was added.

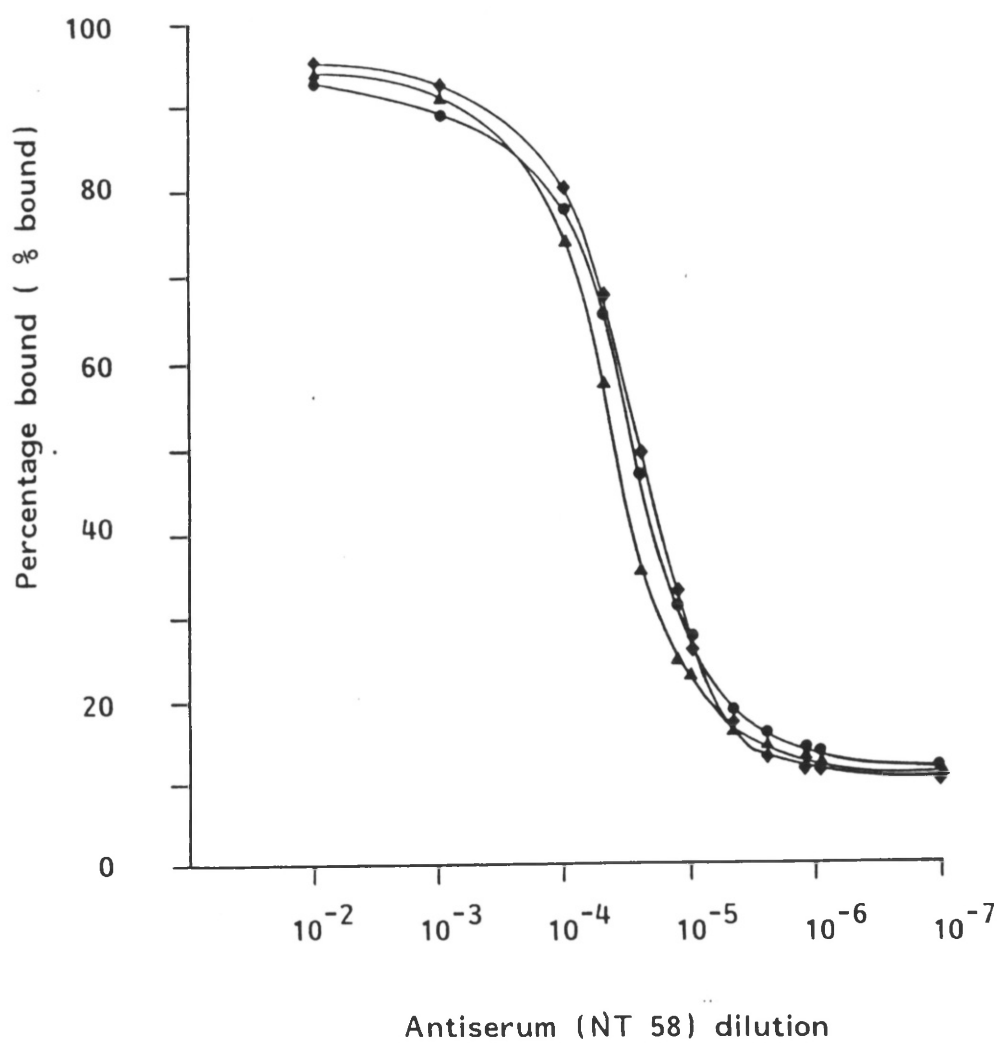


Figure 2:5. Antiserum (NT 58) dilution curves; (●) with 10.0 fmol labelled neurotensin added per tube, (◆) with 1.0 fmol labelled neurotensin and 5.0 fmol synthetic neurotensin added per tube, (▲) with 1.0 fmol labelled neurotensin and 10.0 fmol synthetic neurotensin added per tube.

that described by Blackburn and Bloom (1979).

Radioimmunoassay was performed using antiserum NT 58 with labelled neurotensin which was immunologically identical to synthetic neurotensin in 0.06 M phosphate EDTA (10 mM) with 0.3 % gelatin buffer, pH 7.2. The assay was incubated for five days at 2^0C - 8^0C. The total assay volume was 0.8 ml containing 0.1 ml antiserum NT 58 giving a final dilution of 1 in 80,000 and 0.1 ml [^{125}I] neurotensin (2000 - 2500 cpm, equivalent to 1.0 - 2.0 fmol labelled neurotensin). In the case of assay for plasma, a sample volume of 0.2 ml unextracted plasma was used, whereas in the case of assay for tissue extracts, the sample volumes were 0.01 ml (10 ul) and 0.001 ml (1.0 ul). A standard RIA protocol is shown in Table 2:2.

Each standard curve consisted of ten standards containing 1, 2, 3, 5, 10, 15, 20, 25, 50 and 100 fmol synthetic neurotensin per tube. Duplicate standard curves were set up. The standard was added from the working standard vial containing 1.5 pmol neurotensin reconstituted in 1.5 ml assay buffer, i.e. 1 pmol/ml. In each assay, there were also duplicate tubes without antibody; i.e. blanks, to estimate non-specific binding. In addition, control tubes containing an excess of antibody

Table 2:2. Radioimmunoassay protocol of neurotensin.

Radioimmunoassay Format of Neurotensin

Tube	Buffer (ul)	Antibody (ul)	^{125}I-NT (ul)	Std (ul)	Sample (ul)
1-2	500	-	100	-	200*
3-4	450	100	50	-	200*
5-6	300	100	200	-	200*
7-10	400	100	100	-	200*
11-12	400	100	100	1	200*
13-14	400	100	100	2	200*
15-16	400	100	100	3	200*
17-18	400	100	100	5	200*
19-20	400	100	100	10	200*
21-22	400	100	100	15	200*
23-24	380	100	100	20	200*
25-26	375	100	100	25	200*
27-28	350	100	100	50	200*
29-30	300	100	100	100	200*
31-34	400	100	100	-	200*
35-40	400	100	100	-	200
41-42	400	100	100	-	200
43-44	400	100	100	-	200
etc.					

where tubes 7-10 and 31-34 are "zero tube", thus "zero" plasma (200* ul) are added per tube. Tube 35-40 are quality control (QC) tubes where QC plasma (200 ul) are added.

(0.001 ml neat antiserum) were included in all assays to determine immunoprecipitability of the tracer (labelled neurotensin). At frequent intervals in the assay, "zero" tubes which contained antibody NT 58 and labelled neurotensin were placed to monitor potential "drift" of reference binding, i.e. zero binding, through the assay.

For assay of neurotensin in plasma, neurotensin-free plasma (0.2 ml/tube) was added to the standard curve and the "zero " assay tubes to minimise the matrix effects, i.e. to mimic the effects of constituents of the plasma samples to be assayed.

Neurotensin-free plasma was prepared from a pool of fasting human plasma which had a low initial neurotensin concentration. This pooled plasma was mixed with charcoal (10 g/100 ml) for 30 minutes to remove neurotensin. The plasma was then separated from the charcoal by several cycles of filtration and centrifugation. The plasma was then tested for immunoreactive neurotensin before use.

Separation of the antibody bound and free fractions of neurotensin was performed using dextran-coated activated charcoal. The charcoal was added as a

250 ul slurry in assay buffer to each tube. The tubes were agitated and then centrifuged at 2500g for 20 min at 4^0C. The supernatant was aspirated by hand and both the supernatant (antibody-bound fraction) and the charcoal precipitated (antibody free fraction) were counted sequentially in the same wells of a multidetector gamma counter (Nuclear Enterprises, Scotland). The gamma counters were linked via a microprocessor to a count recorder which was connected to a printer (Oki Microline 182). Background counts of the gamma counters were subtracted from the respective counting wells. The data were expressed as percentage bound calculated as:

$$\frac{\text{Counts in bound fraction}}{\text{Counts in bound fraction} + \text{Counts in free fraction}} \times 100\ \%$$

by a computer programme specially written by the Department of Medical Physics, Royal Postgraduate Medical School, for our laboratory. The percentage bound values were plotted against the concentration of neurotensin added to produce a dose-response curve(Figure 2:1) using a desk-top computer (Act Sirius).

Assays for plasma and tissue neurotensin-like

immunoreactivity were similar, except that the latter sample additions were 0.01 ml and 0.001 ml; as the concentration of immunoreactive neurotensin in tissue extracts is much greater than that in plasma. The detailed description of assay for tissue immunoreactive neurotensin is in chapter 3.

Discussion

Immunoassay has revolutionised endocrinology and has become one of the most common tools used in the measurement of hormones. An array of immunoassay techniques such as the radioimmunoassay (RIA) (Yalow and Berson 1960, Ekins 1960), the solid phase RIA (Catt et al 1967), the immunoradiometric assay (Woodhead et al 1974), the two-site sandwich (Miles et al 1974) and the enzyme-linked immunoadsorbant assay (ELISA) are now available for the measurement of a wide range of substances in biological fluids.

The main advantage of radioimmunoassay over bioassay is its high sensitivity which allows the detection of substances present in very small amounts (femtomols) in biological fluids. Additionally, RIA can offer a highly specific measurement of the substance in question and in principle the system is simple. The simultaneous

estimation of a large number of samples can be performed with good precision. However, RIA is not without its problems, limitations and pitfalls. The measurement of a peptide by RIA is determined by the chemical structure of the molecule rather than by the biological activity of the peptide. Only three or four amino acid residues of a peptide are usually required as an antigenic determinant. These amino acid residues are usually adjacent to one another (continuous epitopes) however the folding of a peptide chain may cause the non-adjacent amino acids (discontinuous epitopes) to be recognised by the antibody. Precursors or peptide fragments (both bioactive or inactive) as well as unrelated proteins or peptides containing a similar sequence to the antigenic determinant may be recognised by the antibody, resulting in these being registered as a measurement of immunoreactivity. For example, cholecystokinin (CCK) and gastrin share a common carboxy-terminal pentapeptide, and an antibody raised to one may react equipotently with both. Antiserum to neuropeptide tyrosine tyrosine (NPY) may react with the structurally related peptides to NPY such as peptide tyrosine tyrosine (PYY) and avian pancreatic polypeptide (APP). Antiserum for the measurement of pancreatic glucagon may also detect enteroglucagon immunoreactivity.

Apart from this specific interference, RIA is also prone to non-specific interference. This includes any factors that may alter the antigen and antibody interaction resulting in errors in the assay, for example, haemoglobin from lysed red cells, high salt concentration, high plasma urea, abnormal plasma proteins and other as yet unidentified factors.

Some research investigators have attempted to overcome some of the problems of non-specific interference by incubating standard curve tubes in buffer alone. However, the difference in the assay conditions of standard and unknown samples may give rise to anomalous results. It has thus been suggested that the peptides in the samples must first be extracted and then assayed under the same conditions as the standards tubes. One of the common extraction techniques is alcohol precipitation (Yalow 1985, Theodorsson-Norheim et al 1987). Sample preparation cartridges (Sep-pak) are also favoured (Eng and Yalow 1981, Theodorsson-Norheim 1987). This is time-consuming, expensive and may lead to inadequate recovery of the peptide or introduce extraction interference. One alternative approach is to generate a reference plasma (zero plasma) as described here in which the peptide to be measured is removed by charcoal or

resin adsorption. However this process may remove other small molecules which should remain if the plasma is to be truly representative of the chemical milieu in plasma, thus creating a new assay environment.

There are other problems with radioimmunassay. These include the adsorption of peptide to glass and polystyrene tubes and the instability of the peptide to be measured in the assay. Adsorption may be minimised by the addition of albumin or gelatin to the incubation medium. Columns used for characterisation of peptide immunoreactivity can be siliconised prior to chromatographic analysis. Degradation of the peptide in assay may be overcome by the addition of bacteriocides (e.g. sodium azide or thiomersal) and proteolytic enzyme inhibitors (e.g. trasylol or EDTA) to buffers and incubation media; in addition the assay may be incubated at 2^0C - 8^0C.

The radioimmunoassay procedure described here allows the direct measurement of immunoreactive neurotensin in unextracted human plasma, and in extracts of tissues (chapter 3). The purification of the [^{125}I]-neurotensin was effective and rapid: by reverse phase HPLC. This may be achieved within two hours and labelled neurotensin was

immunologically indistinguishable from synthetic neurotensin.

Numerous radioimmunoassays for the measurement of tissue and plasma neurotensin-like immunoreactivity (NTLI) have been described (Carraway and Leeman 1976a, Blackburn and Bloom 1979, Kronheim et al 1981, Emson et al 1982, Shulkes et al 1982). The antisera in these RIAs differ considerably in their cross-reactivities with neurotensin, its fragments and other neurotensin related substances.

Despite the variabilities in the specificities of the neurotensin antisera, there is general agreement about the regional distribution of neurotensin-like immunoreactivities in the mammalian species studied to date (chapter 3). The measurement of plasma NTLI, however, is more problematic. With some antisera, extraction of plasma is a prerequisite and different plasma concentrations are reported depending on the method used.

Rosell and Rokaeus (1979) reported a basal immunoreactive neurotensin concentration of 52 pmol/l (n = 6) using an N-terminally directed antiserum (07701) in unextracted human plasma.

However, Petersen et al (1984) reported basal plasma NTLI concentration of 29 $\pm$ 12 pmol/l (n = 5) using the same antiserum, 07701, and unextracted plasma. Theodorsson-Norheim et al (1983a) reported a mean fasting plasma NTLI concentration of 44 pmol/l (n = 9) when they used antisera 07701/9, an N-terminally directed antiserum. However in another paper, they reported that the concentration of neurotensin chromatographically identified as authentic neurotensin 1-13 was estimated to be 3 pmol/l basally when the NTLI was measured in gel filtration eluates using four antisera including the N-terminally directed antiserum 07709 (Theodorsson-Norheim & Rosell,1983 b). In a study of fasting plasma NTLI again using unextracted plasma from 106 human subjects, Holst Pedersen and Fahrenkrug (1986) reported a basal NTLI concentration of 95 pmol/l (median value, range 34-275 pmol/l). They used the same antiserum 07709 as Theodorsson-Norheim and Rosell and co-workers (1983a, 1983b).

When plasma NTLI was measured using antiserum 07709 in unextracted human plasma with the radioimmunoassay procedure described here, a fasting concentration of 44 $\pm$ 9 pmol/l was reported in one study (chapter 6) and 13 $\pm$ 3 pmol/l in another study (chapter 7). However, with antiserum

NT 58 a basal plasma NTLI of 22 ± 1 and 6 ± 1 pmol/l was reported in these two studies respectively. The difference in the basal concentration in these two studies is due to the long fasting period of the volunteers in the second study. This was confirmed by the chromatographic profiles obtained for the basal plasma samples (chapter 7).

When extracted human plasma was used, Shaw and Buchanan (1983) reported a fasting concentration of 6 - 7 pmol/l when an "intact" antiserum (NT 3) was employed. However, this antiserum showed 18.6 % crossreactivity with neurotensin 8 - 13. Shulkes et al (1982) also used extracted plasma and reported basal plasma NTLI concentrations of 29 ± 4 pmol/l (n = 30) using an N-terminally directed antiserum. The same authors, again using extracted plasma, but this time using a C-terminally directed antiserum, reported lower basal concentrations of plasma NTLI, 18 ± 2 pmol/l (n = 10) (Shulkes et al 1984). In contrast, Hammer et al (1982) have reported yet lower basal plasma concentrations, 2.8 - 3.5 pmol/l, with an N-terminally directed antiserum (TG1), compared to that of 19.7 - 24.2 pmol/l (n = 5) with a C-terminally directed antiserum (HC 8) in extracted human plasma. Service et al reported a basal plasma concentration of NTLI

of 24 - 30 pmol/l with an N-terminally directed antiserum using extracted plasma (Service et al 1986).

Differences in the reported basal plasma NTLI may in part be explained by the fact that there is no standardised assay methodology using a common neurotensin antiserum; at present, from the data reported in the literature, the basal fasting plasma NTLI in man may be taken as in the range of 3-95 pmol/l. With the radioimmunoassay procedure described here using a C-terminally directed antiserum NT 58, the fasting human plasma NTLI concentration was found to be less than 25 pmol/l.

The variability in plasma immunoreactive neurotensin concentrations reported by various investigators has demonstrated some of the problems encountered in RIA as discussed above. Different assays will possess distinct intrinsic characteristics and as a result the apparent immunoreactivity measured will vary. There is no perfect assay, however, despite the pitfalls of RIA, it can be a useful tool for the study of the biochemistry and physiology of hormones/peptides.

Chapter 3

DISTRIBUTION OF NEUROTENSIN

Introduction

Following the purification of neurotensin, Carraway and Leeman (1976a) developed a radioimmunoassay to the peptide to study its distribution. Immunoreactive neurotensin was identified in the hypothalamus of a number of mammalian species (Carraway and Leeman 1976a). This initial observation was confirmed and extended when Uhl and Snyder (1976) reported an ubiquitous but uneven distribution of neurotensin in the bovine central nervous system. Although neurotensin was originally isolated from bovine hypothalamic tissue, much larger quantities were detected in the gastrointestinal tract by radioimmunoassay (Carraway and Leeman 1976b, 1978). In the human gastrointestinal tract, neurotensin is mainly localised to the mucosal "N" cells (Polak et al 1977) within the mucosal epithelium of the gut (Ferri et al 1983) and the concentration of immunoreactive neurotensin increases from duodenum to distal ileum (Hammer et al 1980).

The distribution pattern of neurotensin-like immunoreactivity (NTLI) is similar among the mammalian species investigated to date. There are, however, inter-species differences in the absolute concentration of NTLI present. For example, the concentration of NTLI is an order of magnitude greater in extracts of cat tissues than in equivalent tissue extracts from other species (Goedert and Emson 1983a, Holzer et al 1982, Carraway and Mitra 1987a).

The cDNA that encodes neurotensin has been isolated from a cDNA library constructed from the enriched primary cultures of canine enteric mucosal cells (Dobner et al 1987). The deduced amino acid sequence indicates that neurotensin is encoded within a 170 amino acid precursor. This precursor protein not only contains neurotensin but also neuromedin N, a peptide identified from porcine spinal cord that is structurally related to neurotensin (Minamino et al 1984). This may explain, in part, the original observation by Carraway and Leeman (1976b) that an antiserum which cross-reacted with six to seven amino acid residues of the C-terminal portion of the neurotensin molecule provided a much larger measurement of NTLI in extract of the rat stomach than an

antiserum which required both terminals of the neurotensin molecule for recognition.

With the radioimmunoassay described in the previous chapter, the extraction efficiency using different medium on added neurotensin in tissue extract is examined. The peripheral tissue distribution of neurotensin and neuromedin N in rat is studied and compared. The concentration of immunoreactive neurotensin is quantified with region specific antisera. The immunoreactive neurotensin and neuromedin N are characterised with both gel filtration and high pressure liquid chromatography. In addition, the distribution of neurotensin in rat and human central nervous system is reported.

Materials and methods

Guinea pigs (n = 6) were sacrificed by decapitation and the livers were removed onto ice . The liver was minced, and a weighed portion of the tissue was placed into four 10 ml polystyrene tubes containing 6 ml of (1) 0.5 M NaOH (pH 13.54), (2) 0.2 M HCl (pH 0.65), (3) distilled water (pH = 6.98), (4) 0.5 M CH_3COOH (pH = 2.41). The tubes were pre-boiled to 97^0-100^0C. The tissues were boiled for 15 min. After brief cooling, aliquots (1.0 ml) were taken from each tube for the measurement of endogenous

neurotensin immunoreactivity. To the remaining volume (5.0 ml) in each tube, 10 pmol of neurotensin was added and all tubes were re-boiled for a further 15 min. The samples were cooled and an aliquot was taken for the measurement of NTLI.

Adult Wistar rats (n=8) were killed by decapitation. Tissues were dissected within 20 min, weighed, and extracted immediately in boiling 0.5 M acetic acid for 10 min in polystyrene tubes (Nunc tubes) in an approximate extraction volume to wet weight ratio of 10 to 1 (ml/g wet weight). The tissues dissected included different regions of the gastrointestinal tract, liver, kidney, heart and lung. The extracts of the tissues were then cooled and centrifuged for 15 min at 4^{0}C. Aliquots (1.0 ml) of the supernatants were taken and lyophilised. The extracts were subsquently reconstituted in 0.06 M phosphate buffer, pH 7.4, containing EDTA (10mM) and 0.3 % gelatin just prior to assay

Post-mortem normal human brain tissues were obtained from Dr Rossor, MRC, Neurochemical Pharmacology Unit, Cambridge, U.K., and stored at -70^{0}C. Extraction of tissue was performed in pre-weighed capped polypropylene tubes filled with distilled water in a volume approximately 10 times the weight of the tissue. The polypropylene tubes

were placed in a vigorously boiling water bath. The weighed tissues were added when the tubes had reached 97^{0}-100^{0}C and then boiled for 10 min. After brief cooling, the tube contents were homogenised with an Ultra-Turrax blender at 20,000 rpm for 2 min and re-weighed. Glacial acetic acid (30 ul/ml of extract) was then added to the suspension to produce a final concentration of 0.5 M acetic acid, and the extract was placed in the boiling water bath for a further 10 min. After cooling the acid extracts were re-weighed and stored at -20^{0}C.

Radioimmunoassay (RIA)

For extracts of the rat peripheral tissues, aliquots of 10 ul and 1 ul of the reconstituted samples were assayed for neurotensin with the C-terminally directed antiserum NT 58, and an N-terminally directed antiserum (a gift from Dr T W Moody, The George Washington University, Washington DC, U S A). The radioimmunoassay and assay format were identical to that described for plasma neurotensin in chapter 2.

Immunoreactive neuromedin N was measured with a recently developed RIA (Lee et al 1987). Briefly, neuromedin N (Peninsula Laboritories, Merseyside,

England) was conjugated to bovine serum albumin in a molar ratio of 4 to 1 using glutaraldehyde (O'Shaughnessy 1982). Neuromedin N antiserum was raised in rabbits (n = 6) immunised initially with 100 ug of neuromedin N per rabbit in Freund's complete adjuvant and boosted two months later with 50 ug neuromedin N per rabbit in Freund's incomplete adjuvant at 4 week intervals. [^{125}I]neuromedin N was prepared by the chloramine T method (Hunter and Greenwood 1962) and the iodination product was purified by reverse phase high pressure liquid chromatography using a Techsil C18 column eluted with a stepwise gradient of acetonitrile from 20 % to 80 % (vol/vol) containing 0.1 % aqueous trifluoroacetic acid (TFA) in water. Aliquots (500 ul) of the reconstituted rat tissue extracts were assayed for immunoreactive neuromedin N in conditions identical to that described for neurotensin.

Chromatographic analysis

For the chromatographic analysis of rat tissue extracts for neurotensin and neuromedin N, tissue extracts (2 ml each, n=8) were pooled. The pooled tissue extracts were prepared using Sep-pak C18 sample preparation cartridges (Octadecylsilylsilica cartridges) prior to chromatography. The pooled

extracts were loaded onto the Sep-pak C18 cartridges, protein and high polarity compounds were washed off with 10 ml 0.1 % (vol/vol) TFA in distilled water. The cartridges were then eluted with 1.0 ml 50 % (vol/vol) acetonitrile (HPLC grade) in distilled water containing 0.1 % (vol/vol) TFA. The eluents were diluted with 3.0 ml 0.1 % (vol/vol) TFA in distilled water and two aliquots (1.5 ml each) were taken, one for gel filtration and the other for HPLC analyses.

For gel filtration, samples were applied to a Sephadex G-50 superfine column (1.5 X 90 cm) and eluted with 0.06 M phosphate EDTA buffer, pH 7.4, containing 0.3 % bovine serum albumin and 0.2 M sodium chloride at 4^0C. The column was calibrated with dextran blue and [^{125}I]Na as void volume and total volume markers respectively. The elution positions of synthetic neurotensin and neuromedin N were pre-determined in separate runs under identical conditions. Fractions of 2.0 ml were collected and evaporated to dryness in a SpeedVac sample concentrator (Savant, Mass., USA). The fractions were reconstituted in 0.7 ml of assay buffer and 0.05 ml of each fraction was assayed for neurotensin with the C-terminally directed antiserum NT 58. Fractions (0.6 ml of each fraction) were assayed for neuromedin N. Reverse

phase HPLC was performed using a Waters system (Waters Associates Inc, Milford, Mass., USA) which consisted of a U6K sample injector, model 6000A and model M45 solvent delivery pumps, a model 660 solvent programmer for gradient elution and a Frac-100 fraction collector (Pharmacia Fine Chemicals, Uppsala, Sweden) with a Techsil C18 column (0.39 X 30 cm)(HPLC Technology, Cheshire, England). The column was eluted with a stepwise gradient of 20 % to 50 % (vol/vol) acetonitrile in distilled water containing 0.1 % (vol/vol) aqueous TFA for 60 minutes. Fractions (1.0 ml) were collected. Aliquots (0.1 ml) of each fraction were assayed for neurotensin (with both the C- and N-terminally directed antisera) and for neuromedin N.

Results

The comparative distribution of immunoreactive neurotensin and immunoreactive neuromedin N in the rat peripheral tissues is shown in Table 3:1. Both gel filtration and HPLC analyses of the rat ileal extract demonstrated that ileal immunoreactivity co-eluted with synthetic neurotensin and neuromedin N when measured with the respective antisera (Figure 3:1, 3:2, 3:3). In addition, HPLC analysis of rat kidney extract showed that immunoreactive

Table 3:1.

Regional distribution of immunoreactive neurotensin and immunoreactive neuromedin N in rat (pmol peptide/g wet weight, mean ± sem, n = 8)

Region	NTLI*	NTLI**	NNLI
Stomach	< 0.4	< 0.6	1.0 ± 0.6
Duodenum	1.6 ± 0.1	nd	nd
Jejunum	5.7 ± 0.9	5.3 ± 0.8	2.8 ± 1.5
Ileum	24.9 ± 3.1	nd	nd
Ileum(terminal)	89.6 ± 6.4	94.1 ± 8.6	40.8 ± 5.9
Caecum	15.5 ± 1.9	26.3 ± 3.6	7.0 ± 1.4
Asc Colon	15.3 ± 2.2	9.9 ± 2.7	4.5 ± 2.1
Trans Colon	1.9 ± 0.6	1.6 ± 1.2	< 0.8
Desc Colon	< 0.4	< 0.6	< 0.8
Liver	< 0.4	< 0.6	3.3 ± 1.1
Kidney	< 0.4	< 0.6	17.0 ± 0.6
Heart	< 0.4	< 0.6	6.1 ± 1.6
Lung	< 0.4	< 0.6	3.3 ± 1.3

* Immunoreactive neurotensin (NTLI) measured with C-terminal antiserum NT 58

** Immunoreactive neurotensin (NTLI) measured with N-terminal antiserum

nd Not done

neuromedin N was eluted at the position of synthetic neuromedin N (Figure 3:3). The distribution of NTLI in rat and human brain region is shown in Figure 3: 5 and Table 3: 2. Gel filtration of the rat hypothalamic extract demonstrated that immunoreactive neurotensin co-eluted with synthetic neurotensin (Figure 3:4). Serial dilutions of rat hypothalamic extracts were also performed and assayed (Figure 3:6).

Recovery of exogenous neurotensin when boiled in the presence of guinea pig liver tissue was : 96 % using 0.5 M acetic acid, 82 % using 0.2 M HCL and 85 % using distilled water. No neurotensin was detected when extracted in 0.5 M NaOH.

Discussion

The findings in this chapter demonstrate a discrete localisation of neurotensin-like immunoreactivity (NTLI) in rat and human tissue by radioimmunoassay. The assay was considered valid as the serial dilutions of the rat hypothalamic extracts exhibited parallelism with synthetic neurotensin. The extraction medium, 0.5 M acetic acid, gave the maximum recovery of exogenous neurotensin in the presence of guinea pig liver tissue as compared with 0.2 M HCl or distilled water. No neurotensin

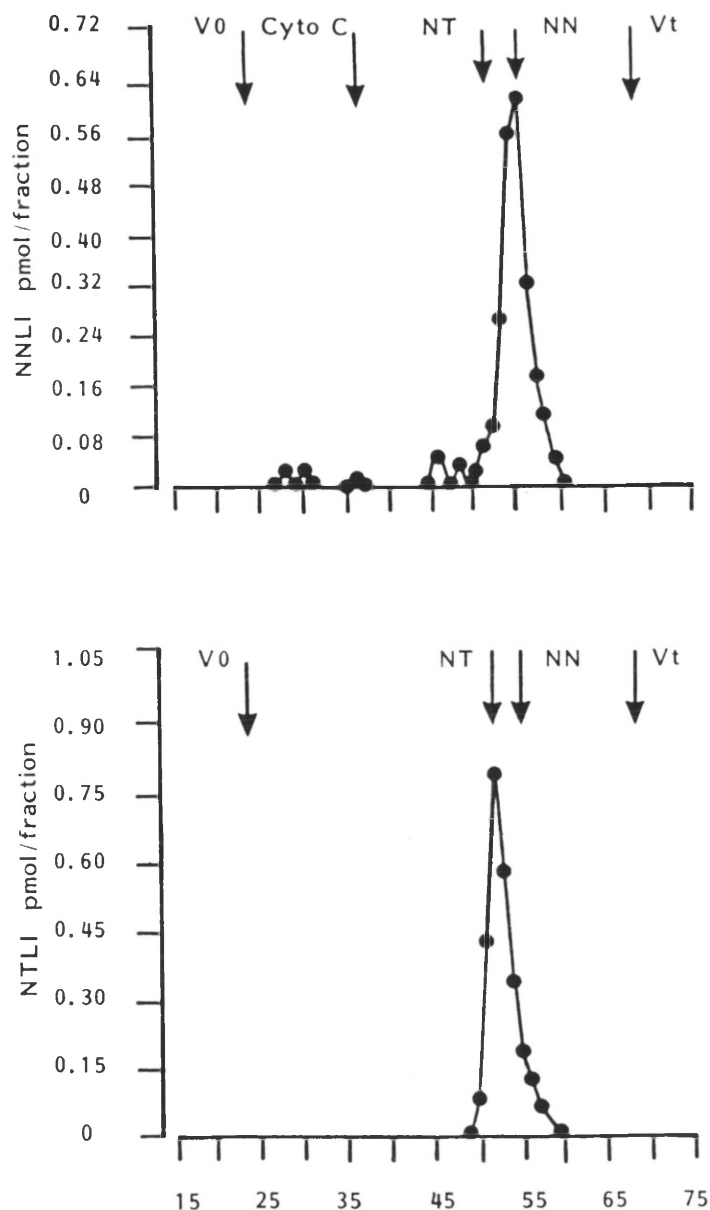


Figure 3:1. Gel-filtration profiles of immunoreactive neuromedin N (upper) and immunoreactive neurotensin(lower) of pooled rat ileal extracts. The G50 Sephadex superfine column (1.5 x 90 cm) was eluted with 0.06 M phosphate buffer, pH 7.4, containing 0.3 % BSA and 0.2 M sodium chloride at 4^{0}C. The elution positions of void volumn (Vo) and total volumn (Vt), synthetic neuromedin N and neurotensin were as indicated.

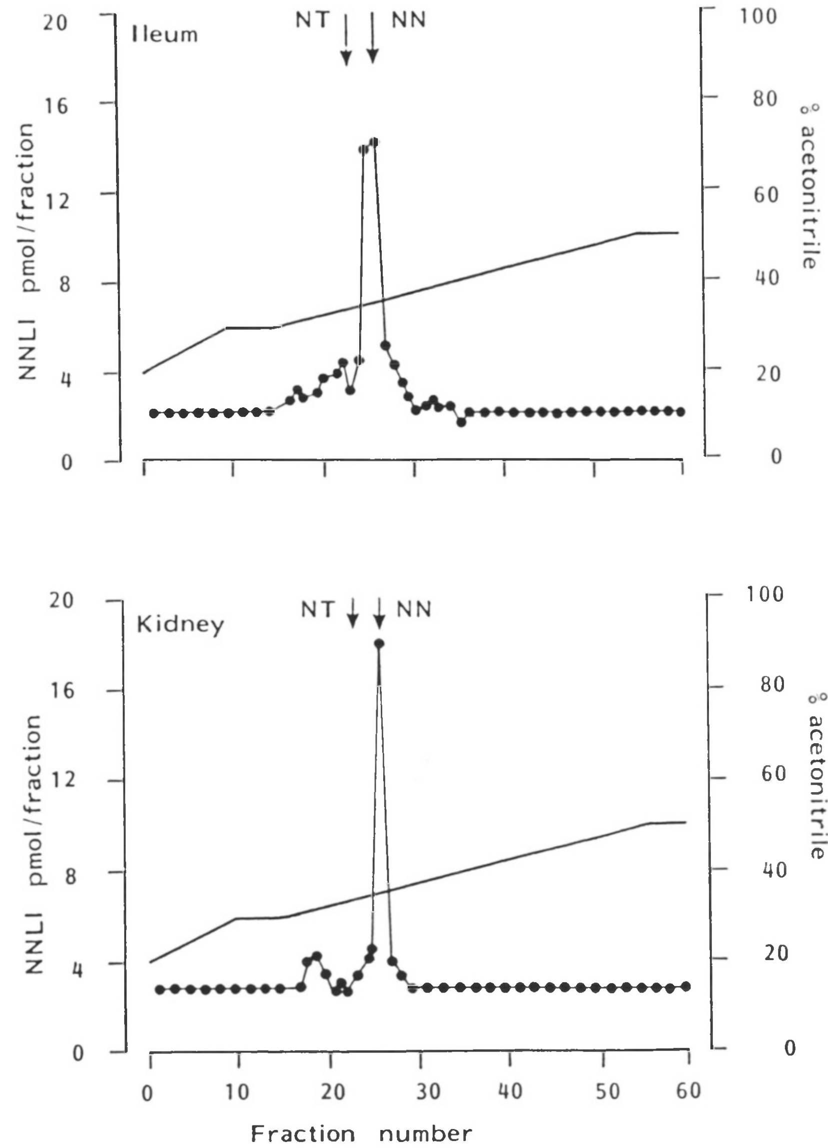


Figure 3:2. HPLC profiles of pooled ileal extracts (upper) and pooled kidney extracts (lower). The techsil C18 column (0.39 x 30 cm) was eluted with a gradient from 20-50 % (0.5 %/min) acetonitrile in distilled water containing 0.1 % trifluoroacetic acid. Fractions of 1.0 ml were collected, and aliquots (0.1 ml) of each fraction were assayed for neuromedin N. The elution positions of neuromedin N and neurotensin were as indicated.

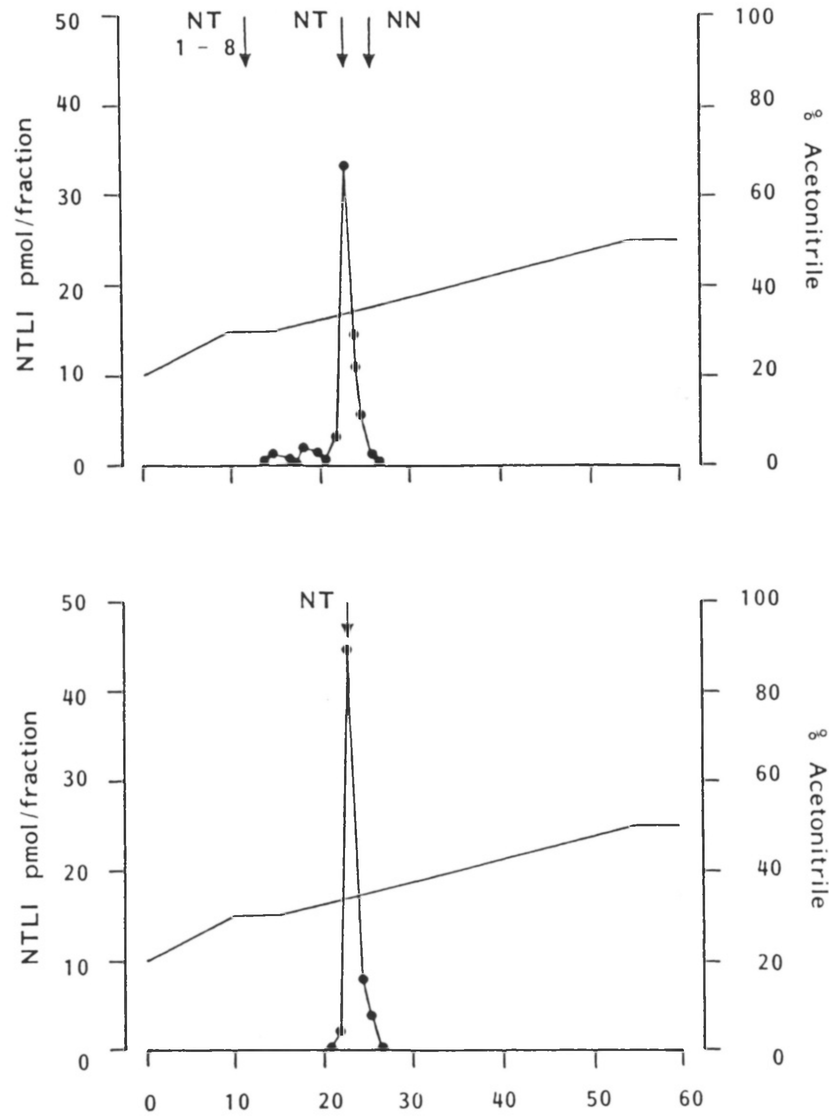


Figure 3:3. HPLC profiles of pooled ileal extract The chromatographic fractions were as those of Figure 3:2. Immunoreactive neurotensin from each fraction were measured with the C-terminally directed neurotensin antiserum (upper) and measured with the N-terminally directed neurotensin antiserum (lower).

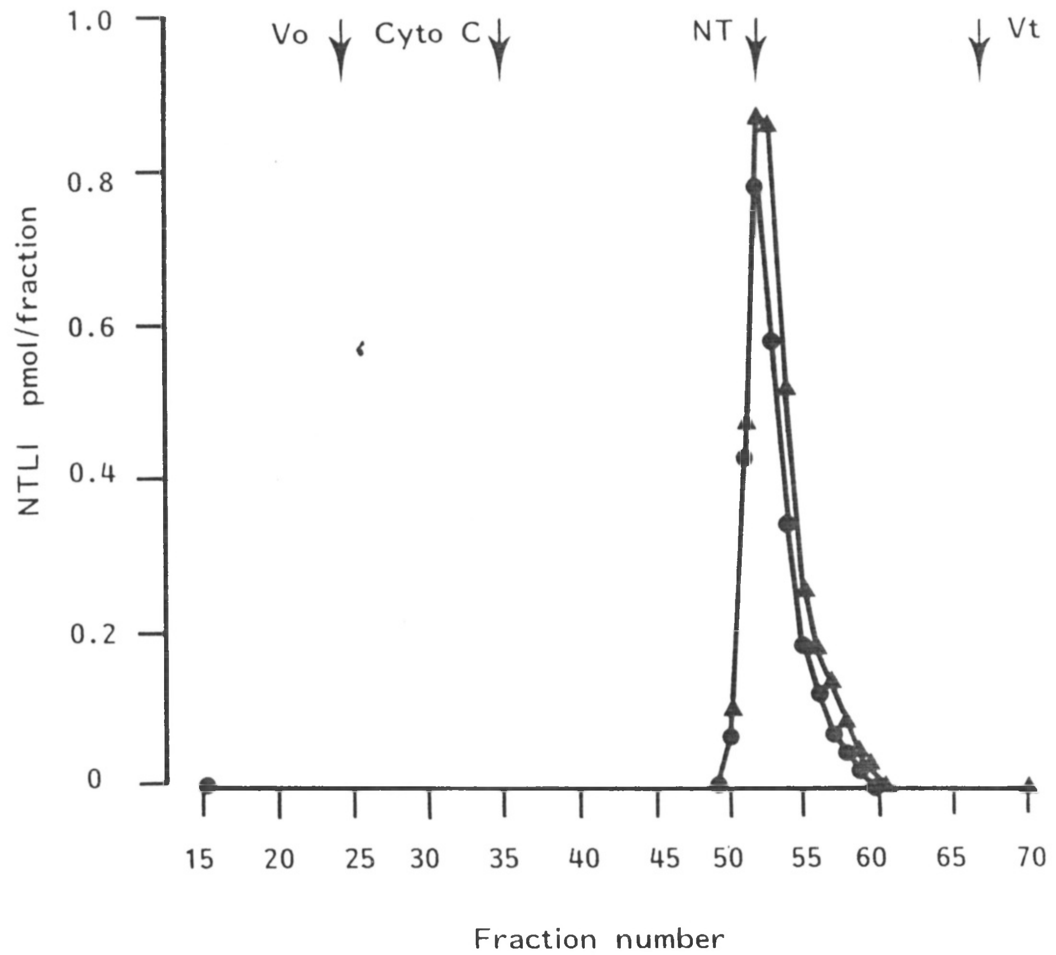
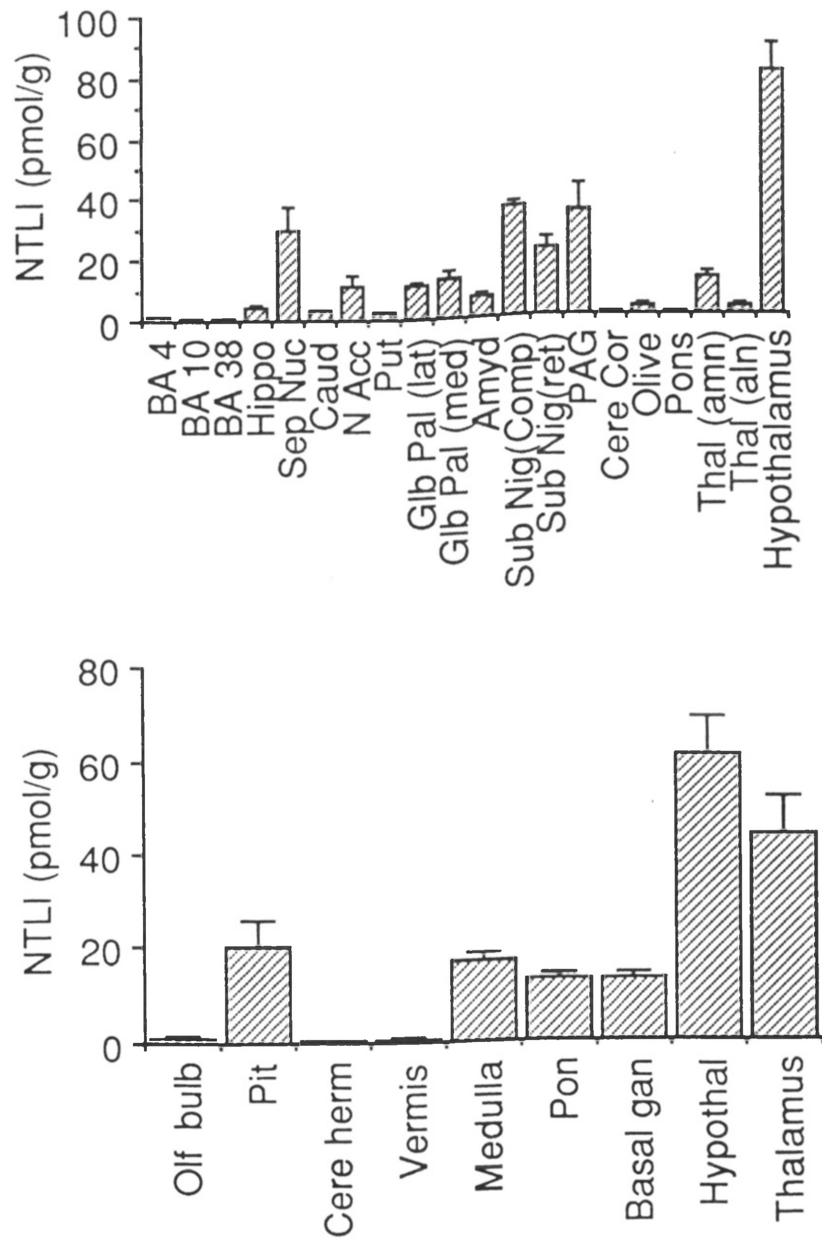


Figure 3: 4. Gel-filtration profile of rat hypothalamic extracts. (●) when NTLI was measured with C-terminally directed antiserum, (▲) when NTLI was measured with N-terminally directed antiserum.

FIgure 3:5. Distribution of immunoreactive neurotensin in human and rat central nervous system.



Upper panel: human CNS extracts (n = 5).
Lower panel: rat CNS extracts (n = 8).
Immunoreactive neurotensin concentrations were expressed as mean ± sem in pmol/g wet weight.

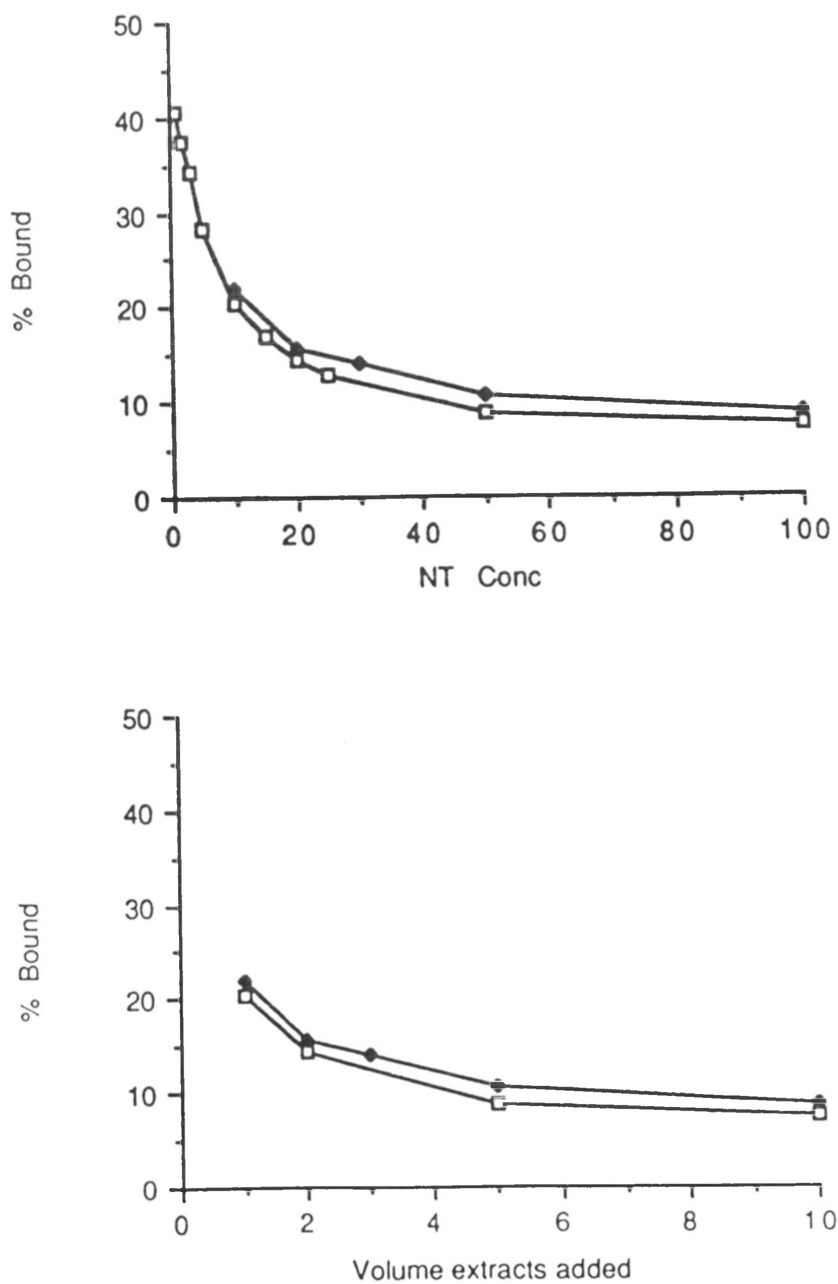


Figure 3:6. Serial dilution curve of rat hypothalamic extracts. (□) synthetic neurotensin; (◆) rat hypothalamic extracts, neurotensin concentration in fmol/tube, tissue extract additions: 10, 20, 30, 50, 100 ul.

Table 3:2. Distribution of immunoreactive neurotensin in human brain (pmol/g) : a comparison.

Regions	Present n = 5	Cooper'81 n = 9-13	Ferrier'83 n = 13	Emson'85 n = 8-12
Brodman Area	< 0.4	0.8 ± 0.1	0.7 ± 0.1	0.6 ± 0.2
Hippocampus	4.7 ± 0.8	4.4 ± 0.6	1.9 ± 0.3	12.3 ± 4.3
Septal Nucei	29.5 ± 8.1	ND	13.0 ± 2.0	ND
Caudate Nucleus	3.0 ± 0.5	2.9 ± 0.4	ND	ND
Nucleus Accumbens	11.2 ± 3.8	ND	5.7 ± 0.7	14.7 ± 4.5
Putaman	2.8 ± 0.2	2.5 ± 0.3	1.5 ± 0.2	3.1 ± 0.9
Globus Pallidus			5.0 + 1.0	
(lateral)	11.2 ± 1.3	9.8 ± 1.1	-	12.1 ± 1.8
(medial)	13.2 ± 2.5	9.7 ± 1.3	-	17.2 ± 3.4
Amygdala	6.8 ± 1.4	5.4 ± 1.1	5.2 ± 0.9	19.6 ± 2.1
Substantia Nigra	-	23.4 ± 2.0	18.0 ± 4.0	-
(compact)	36.4 ± 1.7	-	-	54.5 ± 6.7
(reticular)	22.2 ± 3.5	-	-	36.3 ± 4.0
Hypothalamus	-	-	37.0 ± 5.0	35.2 ± 3.6
(lateral)	22.8 ± 3.6	-	-	-
(posterior)	25.6 ± 4.3	23.6 ± 2.6	-	-
(anterior)	-	33.4 ± 5.0	-	-
(preoptic)	22.0 ± 2.5	-	-	-

Cooper'81 : Brain Res 218: 219-232.
Ferrier'83 : J Neurol Sci 62: 159-170.
Emson'85 : Brain Res 347: 239-244.
ND : not done
Results are expressed as mean ± sem .

was detected when 0.5 M NaOH was used. This may be related to the fact that NT is a basic peptide. The solubilities of peptides vary in different extraction media, for example, the extraction of tissue cholecystokinin in neutral medium (pH = 7.0) is preferred (Marley and Rehfeld 1984). The distribution of neurotensin in extracts of the rat peripheral tissues was comparable with that of neuromedin N. The data show that like neurotensin, neuromedin N was also present throughout the gastrointestinal tract with the exception of the transverse and descending colon. Highest concentrations of immunoreactive neuromedin N were found in the rat terminal ileum, 40.8 ± 5.9 pmol/g (n=8). The concentrations of NTLI in the same extracts of terminal ileum (n = 8) were 89.6 ± 6.4 pmol/g using the C-terminally directed antiserum (NT 58) and 94.1 ± 8.6 pmol/g when measured with an N-terminally directed neurotensin antiserum. It thus appeared that the concentration of neurotensin was twice that of neuromedin N. In a recent report by Carraway and Mitra (1987a), the distribution of immunoreactive neurotensin has also been compared with that of neuromedin N. The authors reported that in the cat the distribution of NTLI and immunoreactive neuromedin N was similar. However, the ratio of these two immunoreactivities varied from 0.5 to 15 depending on the tissue (Carraway

and Mitra 1987a). They also found that the two areas where there were least concentrations of NTLI contained the highest concentrations of immunoreactive neuromedin N. For example, in the feline stomach and frontal cortex there were 7 and 15 fold less NTLI than immunoreactive neuromedin N respectively. . The distribution of neuromedin N in other mammalian species is still unknown.

Considerable concentrations of immunoreactive neuromedin N were measurable in rat kidney extracts, 17.0 $\pm$ 0.6 pmol/g (n = 8), whereas no NTLI was detected in these extracts using both the C- and N-terminally directed neurotensin antisera. The absence of NTLI in rat kidney is in accord with the report by Carraway et al (1976b), and also in agreement with the finding previously reported in cat (Goedert et al 1983a).

One possible explanation for these observations is that neurotensin and neuromedin N may be stored within different cells. However, this could not be the case if neurotensin and neuromedin N were encoded within the same precursor protein as demonstrated in canine mucosal cells (Dobner et al 1987). Differential regulation of release of the two peptides, neurotensin and neuromedin N, from their precursor may occur. Further possibilities

are that of alternative splicing of mRNA or of tissue specific processing of the polyprotein precursor which may result in the formation of different bioactive peptides originating from the same DNA. The processing of polyproteins responsible for calcitonin and calcitonin gene-related peptide (cGRP) (Amara et al 1982, Rosenfeld et al 1983, Morris et al 1984), substance P and substance K (Nawa et al 1983), gastrin releasing peptide (GRP) (Spindel et al 1986), and the processing of preproglucagon mRNA (Mojsov et al 1986) are just a few examples of these phenomena. The preproglucagon mRNAs in pancreatic and intestinal extracts are identical, but different and specific peptides are generated in the two tissues (Mojsov et al 1986). It is of interest that most recently a biologically active peptide, namely PHV (peptides histidine valine), has been isolated, which is encoded within the precursor polyprotein for vasoactive intestine polypeptide (prepro VIP). PHV is absent in tissues where VIP or PHM (peptide histidine methionine) are found in high concentrations (Yiangous et al 1987), suggesting possible tissue-dependent processing of peptide precursor.

The best known example of tissue specific post-translational processing of a pre-prohormone

is that of pre-opiomelanocortin (POMC), a common precursor for several pituitary hormones. The processing of POMC in the anterior pituitary lobe has been demonstrated to be clearly different from that in the neurointermediate lobe thereby giving rise to different bioactive peptides in the respective areas of the pituitary glands (Douglass et al 1984). In contrast, it has been reported that heterologous monkey kidney cells are able to synthesise and secrete POMC but are unable to process this prehormone to its bioactive components (Noet et al 1987).

The tissue concentrations of the C- and N-terminal immunoreactive neurotensin in the present study were similar. The ratio of immunoreactive neurotensin to immunoreactive neuromedin N in the gastrointestinal tract was relatively constant (approximately 2:1). This is consistent with the findings for neurotensin and neuromedin N in extracts of cat gastrointestinal tissues (Carraway and Mitra 1987a).

In the post-translational processing of the neurotensin precursor from the canine enteric mucosal cells, the paired basic amino acids Arg-Arg at position 8 and 9 within the neurotensin molecule must somehow be protected. It has been suggested

that in mammals the proteolytic cleavage of peptide precursors at Lys-Arg sites is preferred over that of Arg-Arg or Lys-Lys sequences (Douglass et al 1984). The cleavage of Arg^{8}-Arg^{9} within the neurotensin molecule has however been implicated in its inactivation in the central nervous system (McDermott et al 1982, Emson et al 1985b) and in the metabolism of neurotensin after its secretion into the circulation (chapter 6,7). The exact mechanism of the processing of the neurotensin precursor in different species and tissues remains to be elucidated.

Chapter 4

REGULATION OF NEUROTENSIN RELEASE

Introduction

Neurotensin is released into the general circulation in response to nutrient ingestion, in particular, fat (Mashford et al 1978a, Rosell and Rokaeus 1979, Blackburn and Bloom 1979, Hammer et al 1982). The release of neurotensin, following the ingestion of 10 % intralipid in man, is rapid, and the rise of plasma neurotensin-like immunoreactivity (NTLI) appears to be biphasic: one peak within 30 min and the second peak at 90 min (Rosell and Rokaeus 1979). This may be due to a differential rate (fast and delayed) of arrival of the fat in the lower gastrointestinal tract where the N cells are mainly localised. The delayed peak of immunoreactivity detected may be the concomitant sum of the neurotensin released from the gut plus its metabolic fragments which are recognised by the antiserum used in the measurement of the plasma immunoreactive neurotensin (chapter 7). Alternatively, it could be due to an initial release of NTLI directly through a neural mechanism or indirectly via other factors which are under neural influences. For example, infusions of

bombesin and its mammalian counterpart, gastrin releasing peptide (GRP) in man and rat have been reported to elevate plasma immunoreactive neurotensin (Ghatei et al 1982a, Rokaeus et al 1982). Both bombesin and GRP are neuropeptides present in the nerve plexuses intrinsic to the gastrointestinal tract (Dockray 1979, Hutchinson et al 1981, Buffa et al 1982). These peptides, however, have yet to be demonstrated in the mucosal endocrine cells or in the systemic circulation under normal physiological conditions. Neurotensin release stimulated by perfusion of the rat gut with oleic acid and alcohol has been reported to be inhibited by a cyclic hexapeptide analogue of somatostatin (Ferris et al 1985a). This analogue also suppressed the appearance of neurotensin 1-8, presumably the metabolite of neurotensin in the rat systemic circulation. Somatostatin analogues are in general much more potent in their actions and have a longer half life (Adrian et al 1981, Bauer et al 1982) than the natural peptide, the physiological action of which is difficult to ascertained because of its short half life under normal condition.

In this chapter, the possible neural influence on the control of release of neurotensin is described using an isolated perfused rat terminal ileum preparation. In addition, the possible interaction

of somatostatin on neurotensin release in human volunteers was studied using an octaopeptide analogue of somatostatin, SMS 201-995 (Sandoz, Basal, Switzerland). The inhibitory effects of SMS 201-995 on gastrointestinal peptide secretions have previously been demonstrated (Kraenzlin et al 1985). Little is known of its effect on neurotensin release.

Materials and Methods

Isolated ileal preparation:

Male Wistar rats (200-250 g) with free access to water were fasted overnight. The rats were anaesthetised with intramuscular injections of sodium pentobarbitone (60 mg/kg). The bulk of the colon was removed after ligating the middle and the right coelic artery and vein. A cannula was inserted and tied into the terminal ileum, 30 cm proximal to which a second cannula was inserted, and the gut lumen was immediatedly perfused with oxygenated modified Kreb-Henseleit solution containing the following in mmol/l: Na^+ 143, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 128, $H_2PO_4^{2-}$ 1.2, SO_4^{2-} 1.2, HCO_3^- 25, glucose 5.5 (luminal perfusate). The remainder of the gut, i.e. upper small intestine, jejunum and duodenum was removed

after ligating the respective arteries and veins. Heparin (0.25 ml, 5000 iu/ml) was injected into the inferior vena cava. A fine bulldog clamp was placed on the aorta above the origin of the superior mesenteric artery, and immediately after, the arterial cannula was inserted through an incision in the aorta into the superior mesenteric artery and secured. The preparation was then perfused with arterial perfusate (modified Kreb-Henseleit solution as above plus bovine serum albumin 0.25 % and dextran 4.6 %). The venous effluent was collected via a cannula tied into the superior mesenteric vein.

The isolated perfused preparation was transferred to a gut bath which was placed inside a 37^0C incubator. The vascular perfusion apparatus is illustrated in Figure 4:1.

The arterial and luminal perfusates were placed in reservoirs (a and b) and pumped along fine nylon tubing (Portex Ltd) to oxygenators (d and e) by means of a multichannel peristaltic pump (c, Technicon Inc, New Jersey, USA) which gave a virtually non pulsatile flow. The flow rates of the arterial and the gut luminal perfusates were 1.5 ml/min and 3.0 ml/min respectively. The isolated rat terminal ileum preparation was placed in the

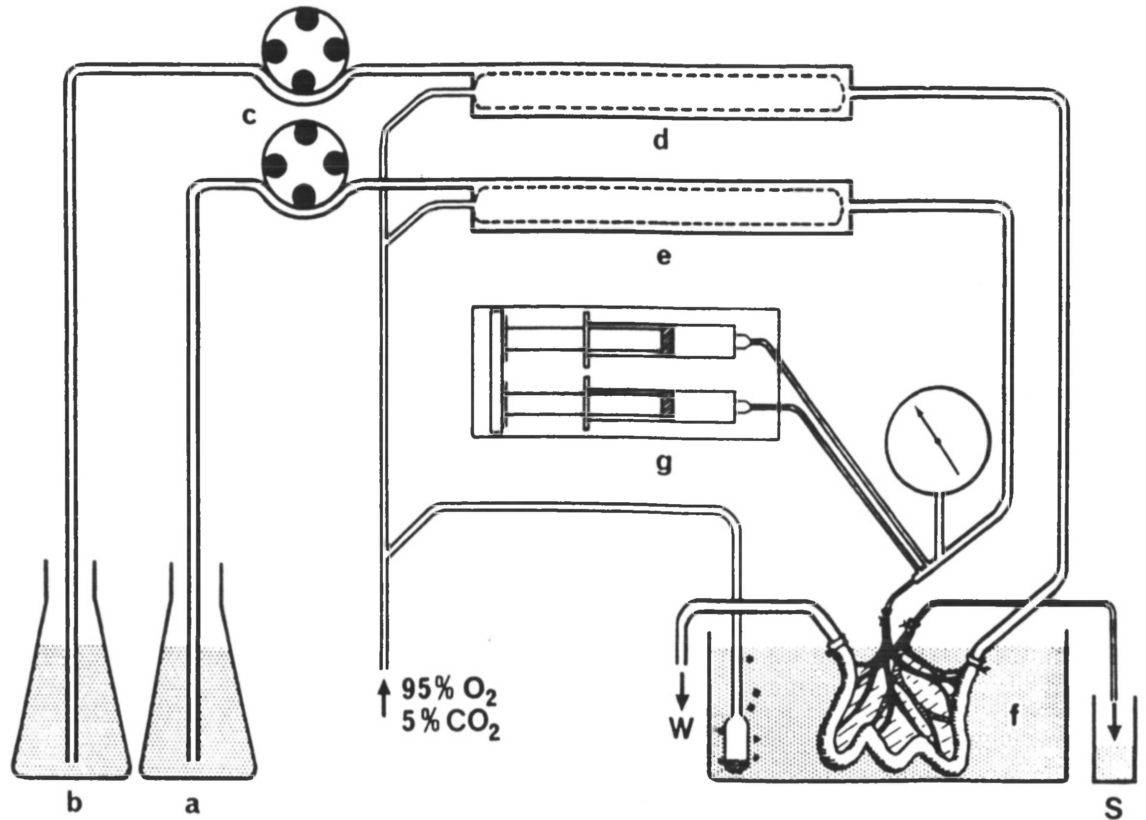


Figure 4:1. Schematic illustration of the isolated perfused rat ileal preparation apparatus. (a) arterial perfusate, (b) luminal perfusate, (c) peristaltic pump, (d) oxygenator, (e) oxygenator, (f) gut bath, (g) infusion pump, (S) sample, (W) waste.

gut bath (f). Test solutions (infusates) were introduced into the side arm of the arterial perfusion line by means of a perfusion pump (g, Harvard Apparatus) at a rate of 0.1 ml/min to give the pre-calculated desired final concentrations. The oxygenators consisted of a tube of dialysis membrane (Visking 8/32) within a sealed plastic casing. The membrane was inflated to a pressure of 49 N/cm^2 with 95 % O_2 and 5 % CO_2 which gave a bubble free perfusate which was pumped along the annular space between the membrane and the casing, at a partial pressure of 4 N/cm^2 (300 mm Hg) O_2 and 0.4 N/cm^2 (30 mm Hg) CO_2.

The test substances used were carbachol BP at final concentrations of 5×10^{-12}, 5×10^{-10}, 5×10^{-9}, 5×10^{-8}, and 5×10^{-7} mol/l; synthetic bombesin (Peninsula Laboratories) at final concentrations of 5×10^{-11}, and 5×10^{-10} mol/l; acid tartrate noradrenaline at final concentrations of 1×10^{-6} and 1×10^{-4} mol/l. The substances were made up to the appropriate concentrations with 0.9 % saline. The test periods for the effect of carbachol and bombesin were 6 min each dose, and for noradrenaline were 15 min each dose. The viability of the isolated gut preparation was tested with 10 % intralipid (an aqueous emulsion of soybean oil, egg phospholipids and glycerol), which was perfused

through the gut lumen over a 45 min period and formed the first series of experiments. Prior to sample collection of the venous effluent, the isolated ileal preparations were allowed to settle for 15 min. Samples were collected over 3 min periods into polystrene tubes containing 0.1 ml aprotinin (Trasylol), frozen at once and stored at - 20^{0}C. The samples were subsequently assayed for neurotensin- and bombesin-like immunoreactivity in duplicate 100 ul aliquots. The immunoreactive bombesin was measured by RIA as previously described (Ghatei 1982).

<u>Infusion of somatostatin analogue (SMS 201-995) in man</u>:

Approval for the study was obtained from the Royal Postgraduate Medical School Ethics Committee. Eight healthy male human subjects (age 20-33 yrs) each gave informed consent and were studied on two separate occasions at least one week apart. The subjects, after fasting overnight, were given a subcutaneous injection of 50 ug SMS 201-995 (Sandoz, Basle, Switzerland) in SMS diluent (sodium chloride in sodium acetate/acetic buffer, pH 4.0), or SMS diluent alone in random order. Thirty minutes after the injection, the volunteers consumed a test breakfast consisting of 90 g white

bread, 10 g butter, two boiled eggs and 200 ml of unsweetened orange juice (62 g carbohydrate, 20 g protein, and 20 g fat, totalling 530 kCal) within five minutes. Blood samples were taken just before the SMS or placebo injection, 30 minutes prior to consuming the test breakfast (0 min) and at 15 min intervals up to one hour, and followed by samples at 90, 120, 180, 240, and 300 min after the consumption of the breakfast. Blood samples (10 ml each) were placed into lithium heparin tubes containing 4000 KIU aprotinin (Trasylol), centrifuged for 5 min at 1600 X g and the plasma separated. The plasma samples were frozen immediately on dry ice and stored at -20^{0}C.

Results

Isolated ileal preparation:

There was a significant release of immunoreactive neurotensin into the venous effluent when the isolated terminal ileal preparation was perfused intraluminally with 10 % Intralipid (Figure 4:2). A mean peak of 200 % above basal concentration of immunoreactive neurotensin was attained at 3-6 minutes after the start of the perfusion, and returned to the basal concentration at 45 minutes. The mean basal concentration of immunoreactive

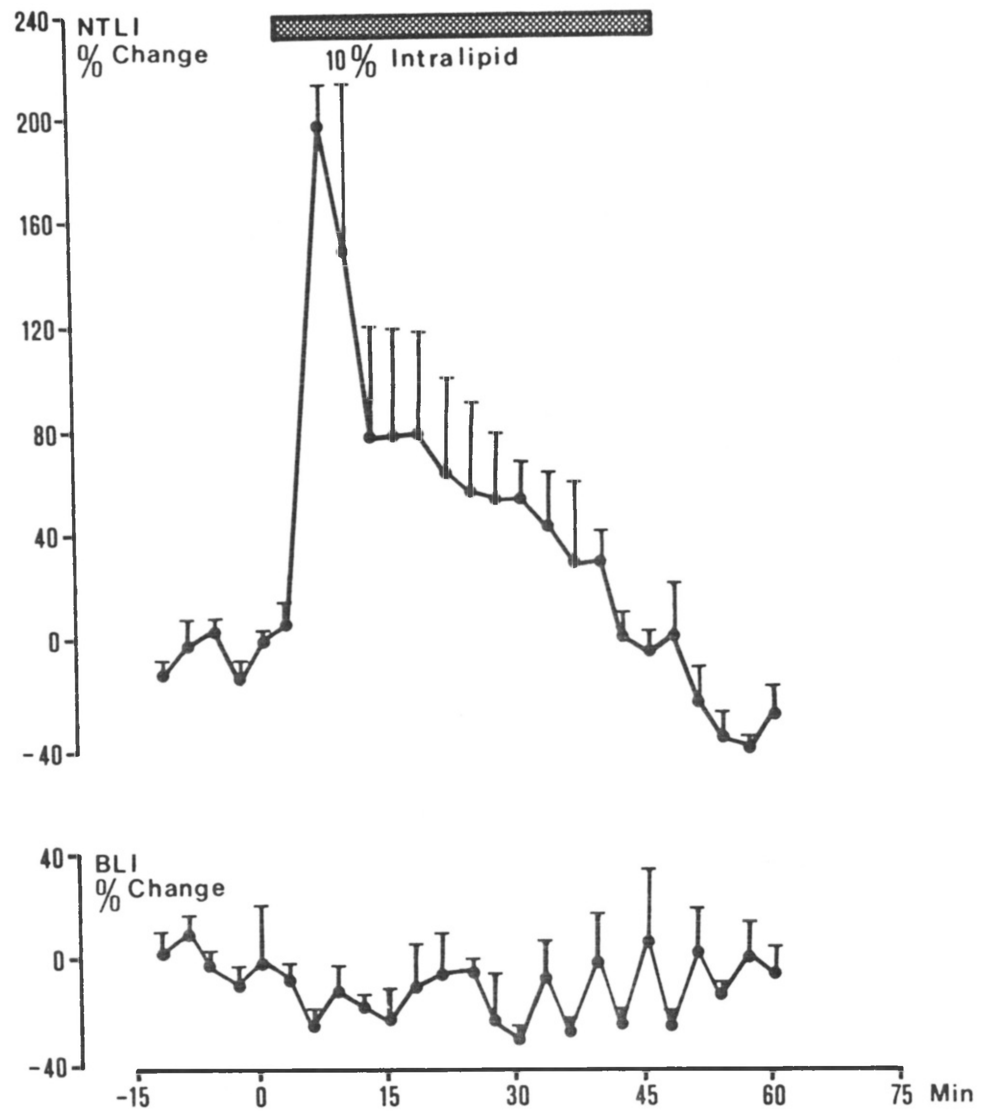


Figure 4:2. Effects of luminal perfusion over 45 min of 10 % intralipid on release of neurotensin-like immunoreactivity (NTLI) and bombesin-like immunoreactivity (BLI). Results are expressed as mean percentage changes from basal and SEM, n=4.

neurotensin was 20 pmol/l (s.d = 0.3 pmol/l, n =4). There was no change in bombesin-like immunoreactivity release throughout the perfusion period of 45 minutes (Figure 4:2). Synthetic bombesin, when infused arterially at final concentrations of 5×10^{-11} and 5×10^{-10} mol/l, caused a marked release of immunoreactive neurotensin into the venous effluent reaching 81 % and 100 % above basal neurotensin levels respectively (Figure 4:3). Carbachol, when infused arterially at a final concentration of 5×10^{-9} mol/l, caused a peak neurotensin release reaching 100 % above basal (Figure 4:4). Bombesin-like immunoreactivity was also released during carbachol infusion. A maximum response of bombesin was observed at a final carbachol concentration of 5×10^{-9} mol/l. However the bombesin response was 4 times as great as that of neurotensin with respect to carbachol infusion. Arterial infusion of nor-adrenaline did not affect the release of immunoreactive neurotensin at the doses tested (final concentrations of 1×10^{-6}, and 1×10^{-4} mol/l); but it had a marked inhibitory effect on the release of immunoreactive bombesin (Figure 4:6). The results in this study were expressed as percentage change from basal. This is because there were considerable variations in absolute basal and stimulated peptide concentrations in the

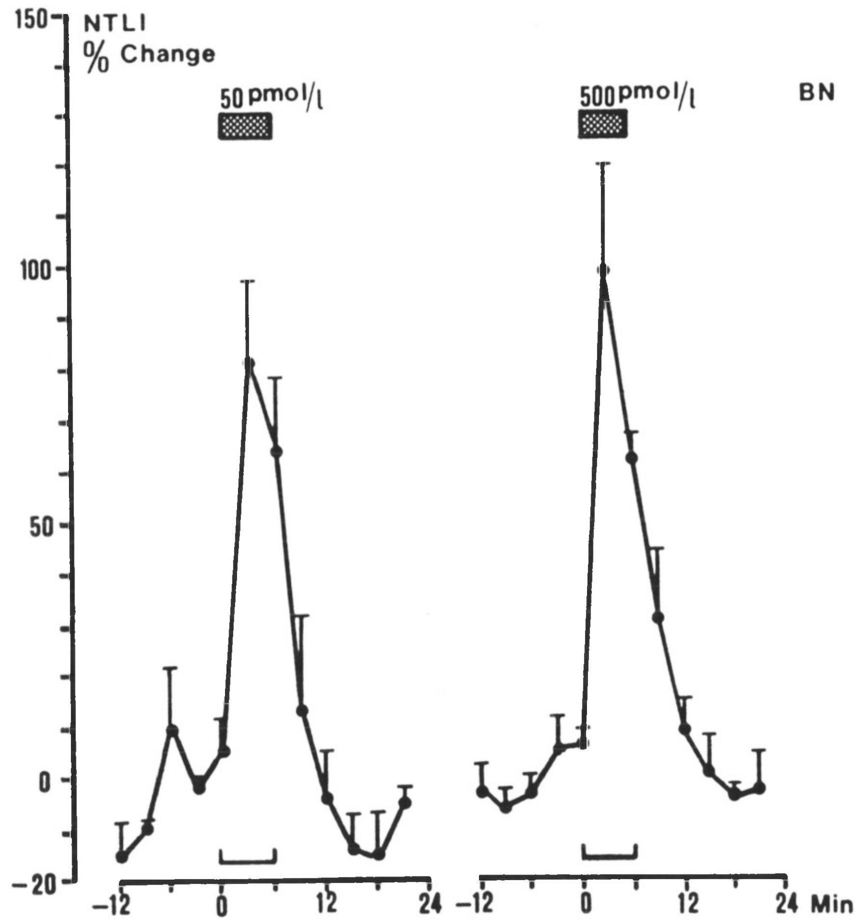


Figure 4:3. Effect of synthetic bombesin (BN) infused arterially over 6 min, (a) 50 pmol/l, and (b) 500 pmol/l on the release of neurotensin-like immunoreactivity (NTLI). Results are expressed as mean percentage change from basal and SEM, n=4.

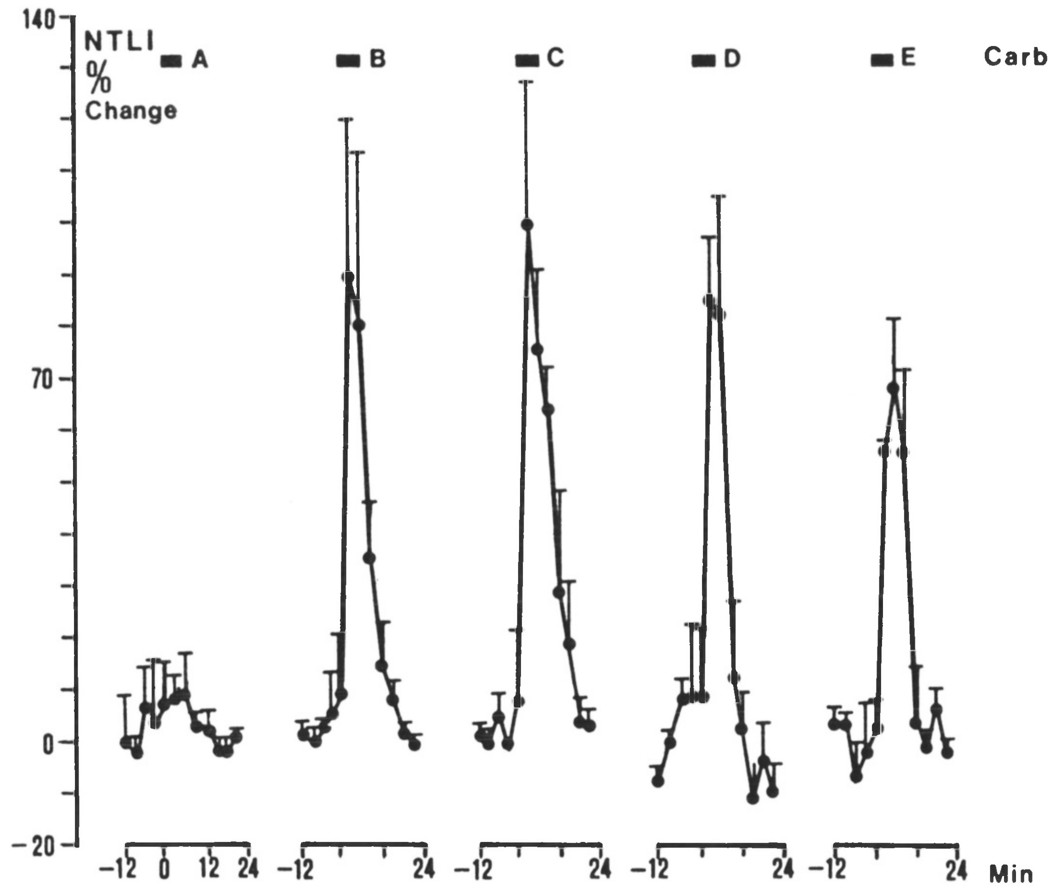


Figure 4:4. Effects of carbachol (Carb) on the release of neurotensin-like immunoreactivity (NTLI). Carbachol was infused arterially over 6 min at (A) 5 X 10^{-12}, (B) 5 X 10^{-10}, (C) 5 X 10^{-9}, (D) 5 X 10^{-8}, (E) 5 X 10^{-7} mol/l.

Results are expressed as mean percentage change from basal and SEM (n = 4-5).

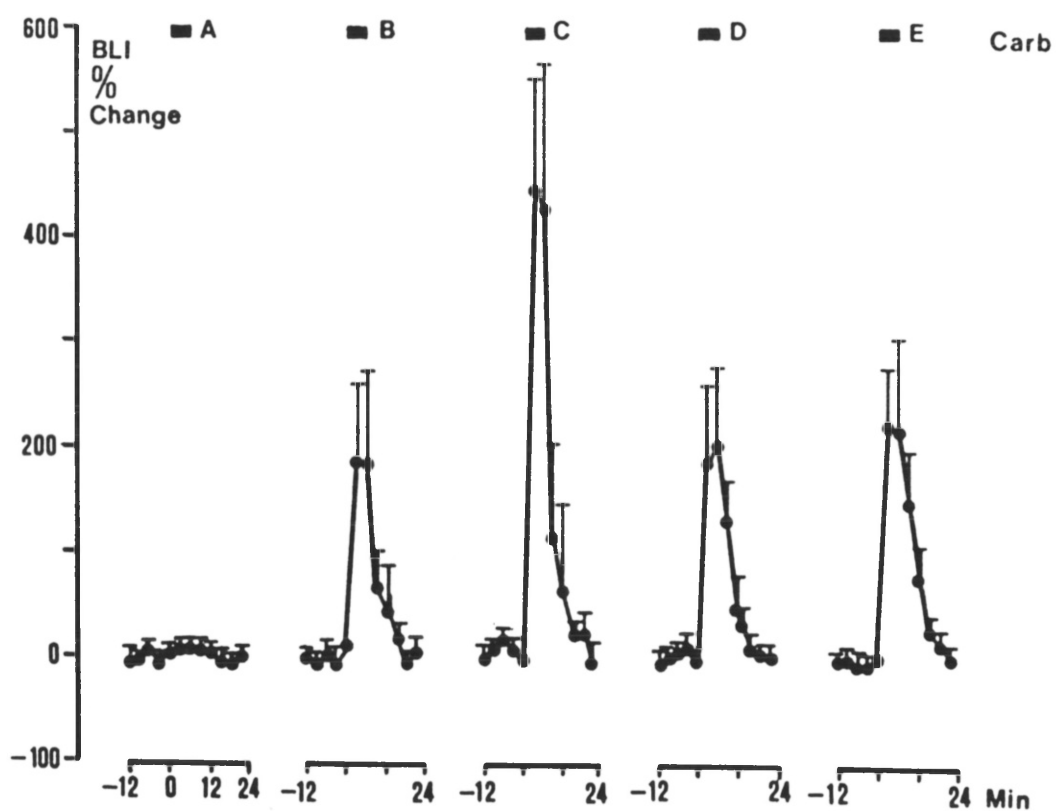
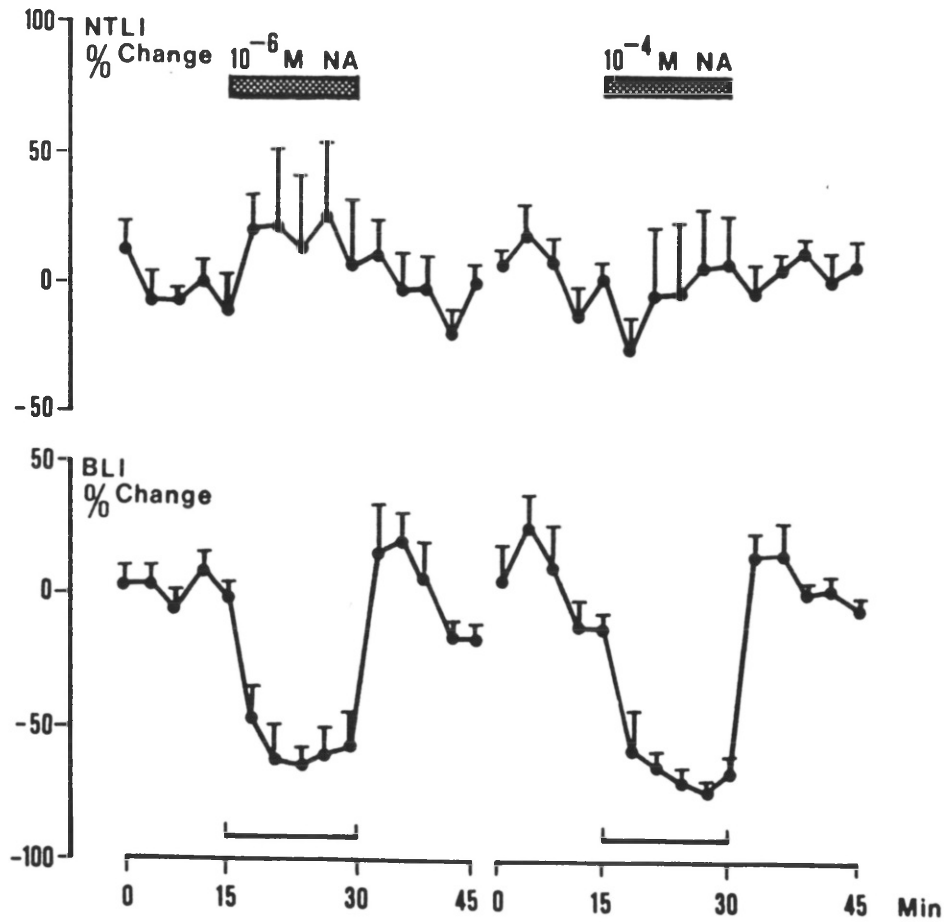


Figure 4:5. Effects of carbachol (Carb) infused arterially over 6 min at doses same as figure 4:4 in mol/l on the release of bombesin-like immunoreactivity (BLI). Results show mean percentage change from basal and SEM.



<u>Figure 4:6</u>. Effects of noradrenaline (NA) infused arterially over 15 min at (a) 1 X 10^{-6} mol/l and (b) 1 X 10^{-4} mol/l on the release of neurotensin- and bombesin-like immunoreactivities (NTLI and BLI). Results are the mean percentage changes from basal and their respective SEMs, n = 4.

venous effluent from individual preparations. However, when expressed as percentage change from basal, reproducible responses were obtained from the different preparations. A single preparation was capable of giving three reproducible responses during the experimental period of 30 minutes under the various infusion conditions.

SMS 201-995 infusion in man:

On control day (SMS diluent alone), following ingestion of a test breakfast, plasma immunoreactive neurotensin rose rapidly reaching a plateau concentration of 16.6 $\pm$ 4.4 pmol/l (mean $\pm$ standard error of the mean [sem], n = 8) and 14.3 $\pm$ 2.8 pmol/l (mean and sem, n = 8) at 15 and 30 min respectively from the mean basal neurotensin concentration of 7.6 $\pm$ 0.8 pmol/l (mean and sem, n = 8). On the SMS day (50 ug SMS 201-995 in SMS diluent), the meal stimulated rise of plasma immunoreactive neurotensin was completely abolished (Figure 4:7). Furthermore, a significant reduction in basal plasma neurotensin was observed following administration of the active compound.

Discussion

The viability of the rat isolated ileal preparation

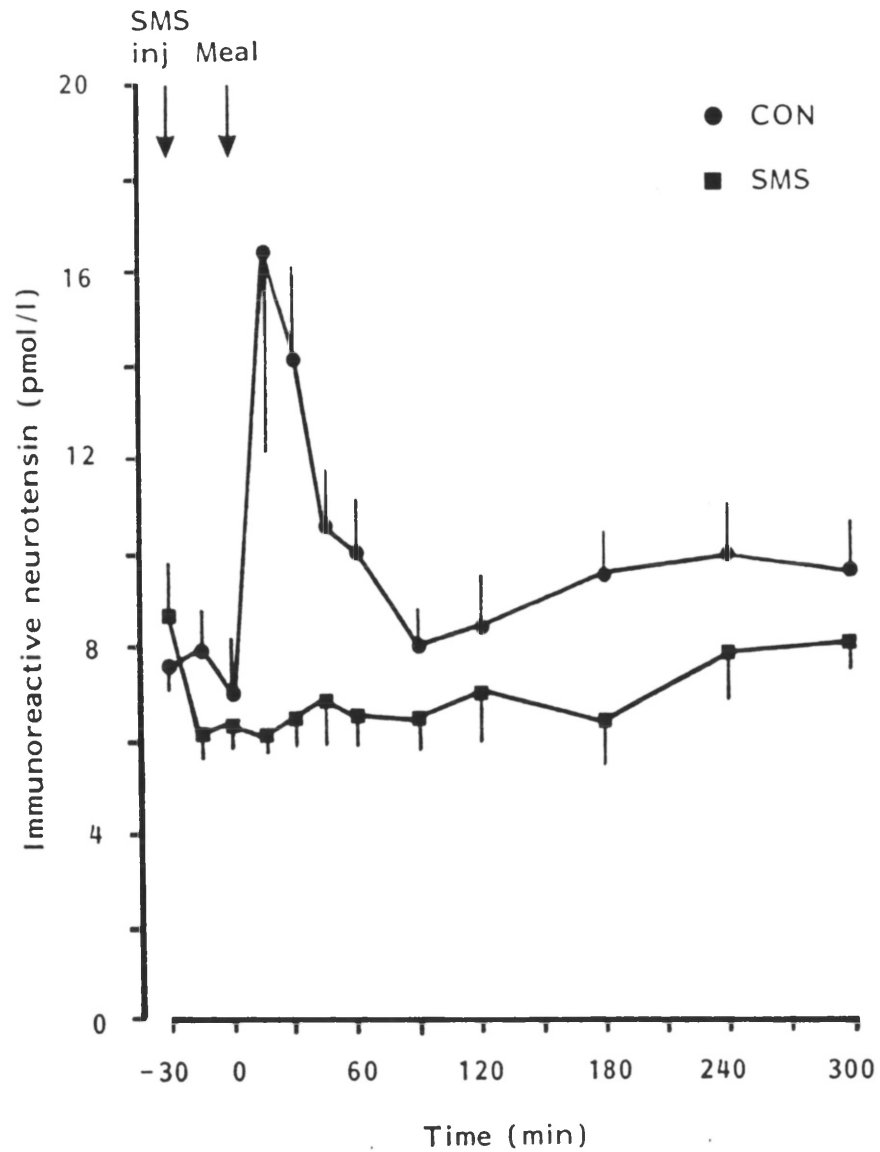


Figure 4:7. Effects of SMS 201-995 (SMS) on the meal-stimulated mean plasma concentrations of immunoreactive neurotensin in man. (●) control day, test breakfast. (■) SMS day, test breakfast plus 50 ug bolus SMS injection.

experiment was established by the intraluminal perfusion with 10 % Intralipid which caused a marked release of NTLI in the venous effluent. The release of immunoreactive neurotensin in response to luminal fat in the rat is in agreement with the findings of Ferris et al (1981). The venous effluent samples were also measured for immunoreactive bombesin, but no release was noted during luminal fat instillation. This suggests that the effect on neurotensin release by luminal fat was not a non-specific one. In the experiment described in this chapter, it has been demonstrated that the arterial infusion of bombesin stimulates the release of neurotensin, while a cholinergic stimulus such as carbachol when infused arterially increases the release of both neurotensin and bombesin, although the neurotensin response is an order of magnitude smaller. It is possible that the release of neurotensin from the rat gastrointestinal tract is controlled by peptidergic and cholinergic stimuli and/or the interaction of these two influences. Al-Saffer et al (1984) reported that the release of neurotensin from the small intestine of the rat was influenced by hexamethonium and morphine both of which abolished fat (oleic acid)-induced neurotensin release from the rat small intestine. However, atropine, at a dose which blocked muscarinic

receptors in the rat, did not inhibit the release of plasma neurotensin. In contrast to this, Feurle et al (1982) reported that in the dog, atropine significantly abolished the fat-induced release of plasma neurotensin. In man, Fletcher et al (1983a) reported that atropine inhibited basal as well as meal-stimulated release of plasma neurotensin when the plasma immunoreactivity was measured with an N-terminally directed neurotensin antiserum. The same authors (Fletcher et al 1983b) in another report demonstrated that in man infusion of bombesin but not of gastrin releasing peptide increased the plasma NTLI. Atropine, however, did not affect the release of neurotensin stimulated by bombesin infusion. It is not clear from this report if atropine suppressed the bombesin release or whether the bombesin infused reflected the "true" amount of the exogenous bombesin used, as immunoreactive bombesin was not measured in the study. The data presented here together with the other data discussed above demonstrates the complexity of the regulation of the release of neurotensin from the gastrointestinal tract. However it is clear that a luminal factor plays an important part in neurotensin release. Whether there is a direct or indirect neural influence in the regulation of neurotensin release from the gut requires further investigation. The neural control

is likely to be a local reflex originating from the enteric nervous system. The release of plasma NTLI is not affected by bilateral vagotomy as shown in animals and modified sham feeding and insulin hypoglycaemia in man (Rokaeus et al 1982, Eaves et al 1985) which suggests that a cholinergic non-vagal mechanism may be involved.

When the somatostatin analogue, SMS 201-995, was given as a bolus injection in human subjects, both basal and meal-stimulated release of neurotensin in plasma were significantly suppressed. It is therefore possible that in addition to the luminal factor, the release of neurotensin may also be controlled and/or modified by peptides such as somatostatin released locally in the gut, i.e. a peptidergic influence.

Chapter 5

NEUROTENSIN IN PC 12 CELLS

Introduction

Apart from the central nervous system and the gastrointestinal tract, neurotensin is also found in peripheral tissues such as the adrenals (Lee et al 1981, Goedert 1983b, 1984, Rokaeus et al 1984) and pituitary glands (Goedert et al 1982). In the cat adrenal gland, neurotensin is immunologically and histologically concentrated in a sub-population of the noradrenaline-containing cells within the adrenal medulla (Terenghi et al 1983). Upon splanchnic nerve stimulation of the cat adrenal, immunoreactive neurotensin, which has the same chromatographic characteristics as authentic neurotensin, is released into the circulation (Rokaeus et al 1984, Ferris et al 1986). The adrenal medulla is unique in that it not only acts as an endocrine organ but also represents part of the sympathetic nervous system which regulates involuntary functions such as heart rate, intestinal movements, the dilation of the pupil and the classic "fight or flight" responses. In addition to the classical neurotransimitters,

adrenaline and noradrenaline, the adrenal medulla stores and releases neuropeptides, for example, the opioid peptides. The adrenal chromaffin cells, because of their kinship with sympathetic neurons, have been widely used for the studies of production and secretion of neurotransmitters and the processing of regulatory peptides (Pruss et al 1985, Wilson et al 1980, 1984). Recently, it has been demonstrated that the cellular content and release of neurotensin is modulated synergistically by dexamethasone and nerve growth factor (NGF) in a neoplastic chromaffin cell line, the PC12 cell line (Tischler et al 1982, 1983). The PC12 clonal cell line was established from a transplantable rat adrenal phaeochromocytoma (Warren and Chute 1972, Greene and Tischler 1976). In their original report, Greene and Tischler demonstrated that the PC12 cells synthesised and stored dopamine and noradrenaline but not adrenaline. The cell line responded reversibly to NGF. In the presence of NGF, these cells exhibited processes branching in a similar manner to those produced by sympathetic neurones in culture. Removal of NGF from the culture medium caused the degeneration of these processes and cell multiplication resumed (Greene and Tischler 1976). Nerve growth factor (NGF) is a polypeptide involved in the regulation of growth and differentiation of sensory and sympathetic

neurons. It represents one of the most important trophic factors discovered in recent years (Levi-Montalcini 1987). NGF receptors have been demonstrated in PC12 cells, human melanoma cells, human neuroblastoma cells and cultured sympathetic neurons (Grob et al 1983, Greene and Greene 1986). Recently, Buck et al have suggested that NGF receptor mRNA expression is developmentally regulated in specific tissues of the nervous system in a differential fashion (Buck et al 1987). The exact mechanism of action of NGF is still far from clear. Glucocorticoid and related steroid hormones such as oestrogen and progesterone represent another group of substances which may be involved in the regulation of protein gene expression. For example, Evans et al reported that growth hormone gene expression was regulated by both glucocorticoid and thyroid hormones (Evans et al 1982). Dexamethasone has been reported to increase the cellular content of enkaphalins in neuroblastoma X glioma hybrid cells (Glase et al 1981).

Because of the synergistic effect of NGF with dexamethasone on the cellular concentration of neurotensin in PC12 cells (Tischler et al 1982, 1983), some aspects of the regulation of neurotensin biosynthesis have been examined, in

collaboration with Dr Arthur Tischler and his colleagues at Tufts University School of Medicine, Boston, USA, using the PC12 cell line.

Materials and Methods

Cell culture:

Cell cultures were performed by Dr Arthur Tischler from his stock PC12 cell cultures at his laboratory in Boston as previously described (Tischler 1982, 1983). Briefly, stock cultures of PC12 cells (passage below 40) were routinely grown on polystyrene tissue culture dishes. The cells were grown in RPMI 1640 medium (Gibco, Grand Island, N.Y.) containing 10 % heat-activated horse serum (K.C. Biologicals, Lenexa, Kansas), 5 % fetal bovine serum (Hyclone Inc), 50 mg/ml streptomycin, and 50 U ml penicillin (Gibco). For experimental manipulation, cells from near-confluent stock culture dishes were detached with a rubber policeman and plated on Falcon dishes coated with air dried rat tail collagen. Both the stock and experimental cultures were maintained at 37^0C in a water-saturated atmosphere of 93 % air and 7 % CO_2. The medium was changed three times per week.

Experimental conditions:

Triplicate cultures of PC12 cells plated in medium supplemented with the following agents at various concentrations were maintained for 14 days and up to 4 weeks: (1) 2.5 S mouse salivary gland nerve growth factor. (2) dexamethasone disodium phosphate (Merck Sharp & Dohme Research Laboritories, West Point, PA). (3) forskolin (Calbiochem). (4) cholera toxin (Boehringer- Mannheim). (5) 12-O-tetradecanoyl-phorbol-13- acetate (TPA).

The triplicate cultures were either supplemented with a single agent or in combination with another agent or with all the agents together. The final medium change was 24 hours prior to the end of the experimental period. After the removal of the culture medium, the cells were harvested by washing in Hanks' balanced salt solution (Gibco) three times, and were scraped from the culture dishes in ice-cold phosphate buffered saline (PBS), pH 7.2, containing 500 KIU/ml Aprotinin (Sigma), sonicated, and boiled for 5 minutes. Aliquots were taken for protein determination, and to the remainder, bovine serum albumin (Radioimmunoassay grade, Sigma) at a final concentration of 1.5 % was added. The samples were frozen and lyophilized in a Speedvac concentrator (Savant, Mass, USA). The lyophilised

samples were reconstituted in distilled water (1.0 ml), and aliquots (0.05 and 0.1 mls) assayed for immunoreactive neurotensin in duplicate.

Results

The cellular concentration of immunoreactive neurotensin in PC 12 cells was affected by NGF, dexamethasone, forskolin and cholera toxin synergistically. The dose-response curves of NGF, dexamethasone and forskolin on cellular immunoreactive neurotensin concentration are shown in Figure 5:1, 5:2, 5:3. The cultures were maintained for two weeks before the harvest of cells. In the presence of dexamethasone (1uM), a plateau concentration of immunoreactive neurotensin (pmol/mg protein) was achieved at a NGF concentration between 50 and 100 ng/ml (Figure 5:1). In the presense of NGF (200 ng/ml), a plateau concentration of immunoreactive neurotensin was reached with a concentration of dexamethasone between 10^{-6}M and 10^{-5}M. With forskolin, in the presence of NGF (200ng/ml) and dexamethasone (1 uM), the dose-response curve showed a peak concentration of immunoreactive neurotensin at a forskolin concentration of 0.25×10^{-6} M (Figure

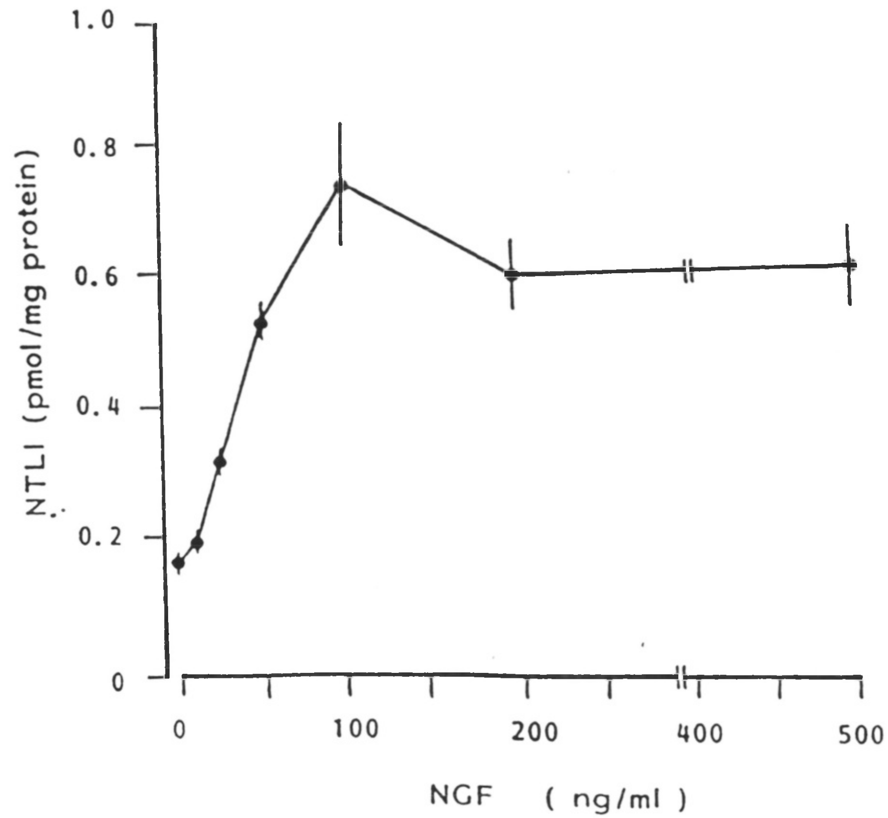


Figure 5:1. Dose response curve for the effects of nerve growth factor (NGF) on the neurotensin-like immunoreactivity (NTLI) of triplicate culture dishes in the presence of dexamethasone (1 uM). PC 12 cell cultures were maintained for 14 days before harvest. NTLI contents are expressed as pmol/mg protein (mean ± SEM, n = 3).

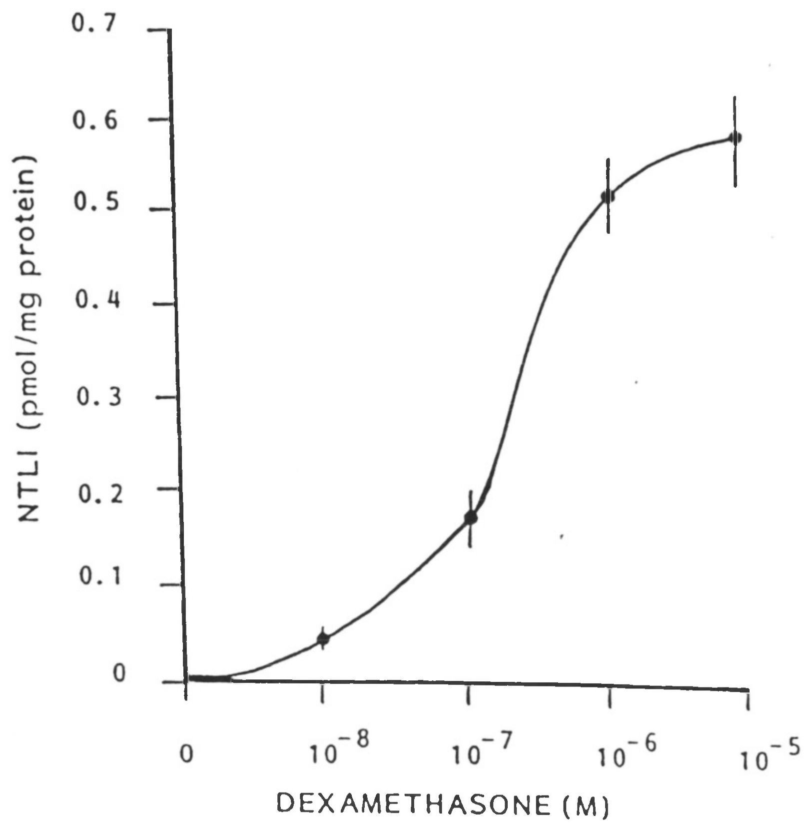


Figure 5:2. Dose response curve for the effects of dexamethasone on the neurotensin-like immunoreactivity (NTLI) concentration of triplicate culture dishes in the presence of nerve growth factor (NGF). Cultures were maintained for 14 days before harvest. NTLI contents are expressed as pmol/mg protein (mean ± SEM, n = 3).

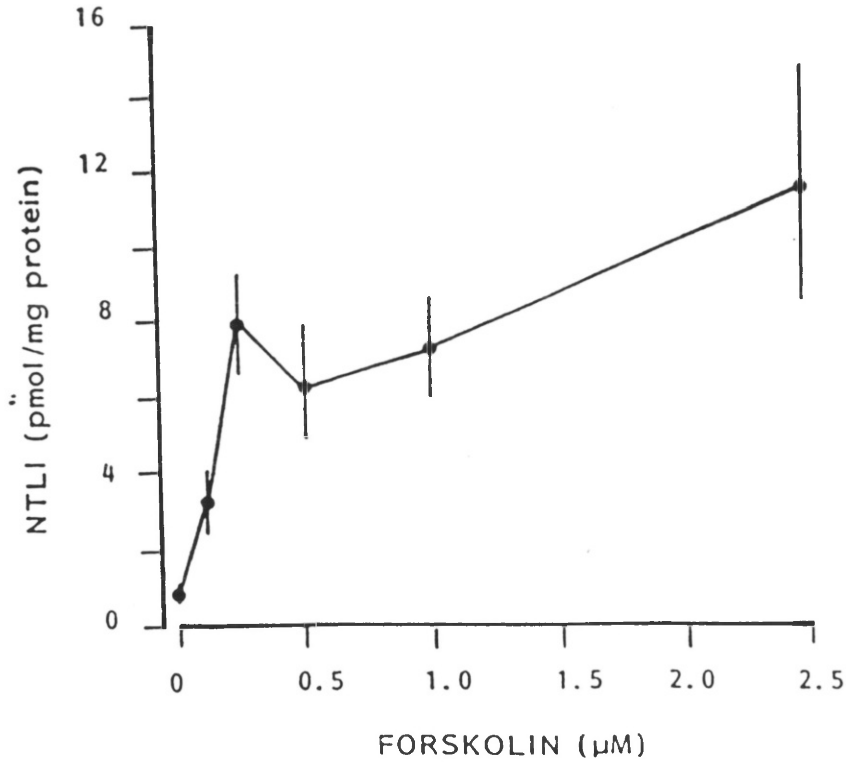


Figure 5:3. Dose response curve for the effects of forskolin on the neurotensin-like immunoreactivity (NTLI) concentration of triplicate culture dishes in the presence of nerve growth factor (200 ng/ml) and dexamethasone (1 uM). Cultures were maintained for 14 days before harvest. Results are expressed as pmol/mg protein (mean ± SEM, n = 3).

5: 3). The early synergistic stimulatory effects of NGF, dexamethasone, forskolin, cholera toxin , Li and TPA are summarised in Figure 5: 4 and Figure 5: 5. The marked increase in PC12 cellular immunoreactive neurotensin concentration in the presence of NGF, dexamethasone and forskolin in PC12 cells lasted up to a period of 28 days when the experiment stopped (Figure 5: 6). The magnitude of the increase of neurotensin immunoreactivity in the PC 12 cells with NGF, Dexamethasone and forskolin was ten fold greater than that of NGF and Dexamethasone in the PC12 cells (Figure 5: 6).

Discussion

The data presented in this chapter demonstrate that NGF, dexamethasone, forskolin and cholera toxin act synergistically to increase the cellular content of NTLI in PC12 cells. This additive effect was maintained for up to 28 days when the experiment was stopped. The increase in cellular neurotensin content was most spectacular when the PC12 cells were treated with forskolin or cholera toxin in the presence of NGF and dexamethasone. This increase was an order of magnitude greater than when the cells were treated with NGF and dexamethasone only.

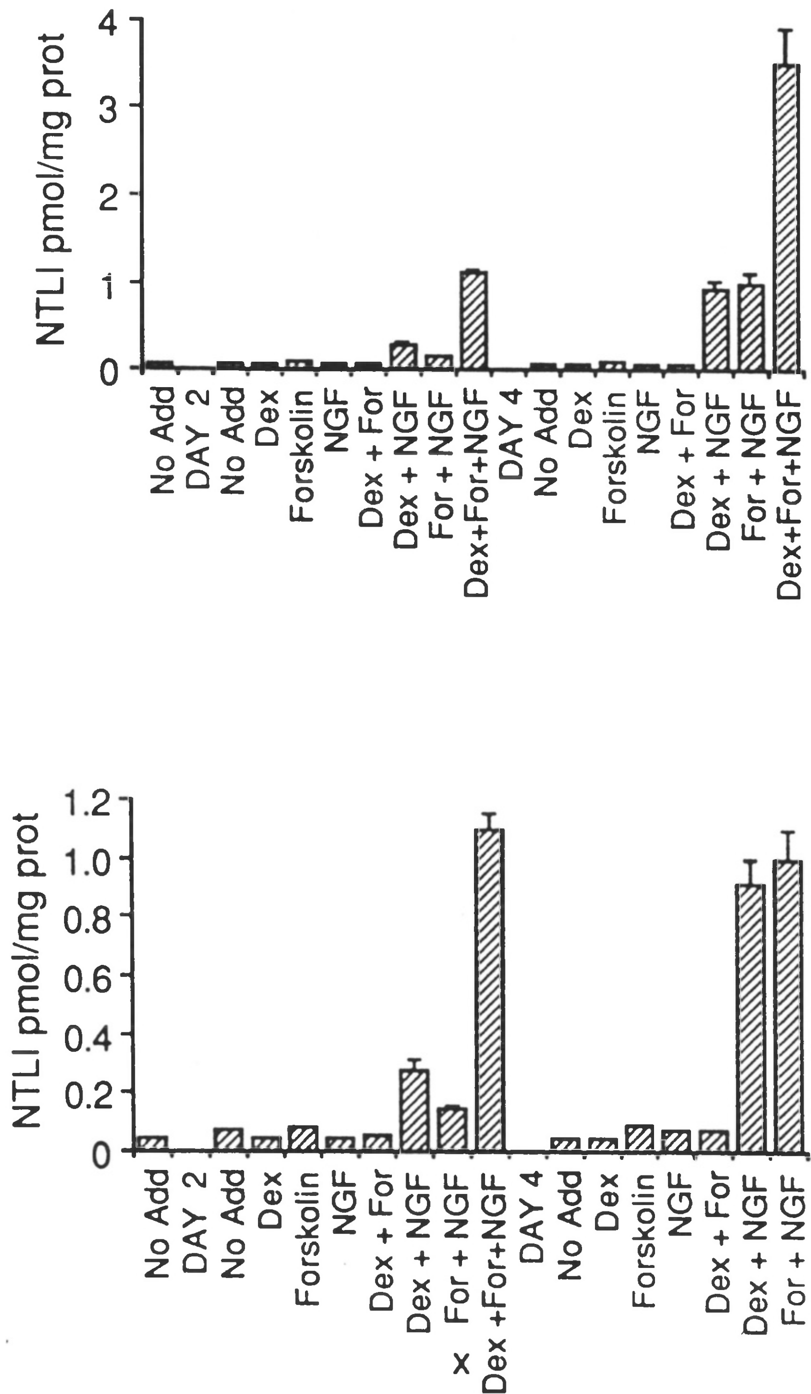


Figure 5: 4. Early effects of nerve growth factor (NGF), dexamethasone (Dex), forskolin (For) on the neurotensin-like immunoreactivity (NTLI). Lower panel is the same as the upper panel except that on the lower panel result for Dex + For + NGF is not shown.

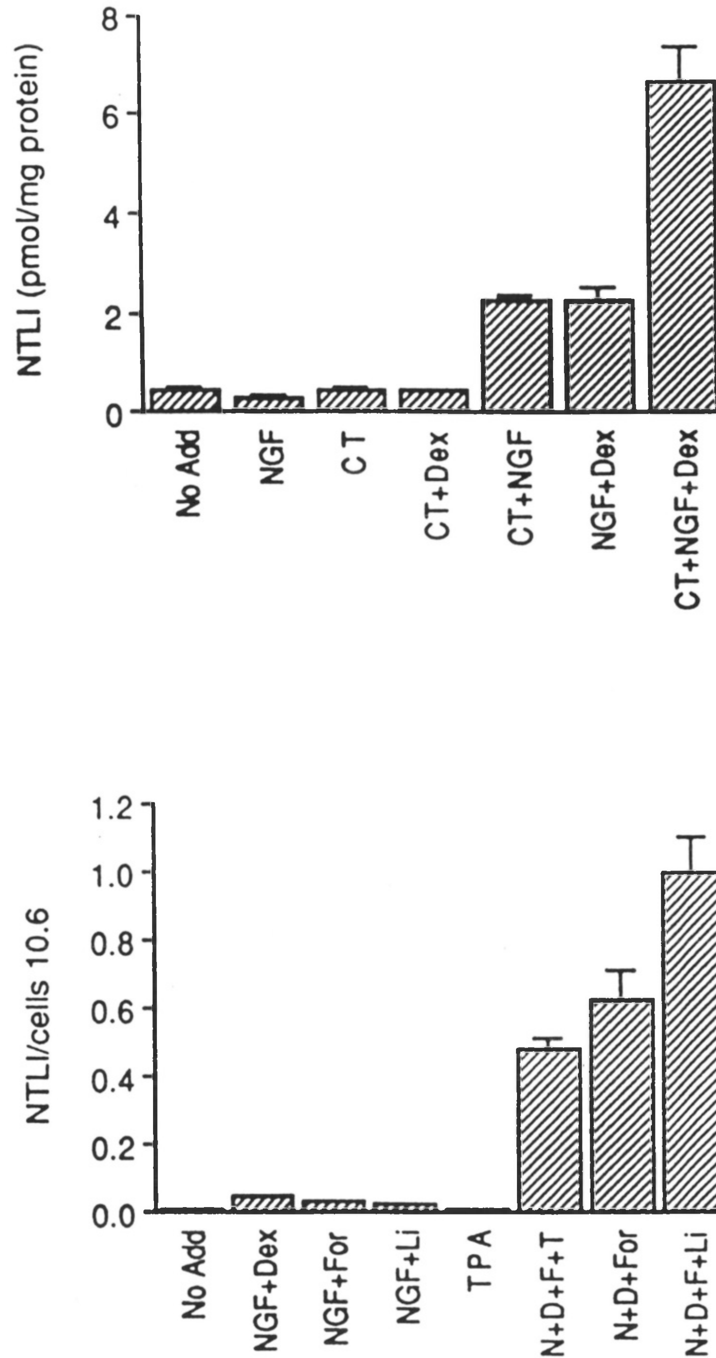


Figure 5: 5. Early effects of nerve growth factor (NGF, N), dexamethasone (Dex, D), forskolin (F), cholera toxin (CT), TPA, and Lithium (Li) on the neurotensin-like immunoreactivity (NTLI).

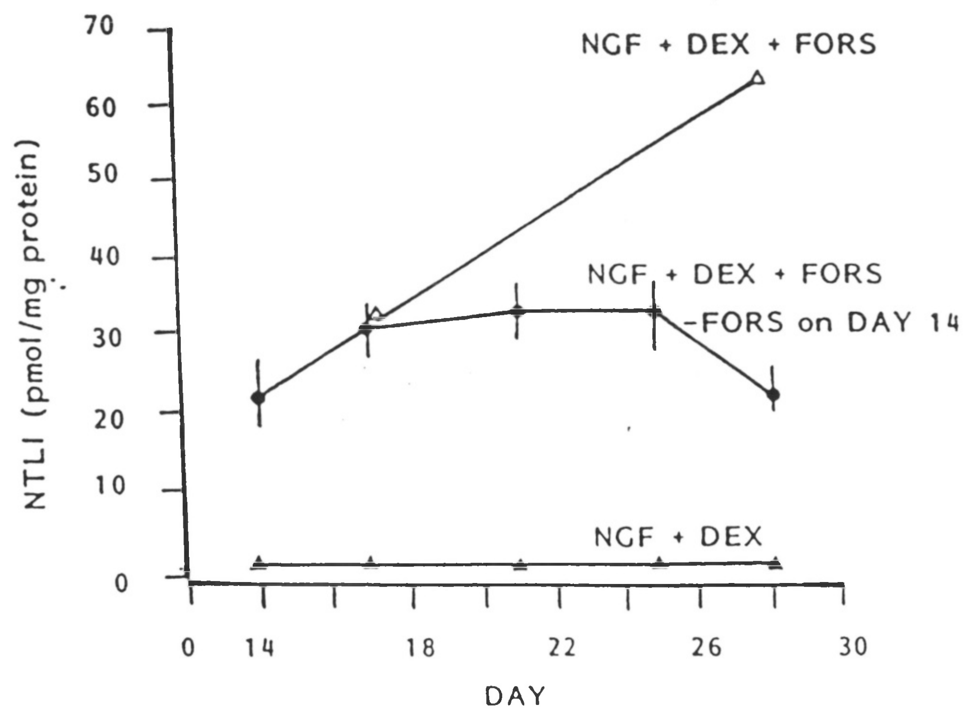


Figure 5:6. Effect of removing forskolin from cultures pre-treated for 14 days with forskolin (FORS, 1uM), dexamethasone (DEX, 1 uM) and nerve growth factor (NGF, 200 ng/ml) on the neurotensin-like immunoreactivity (NTLI) content. The concentration of NTLI 14 days after forskolin removal remains as high as at the time of the removal. Results are expressed as mean ± SEM (n = triplicate culture dishes).

It is well recognised that protein phosphorylation is one of the important general mechanisms which control the intracellular events in mammalian tissues in response to external physiological stimuli. The agents used in the present study are known to have potent biochemical effects in the regulation of the intra-cellular activity. Forskolin, isolated from the roots of the Indian herb, coleus forskohlii, activates adenylate cyclase. Adenylate cyclase is required to transform adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) which in turn activates protein kinase A and subsequent protein phosphorylation. Cholera toxin, produced by cholera bacilli, plays an inhibitory role in the hydrolysis of guanosine triphosphate (GTP) to guanosine diphosphate (GDP) by inhibiting the enzyme GTPase. GTP is important in the activation of guanine nucleotide binding proteins (G proteins) which serve as a link between the physiological stimuli or environmental factors and the receptors on the cell membrane and the subsequent intra-cellular response. The phorbol ester, TPA, a tumour promoting agent, is an activator of protein kinase C. Protein kinase C is part of the "second message" pathway which originates from the membrane lipid, phosphatidylinositol 4,5 biphosphate. The biochemical cascades of this "second messenger" are

depicted in Figure 5.7.

The present study shows that forskolin or cholera toxin in conjunction with NGF and dexamethasone increases markedly the cellular content of NTLI in PC 12 cells, while the phorbol ester TPA has no effect in this respect either alone or together with NGF and dexamethasone. This implies that the regulation of neurotensin biosynthesis in PC12 cells may be adenylate-cyclase mediated. In normal bovine chromaffin cells Pruss et al (1985) reported that TPA increases the cellular store of vasoactive intestinal polypeptide (VIP) but not of enkephalins, they concluded that protein kinase C was involved in the biosynthesis of VIP.

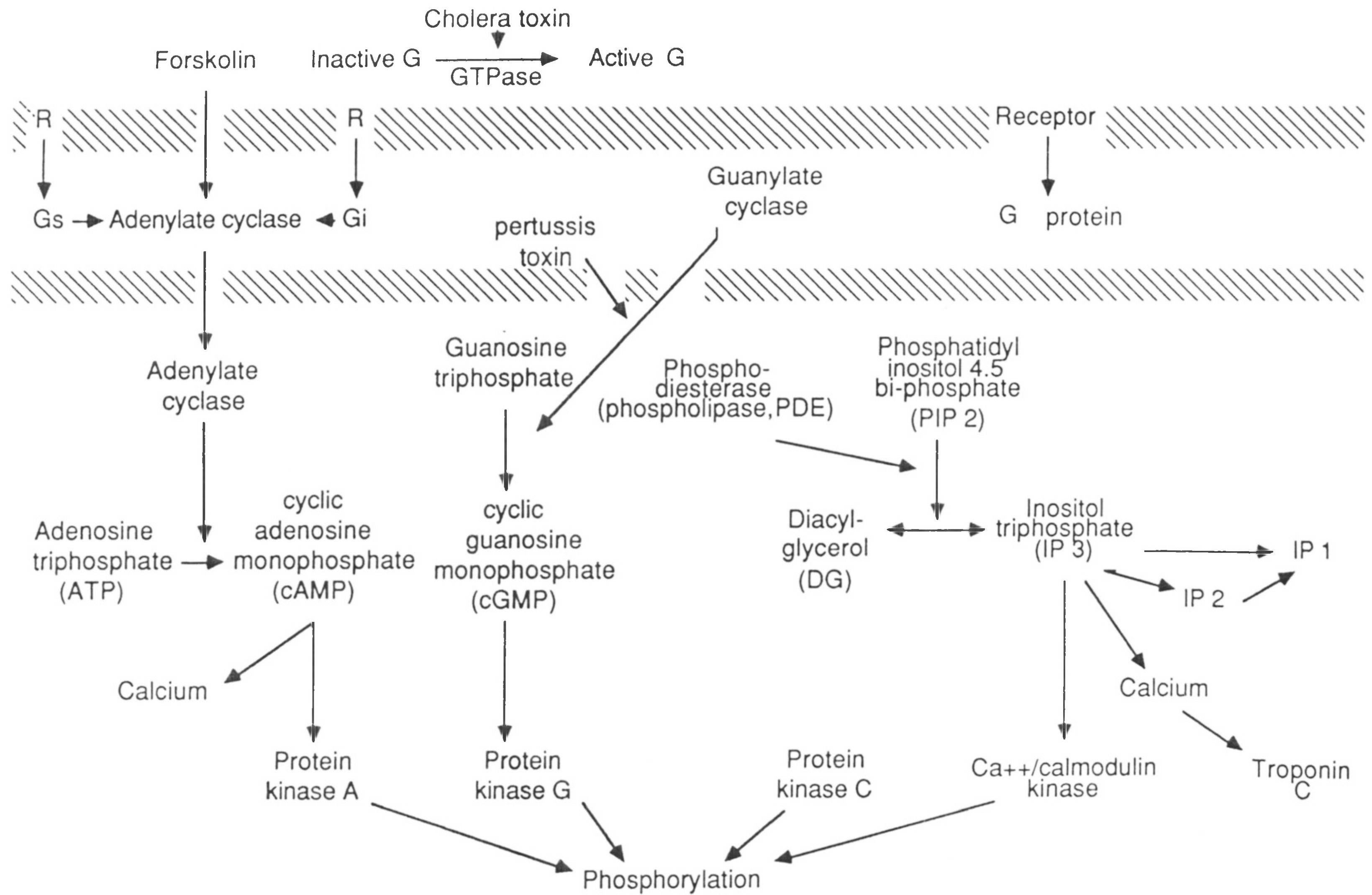


Figure 5:7. Biochemical cascade.

Chapter 6

PHARMACOKINETICS AND METABOLISM OF EXOGENOUS INFUSED NEUROTENSIN

Introduction

It has previously been demonstrated that exogenously administered neurotensin is rapidly degraded in the circulation of rat and sheep to various metabolites, particularly to the relatively stable N-terminal octapeptide fragment, neurotensin 1-8 (Aronin et al 1982, Shulkes et al 1983). The metabolism of neurotensin in man is not known and the present study was undertaken to investigate the metabolic fate of intravenously infused neurotensin in six healthy human subjects. The disappearance half life ($t_{1/2}$) of neurotensin in circulating plasma has been determined and compared with that of infused neurotensin 1-8 in the same subjects. Molecular forms of plasma immunoreactive neurotensin have been characterised by gel filtration and high pressure liquid chromatography (HPLC).

Methods and Materials

Approval of infusions of neurotensin and

neurotensin 1-8 in human subjects was obtained from the Research Ethics Committee of the Royal Postgraduate Medical School and all the volunteers gave their informed written consent.

Six healthy fasting subjects, four men and two women, aged 24-39, were studied. Each subject underwent two separate infusions at an interval of one week: one of synthetic neurotensin and another of synthetic neurotensin 1-8 in random order. Indwelling cannulae were inserted in both antecubital veins, one for blood sampling and the other for the infusion of peptide. Two basal blood samples were taken before the start of the infusion. Neurotensin was administered at a calculated rate of 2.4 pmol/kg/min. During the infusion period of 45 minutes, blood samples were taken at 15, 30, 40, 42 and 45 min. Futher blood samples were taken 1, 2, 4, 6, 8, 10, 15, 20 min after the end of the infusion. Blood samples of 5-10 ml were collected into lithium heparin tubes containing 4000 kallikrein inhibitory units of aprotinin (Trasylol). The samples were centrifuged immediately and the separated plasma was frozen and stored at -20 C within half an hour.

Plasma immunoreactive neurotensin was determined by radioimmunoassay as described in chapter 2 with the

C-terminally directed antiserum NT 58 and the N-terminally directed antibody 07709. The characteristics of the N-terminally directed antiserum 07709 have been described previously (Theodorsson-Norheim et al 1983a). Briefly, the cross-reactivity of antiserum 07709, with respect to neurotensin taken as 100 %, was 152 % and 160 % with neurotensin 1-8 and neurotensin 1-9 respectively, and less than 1 % with neurotensin 1-7, 8-13 and 9-13. The plasma concentrations of pancreatic polypeptide, insulin, glucose and cholesterol in the samples were also measured by established methods (Adrian et al 1976, Albano et al 1972, Barham and Trinder 1972, Lie et al 1976).

Chromatographic analysis

For gel filtration, two plasma samples were used taken from one subject: one (4 ml) during the basal period and one (3 ml) after 45 minutes of neurotensin infusion. Each sample was applied directly to a Sephadex G-50 superfine column (1.5 X 90 cm) as consecutive runs. The column was eluted and calibrated as described previously (chapter 2). Fractions of 2.0 ml were collected and 0.5 ml of each fraction was assayed for immunoreactive neurotensin with both antisera NT 58 and 07709.

HPLC was performed on a Waters system (Waters Associates Inc., Milford, Mass., USA) as previously described (chapter 3) with an octadecylsilylsilica column (u Bondapak C18, 0.39 X 30 cm). Two pooled samples from three subjects were obtained: one basal plasma (24.5 ml) and the other from the plateau period of the neurotensin infusion (plasma volume 19.0 ml). These two plasma pools were then extracted separately on octadecylsilylsilica cartridges (Sep-pak C18) prior to HPLC analysis. Proteins and high polarity compounds were washed off with 10.0 ml 0.2 % (vol/vol) trifluoroacetic acid (TFA) in distilled water. The cartridges were then eluted with 1.0 ml 40 % (vol/vol) acetonitrile (HPLC grade) in distilled water containing 0.2 % (vol/vol) TFA and the eluents centrifuged. The eluents (0.8-0.9 ml) were diluted with an equal volume of 0.2 % (vol/vol) TFA in distilled water and applied to the u-Bondapak C18 column and eluted over 40 minutes with a linear gradient from 0 to 40 % (vol/vol) acetonitrile in distilled water containing 0.2 % (vol/vol) TFA, followed for a further 10 minutes at 40 % of acetonitrile with 0.2 % TFA. The flow rate was set at 1 ml/minute, and fractions of 1 ml were collected. Synthetic neurotensin and neurotensin 1-8 were run on separate occasions using identical conditions. Aliquots (200 ul) of each fraction were assayed

with the two neurotensin antisera NT 58 and 07709.

Treatment of the data

Peptide concentrations are expressed as mean and standard error of the mean (SEM) in pmol/l. Glucose and cholesterol concentrations are expressed as mean and SEM in mmol/l. The half-life ($t_{1/2}$) of neurotensin and neurotensin 1-8 was determined by the method described by Rowland and Tozer (1980). The results for both infusions were expressed as the difference from the basal concentration for each time point for each subject and expressed as the mean and SEM. The peak peptide concentration and the six peptide concentrations of samples collected immediately after the cessation of the infusions were plotted on a logarithmic scale against time. From the slope of the plot the time required for the the plasma immunoreactive neurotensin concentration to decline to one half of peak concentration was taken as its disapperance half life. Statistical analysis was performed by the Wilcoxon rank sum test.

Results

Intravenous infusion of neurotensin produced a rapid increase in the plasma concentration of

immunoreactive neurotensin when measured with the C-terminally directed antiserum NT 58, reaching a plateau concentration within 15 minutes (Figure 6:1). This level was maintained for the duration of the infusion. When the same plasma samples were measured with the N-terminally directed antiserum 07709, a slower rise of plasma NTLI was demonstrated (Figure 6:2), the highest concentration being attained between 40 and 45 minutes after the start of the infusion. The basal and peak concentrations of plasma NTLI when measured with NT 58 were 22 ± 1 and 126 ± 17 pmol/l and when measured with antiserum 07709 were 44 ± 9 and 401 ± 60 pmol/l respectively. The $t_{1/2}$ of plasma NTLI was calculated to be 1.4 minutes as measured with antiserum NT 58 and 6.0 minutes as measured with antiserum 07709 (Figure 6:3). When neurotensin 1-8 was infused, a slow increase of plasma concentration of NTLI was demonstrated when samples were measured with antiserum 07709. The $t_{1/2}$ of neurotensin 1-8, as measured with antiserum 07709, was calculated to be 30.0 minutes (Figure 6:4). Infusion of neurotensin and neurotensin 1-8 at a rate of 2.4 pmol/kg/min produced no unpleasant effects in any subjects and no cutaneous flushing or significant changes of pulse rate and blood pressure were noted.

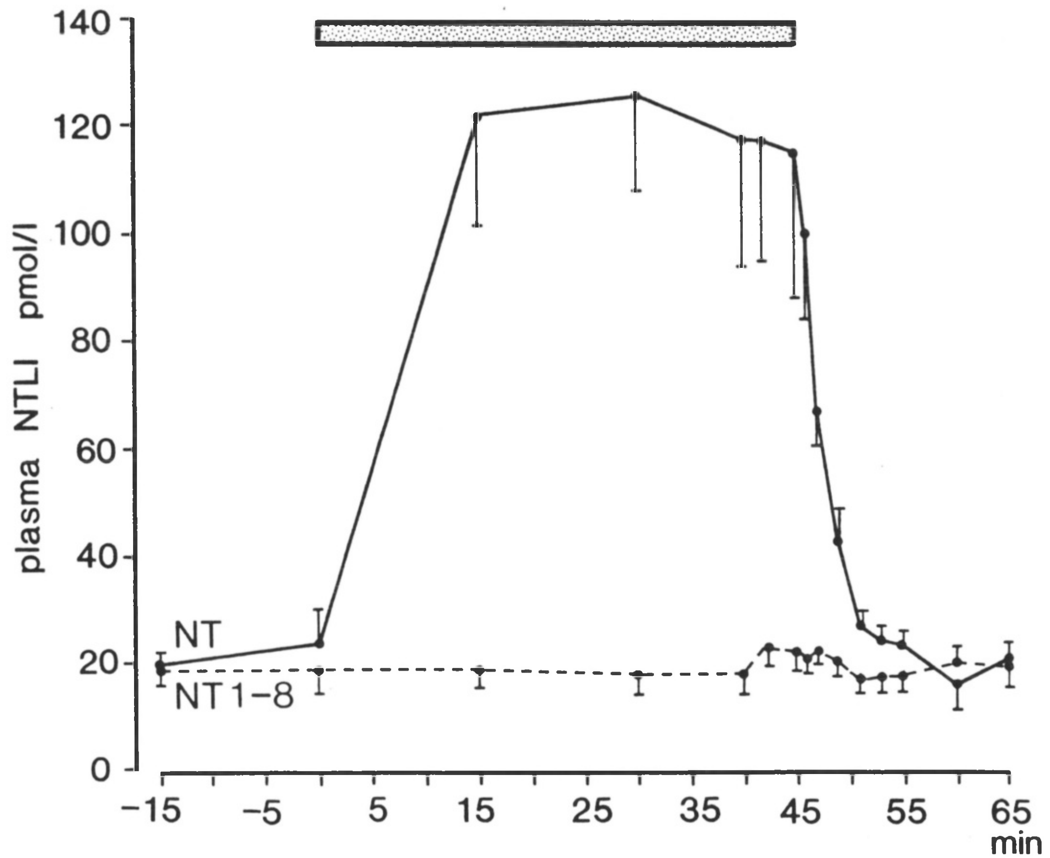


Figure 6:1. Plasma neurotensin-like immunoreactivity (NTLI) in pmol/l (mean ± SEM) measured with C-terminally directed antiserum NT 58 during infusion of NT (————) and NT 1-8 (-------) at 2.4 pmol/kg/min (n = 6).

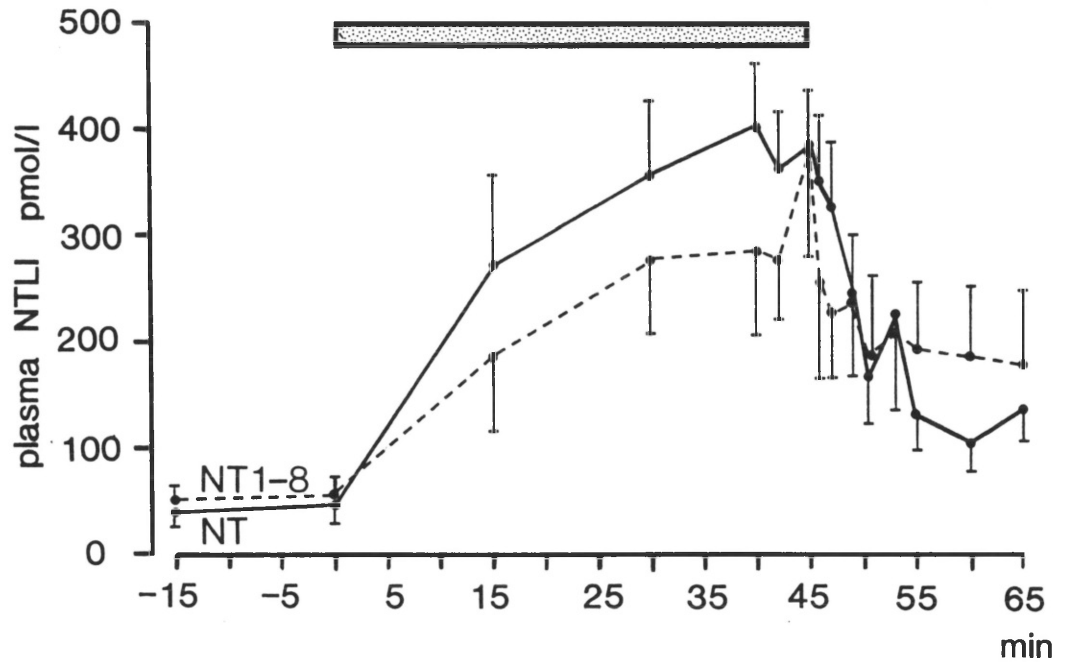
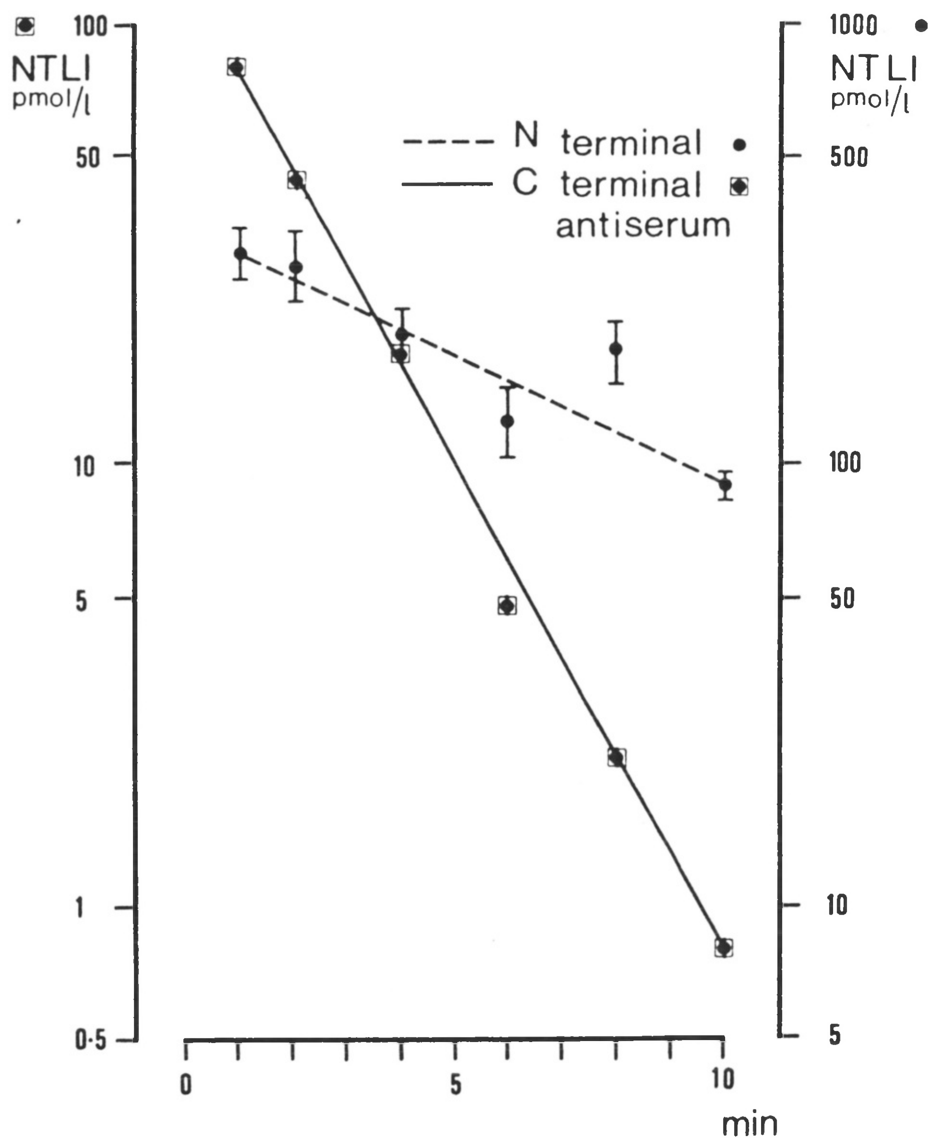


Figure 6:2. Plasma neurotensin-like immunoreactivity (NTLI) in pmol/l (mean ± SEM) measured with N-terminally directed antiserum 07709 during infusion of synthetic NT (————) and NT 1-8 (------) at 2.4 pmol/kg/min (n = 6).



<u>Figure 6:3</u>. Disappearance half-time plots of infused neurotensin (NT) measured with C-terminally directed antiserum NT 58 (————) and N-terminally directed antiserum 07709 (------). The plasma immunoreactivity (NTLI) in pmol/l was expressed as the difference from the basal for each timed point and half-time was determined from the slope. The NTLI (pmol/l) measured with NT 58 is on the left vertical axis and the NTLI (pmol/l) measured with 07709 is on the right vertical axis. Results were expressed as the mean and SEM (n ± 6).

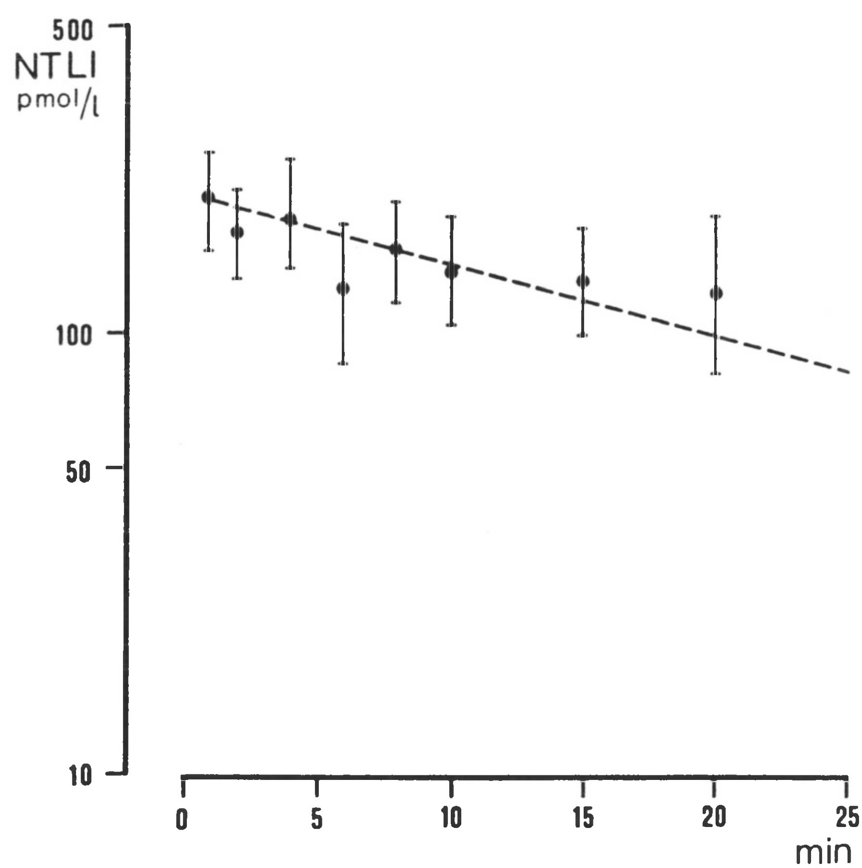


Figure 6:4. Disappearance half-time plot of infused neurotensin 1-8 measured with antiserum 07709. The plasma neurotensin-like immunoreactivity (NTLI) was expressed as mean ± SEM in pmol/l (n = 6).

Infusion of neurotensin and neurotensin 1-8 produced no changes in the mean plasma insulin, glucose or cholesterol concentrations. However, infusion of neurotensin caused a significant rise in plasma pancreatic polypeptide concentrations at 15 and 30 minutes ($p < 0.05$) which was not observed during infusion of neurotensin 1-8 (Table 6:1).

Gel filtration of the basal plasma sample and of the plasma sample taken at 45 minutes of the neurotensin infusion from one subject demonstrated that immunoreactive neurotensin eluted in the positions of synthetic neurotensin and neurotensin 1-8. In addition, material emerging at and just after the void volume position was also noted (Figure 6:5).

In basal plasma, the majority of non-void volume NTLI was eluted in the position of neurotensin 1-8 as measured with antiserum 07709; much less immunoreactivity (less than 6 % of the neurotensin 1-8 immunoreactivity) was eluted in the position of neurotensin as measured with antiserum NT 58. However, in the plasma taken 45 minutes after starting the neurotensin infusion, there was a

Table 6: 1 Effect of (a) neurotensin (b) neurotensin 1-8 on plasma pancreatic polypeptide (PP), insulin (Ins), glucose(Glu), and cholesterol (CHO) concentrations.

Time (mins)	-15	0	15	30	45	60
(a)						
PP	44.0±5.0	49.0±7.0	92.0±28.0*	89.0±24.0*	79.0±25.0	46.0 ±11.0
Ins	46.0±8.0	46.0±8.0	41.0± 4.0	39.0± 6.0	36.0± 5.0	34.0 ± 4.0
Glu	5.1±0.1	5.1±0.1	5.1± 0.1	5.1± 0.1	5.0± 0.1	4.9 ± 0.1
CHO	4.7±0.4	4.5±0.3	4.5±0.3	4.5± 0.4	4.4± 0.4	4.5 ± 0.4
(b)						
PP	41.0±4.0	42.0±5.0	54.0±14.0	46.0± 7.0	56.0±20.0	61.0±21.0
Ins	48.0±9.0	52.0±6.0	43.0± 7.0	37.0± 4.0	43.0± 8.0	37.0± 6.0
Glu	5.1±0.1	5.1±0.1	5.0± 0.1	5.1± 0.1	4.9± 0.1	4.8± 0.1
CHO	4.7±0.4	4.7±0.4	4.6± 0.4	4.6±0.6	4.6± 0.4	4.6± 0.4

The peptide concentrations (pmol/l) were expressed as mean ± sem, n = 6.
Glucose and cholesterol concentrations were quoted as mean ± sem in mmol/l, n = 6.
* denotes $p < 0.05$

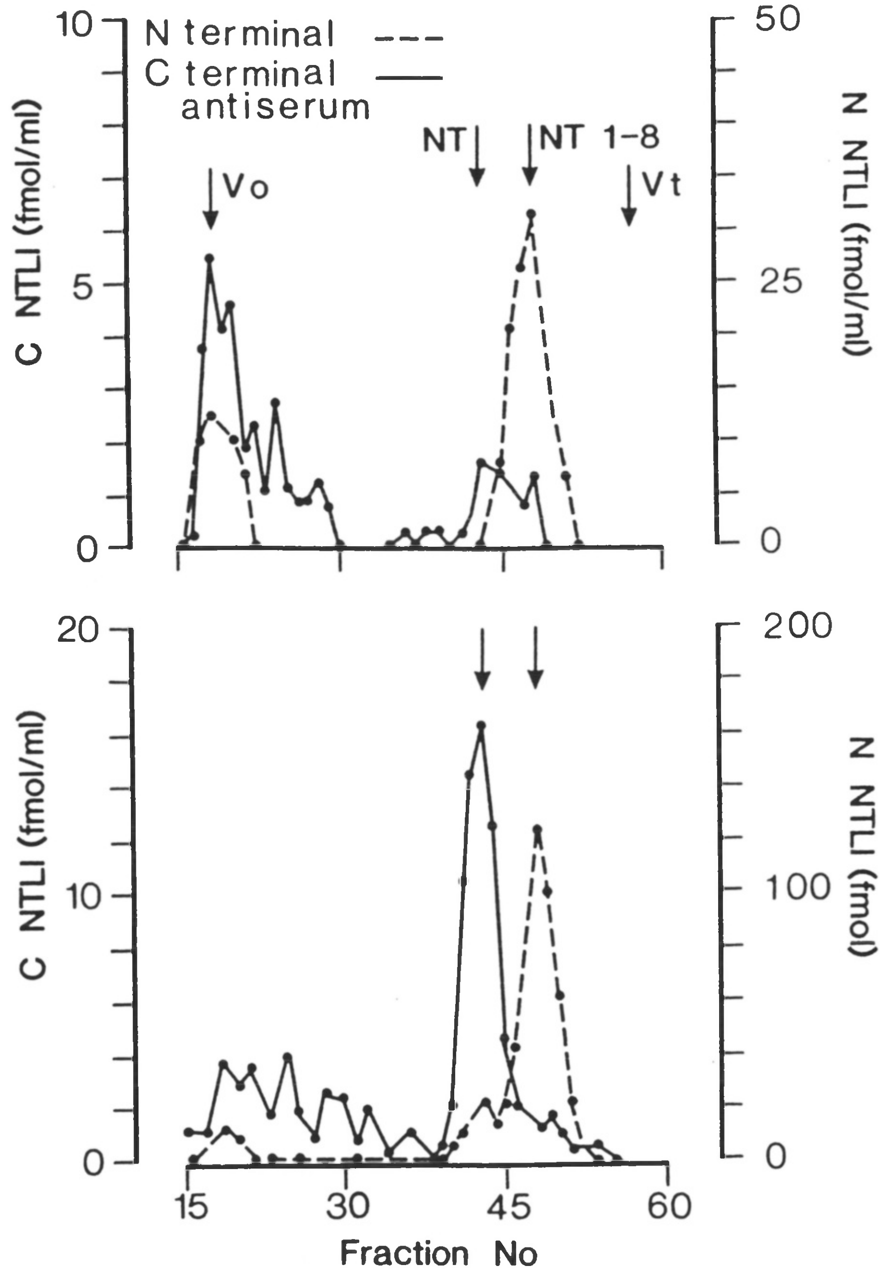


Figure 6:5. Gel filtration chromatographic profiless of basal (upper) and peak neurotensin (NT) infusion (lower) samples from one subject. The G-50 Sephadex superfile column (1.5 X 90 cm) was eluted with 0.06 M phosphate buffer, containing 0.3 % BSA and 0.2 M sodium chloride at 4 C. Fractions of 2.0 ml were collected for assay of immunoreactive neurotensin (NTLI), (————) when measured with C-terminally directed antiserum, and (------) when measured with N-terminally directed antiserum. The elution positions of void volume (Vo), total volume(Vt), synthetic NT and NT 1-8 were as indicated. NTLI (fmol/ml) with antiserum NT 58 on the left vertical axis and NTLI with antiserum 07709 on the right vertical axis

relatively greater amount of neurotensin as measured with NT 58, but this only constituted about 13 % of the amount of neurotensin 1-8 immunoreactivity detected as measured with 07709 (Figure 6:5). The recovery of the added neurotensin and neurotensin 1-8 standards in identical chromatographic runs ranged from 71-83 %. HPLC analysis of the pooled plasma samples gave similar results: in basal plasma, neurotensin-like immunoreactivity co-eluting with neurotensin constituted 5 % of that of neurotensin 1-8 immunoreactivity whereas in plasma after 45 minutes of neurotensin infusion, it constituted 15 % of the neurotensin 1-8 immunoreactivity (Figure 6:6).

Discussion

The infusion rate chosen was based on that previously reported by Blackburn and Bloom (Blackburn et al 1980b) and mimicked the neurotensin plasma concentration obtained after the ingestion of food (Rosell et al 1979). The length of the infusion (45 minutes) was chosen in order to reach a plateau concentration of NTLI in plasma and thus to evaluate its disappearance half life in the circulation.

The present study showed that the metabolism of

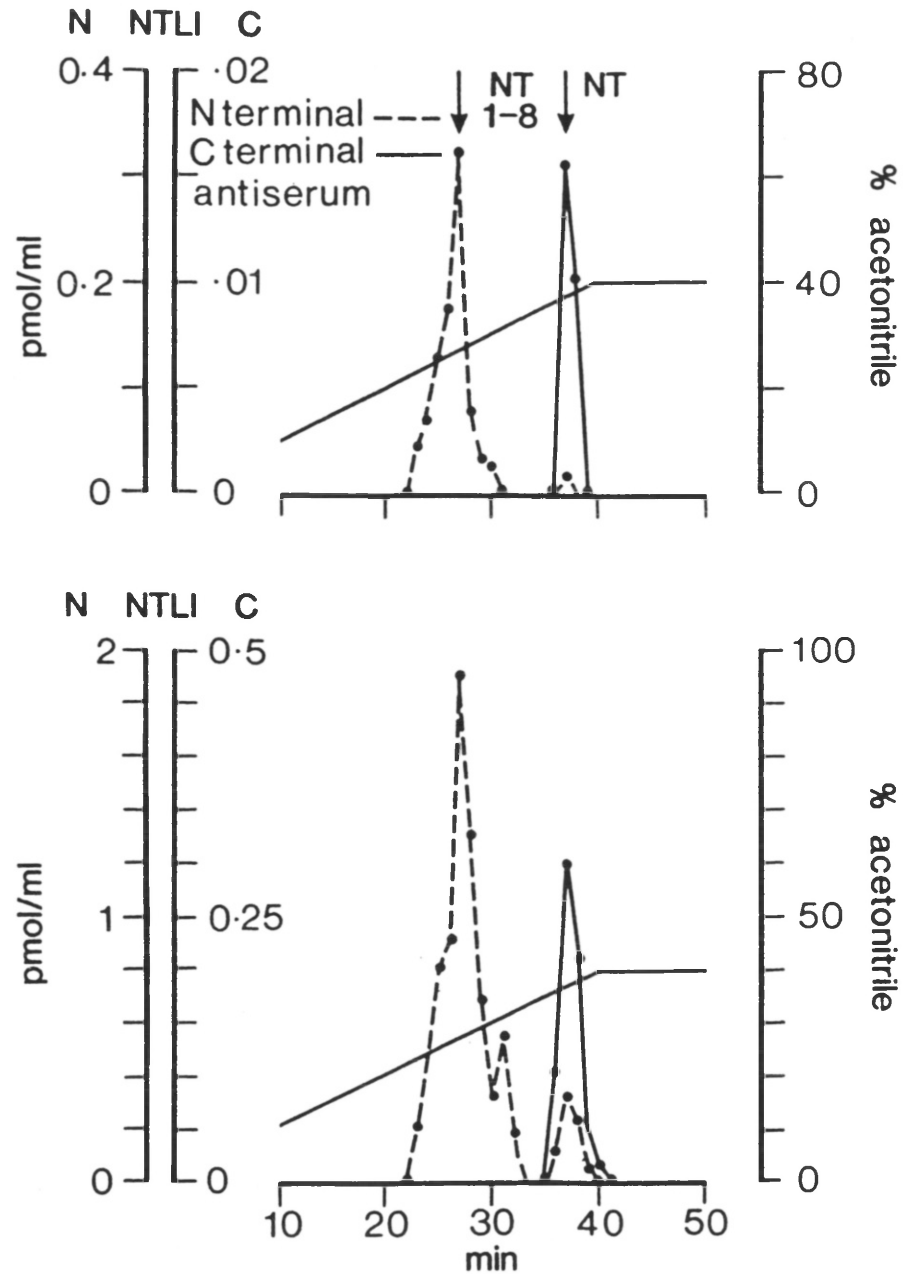


Figure 6:6. HPLC of the pooled basal (upper) and pooled peak infused neurotensin (NT) (lower) samples. Plasma NTLI was extracted by Sep-pak C18 (Waters Associate) before being applied to u-Bondapak C18 column (Waters) and eluted with a gradient from 0 - 40 % (1 %/min) acetonitrile in distilled water containing 0.2 % TFA. Flow rate was 1 ml/min and 1 ml fractions were collected. Assay of the fractions for NTLI was by C-(———) and N-(------) terminally directed antisera.

exogenous neurotensin in the human circulation is rapid and that it is readily degraded to its N-terminal fragment, neurotensin 1-8. When measured with the C-terminally directed antiserum NT 58, the disappearance half life of neurotensin was calculated to be 1.4 minutes. When the N-terminally directed antibody 07709 was employed, the disappearance half life was estimated to be 6.0 minutes. Plasma NTLI concentrations measured with the C-terminally directed antiserum NT 58 could be considered to have reached the plateau level (Figure 6:1). However, when measured with the N-terminally directed antiserum 07709, uncertainty exists whether a plateau level of plasma NTLI was attained within the infusion period of 45 min (Figure 6:2). Thus, the disappearance half life calculated from data obtained with the antiserum 07709 may be only approximate.

When neurotensin 1-8 was infused, its disappearance half life was estimated to be 30 minutes, demonstrating that neurotensin 1-8 is considerably more stable than the intact molecule in the human circulation. The disappearance half life of neurotensin measured with antiserum 07709 can thus be interpreted as the resulting product of the rapid degradation of neurotensin with the concomitant formation and slow degradation of the

neurotensin fragment, neurotensin 1-8, which was also recognised by the antibody.

Both gel filtration and HPLC indicated that major peaks of immunoreactive neurotensin co-eluted with neurotensin and neurotensin 1-8. Most of the plasma neurotensin immunoreactivity detected by antiserum 07709 co-eluted with neurotensin 1-8, both basally and after neurotensin infusion, suggesting that this fragment represents the majority of the circulating plasma neurotensin immunoreactivity. This may explain why the N-terminally directed antiserum 07709 measured higher concentrations of immunoreactive neurotensin in unextracted human plasma than the C-terminally directed antiserum NT 58. This is in accordance with the pharmacokinetic data obtained and is consistent with the findings of Aronin et al (1982). They reported that in rat neurotensin 1-8 has a longer half life in the circulation than neurotensin. The rapid breakdown of intact neurotensin with the generation of neurotensin 1-8 results in the relatively low plasma concentrations obtained when measured with the C-terminally directed antiserum NT 58, as compared with those obtained with the N-terminally directed antiserum 07709. However, these observations are in contrast to a previous report by Carraway et al (1980b) that antisera directed

against the C-terminus of neurotensin measured more immunoreactive neurotensin in extracted plasma than N-terminally directed antisera. This suggests that some C-terminal fragments of neurotensin or neurotensin related substances were produced, or alternatively the N-terminal immunoreactive materials were removed during plasma extraction procedure.

Immunoreactive neurotensin extracted from gastrointestinal tissues has been characterised as the thirteen amino acid polypeptide i.e. neurotensin 1-13 (Holzer et at 1982, chapter 3). Samples from the portal vein (venous drainage from the gastrointestinal tract) contain predominantly neurotensin 1-13 (Ferris et al 1981). The data in the present study shows that when exogenous neurotensin is administered in man neurotensin 1-8 is generated in plasma. It is of interest that hepatic breakdown of neurotensin has been reported recently in rat (Brook et al 1987).

It has been reported that neurotensin infused at a rate of 13.5 pmol/kg/min stimulates defaecation in man (Calam et al 1983). In our volunteers at the dose (2.4 pmol/kg/min) used in this study no such effect was found. No significant effects of neurotensin were demonstrated on the mean plasma

insulin, glucose or cholesterol concentrations. Many of the reported biological actions of neurotensin appear to occur only with pharmacological doses (Brown and Vale 1976, Kitabgi-Dolais et al 1979, Peric-Golia et al 1979, Saito and Saito 1980). However, at the infusion rate of the present study (2. 4 pmol/kg/min), neurotensin caused a significant rise of plasma pancreatic polypeptide concentration. This is in agreement with the findings of others (Blackburn et al 1980b, Fletcher et al 1981, Shulkes et al 1983) in both man and sheep. The infusion of neurotensin 1-8 had no effect on plasma pancreatic polypeptide concentrations in the same human subjects, indicating that the presence of the C-terminal region of the neurotensin molecule is required for this biological activity. Structure-activity studies in animal models have confirmed that the C-terminal region of the neurotensin molecule is responsible for its biological effects (Carraway et al 1975b, Quirion et al 1980).

Chapter 7

METABOLISM OF MEAL-STIMULATED NEUROTENSIN IN MAN

Introduction

The data presented in the previous chapter has shown that exogenously infused neurotensin is exceedingly unstable in human circulation. The infused neurotensin has a very short half life ($t_{1/2}$) of disappearance in plasma and the majority of plasma immunoreactive neurotensin co-elutes at the position of neurotensin 1-8 as demonstrated by both gel filtration and reverse phase high pressure liquid chromatography. Neurotensin 1-8 is relatively stable in human circulation (chapter 6, Shulkes et al 1984) and in rat plasma (Aronin et al 1982), and has been demonstrated to be biologically inactive (Leeman and Carraway 1982, Kitabgi 1982).

A marked elevation of plasma immunoreactive neurotensin by ingestion of nutrients, particularly fat, is well documented (Mashford et al 1978, Rosell and Rokaeus 1979, Go and Demol 1981, Hammer et al 1982, Theodorsson-Norheim and Rosell 1983b). Rosell and Rokaeus (1979) reported that the rise in plasma NTLI was biphasic with an early peak

response followed by a prolonged elevation of immunoreactive neurotensin in plasma lasting several hours after a meal. The uncertainty concerning the "true" half life of neurotensin 1-8 in the human circulation (as shown in the previous chapter) raises the question whether accumulation of this fragment peptide is the cause of the second peak of plasma NTLI seen in response to a meal or whether a second phase of neurotensin secretion is involved. This question can be partly answered by chromatographic fractionation of plasma samples obtained at various time points after a test meal. In the present study the plasma NTLI response to a test meal in five healthy human subjects has been investigated. Plasma immunoreactive neurotensin of the timed samples following stimulation of release has been quantified and characterised by gel filtration chromatography.

Materials and Methods

Approval from the Ethics Committee of the Royal Postgraduate Medical School and informed consent from the participants for the present study were obtained prior to the study.

Experimental protocol

Five healthy volunteers, three men and two women, aged 18-27, fasted overnight for 14-15 hours and then consumed a test meal within 15 min, starting at 1 PM . The meal consisted of a cheese omelette, two slices of toast with unsalted butter, a glass of milk and a slice of home-made cheese-cake (fat 126 g, protein 46 g, carbohydrate 77 g, totalling 6.77 megajoules, 1617 calories). An indwelling cannula was inserted into an antecubital vein for blood sampling. Three basal blood samples were taken at -30, -15 and 0 minutes, followed by further blood samples at 10, 20, 30, 40, 50, 60, 75, 90, 105, 120, 150 and 180 minutes after the start of the meal. Blood samples (7-10 ml) were collected into lithium-heparin tubes containing 4000 kallikrein inhibitor units of aprotinin (Trasylol). The samples were immediately centrifuged and the separated plasma was frozen and stored at -20^{0} C within 15 min.

Radioimmunoassay of plasma NTLI with the C-terminally directed antiserum NT 58 and the N-terminally directed antiserum 07709 was as previously described (Chapter 6).

Equal volumes of plasma from each of the five subjects were pooled for each of the time points -15, 20, 40, 60, 180 min. The pooled plasma samples (average volume 3.0 ml) were applied directly to a column of Sephadex G-50 superfine (1.4 X 90 cm) in separate runs. The column was eluted and calibrated as previously described (chapter 3). Fractions of 2.2 ml were collected and 700 ul and 500 ul of each fraction were assayed for NTLI with the C-terminally directed antiserum NT 58 and the N-terminally directed antiserum 07709 respectively.

Treatment of Data

Plasma NTLI was expressed as the mean and standard error of the mean (SEM) in pmol/l. The average concentration of plasma NTLI at -30, -15, and 0 min was taken as the basal level. For statistical analysis, the results were logarithmically transformed and groups were compared using the Student's paired t test.

Results

Plasma immunoreactive neurotensin in all subjects rose rapidly in response to the test meal, reaching a plateau level at 20 minutes when measured with antiserum NT 58 and at 30 min when measured with

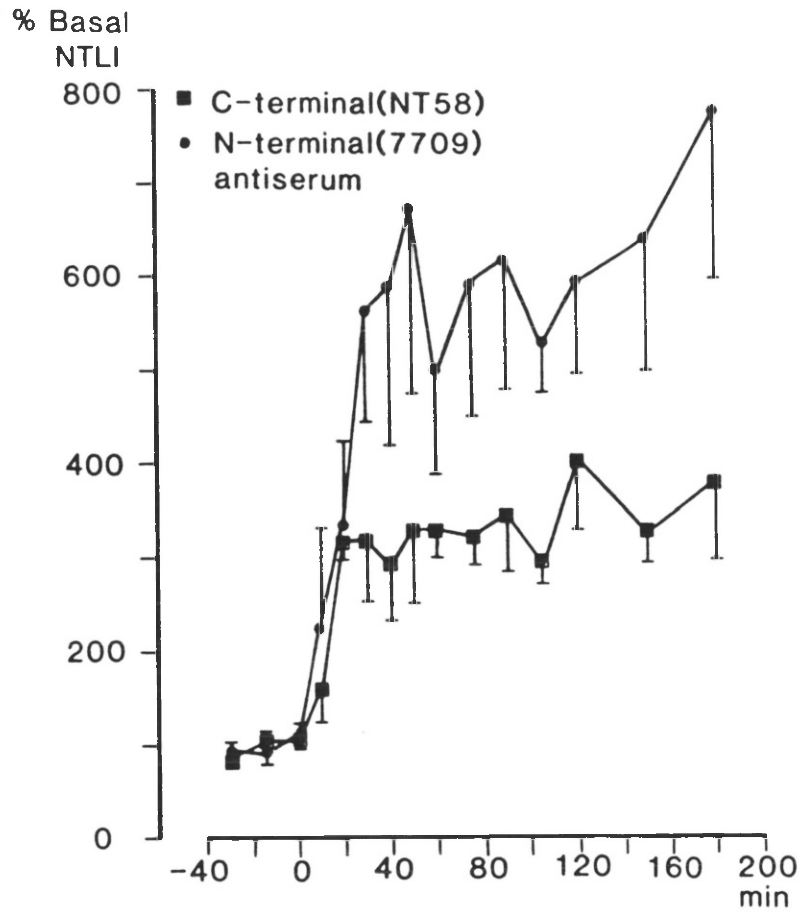


Figure 7:1. Percentage changes of plasma neurotensin-like immunoreactivity (NTLI) from basal (mean ± SEM, n = 5). ■ when measured with the C-terminally directed antiserum NT 58, and ● when measured with N-terminally directed antiserum 07709. 100 % was taken as the basal level.

antiserum 07709 (Figure 7:1). With antiserum 07709 the rate of attaining a plateau plasma NTLI concentration was slower than that seen with antiserum NT 58. The mean basal and plateau (30 min samples) concentrations of plasma immunoreactive neurotensin were 6.0 ± 1.0 and 21.0 ± 7.7 pmol/l (± SEM, n = 5) respectively when measured with antiserum NT 58. This represents a rise at 30 min of 319 ± 65 % of the basal (Figure 7:1). When the same samples were measured with antiserum 07709, the concentrations were 12.9 ± 2.8 and 64 ± 17 pmol/l (± SEM, n = 5) respectively. This represents a rise at 30 min of 564 ± 118 % of the basal (Figure 7:1).

Once plateau plasma concentrations had been attained, the elevation of plasma NTLI was maintained throughout the experimental period. At 180 min when the experiment was terminated, the plasma NTLI concentration was 24.2 ± 7.5 pmol/l measured with antiserum NT 58 and 87 ± 22 pmol/l measured with antiserum 07709 (Table 7:1). The mean concentrations of plasma NTLI of the timed samples of the present study when measured with these two antisera are as shown in Table 7:1.

Table 7:1 Plasma immunoreactive neurotensin (NTLI) concentrations measured with C-(NT 58) and N-(07709) terminally directed antisera after a meal

	Time after meal (mins)							
	Basal	10	20	30	60	90	120	180
NT 58	6±1	9±2	18±2 a	21±8 b	21±6 a	21±6 b	26±8 c	24±8 c
07709	13±3	18±2	34±7 d	64±17 b	60±21 c	69±16 b	70±15 a	87±22 b

The NTLI concentrations (pmol/l) were expressed as mean ± sem , N=5;
a indicates $p < 0.001$; b, $p < 0.005$; c, $p < 0.01$; d, $p < 0.02$.

Gel filtration chromatography

Gel filtration of the pooled timed samples indicated that immunoreactive neurotensin peaks eluted in the positions of both synthetic neurotensin and neurotensin 1-8. In addition, immunoreactive material eluting at the void volume position was also detected, the nature of which is still unclear. For the basal pooled sample, however, no immunoreactive peak eluting at the neurotensin position was detected when measured with either C- or N-terminally directed antisera (Figure 7:2). The chromatographic profiles of the 40- and 60-min samples were similar to those at 120- and 180-min (Figure 7:3, 7:4). For the 20-, 40-, 60-, 120- and 180-min pooled samples, the C-terminal NTLI material co-eluting with neurotensin when expressed as a percentage of N-terminal NTLI at the neurotensin 1-8 position was calculated to be 14, 16, 13, 15, and 14 %. Taking into account the cross-reactivity of neurotensin 1-8 of 152 % against neurotensin standards, this represents a mean molar ratio of neurotensin to neurotensin 1-8 of 1 to 4.6. The recoveries of intact neurotensin and neurotensin 1-8 standards added to charcoal-stripped plasma in identical chromatographic runs were 83 % and 82 % respectively.

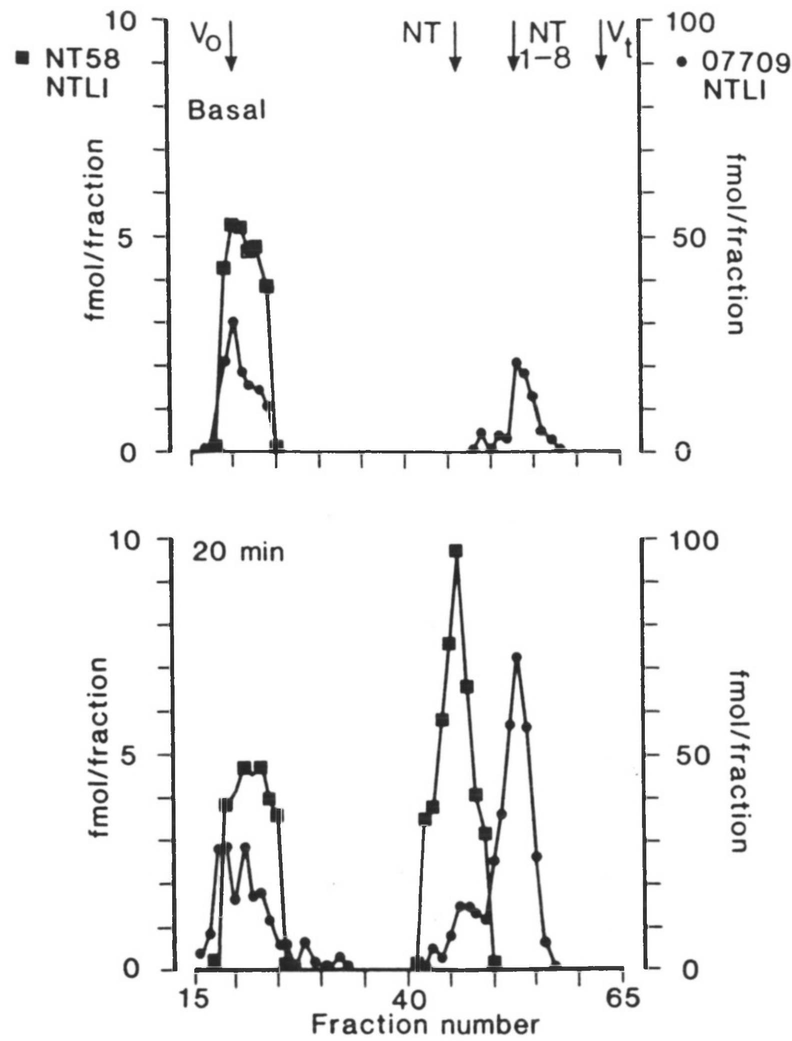


Figure 7:2. Gel filtration profiles of pooled basal (upper) and pooled 20-min (lower) plasma samples from five subjects. The G-50 Sephadex superfine column (1.4 X 90 cm) was eluted with 0.06 M phosphate buffer, containing 0.3 % BSA and 0.2 M sodium chloride at 4 °C. Fractions of 2.2 ml were collected for RIA of immunoreactive neurotensin (NTLI). ■ when measured with C-terminally directed antiserum NT 58, ● when measured with N-terminally directed antiserum 07709. The concentration of NTLI (NT 58) shown on the left vertical axis and NTLI (07709) on the right vertical axis. The elution positions of void volume (Vo), total volume (Vt), synthetic neurotensin (NT, and NT 1-8 were as indicated.

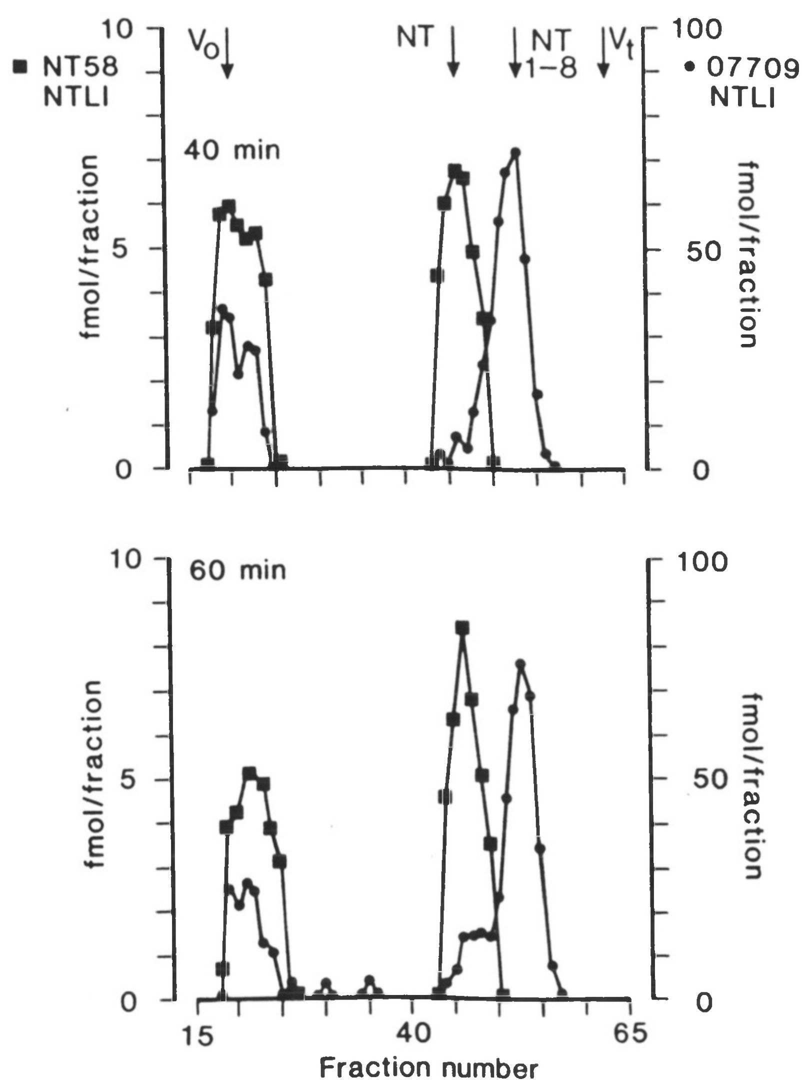


Figure 7:3. Gel filtration profiles of pooled 40-min (upper) and pooled 60-min (lower) plasma samples from five subjects. The G-50 Sephadex superfine column (1.4 X 90 cm) was eluted with 0.06 M phosphate buffer, containing 0.3 % BSA and 0.2 M sodium chloride at 4 ^{0}C. Fractions of 2.2 ml were collected for RIA of immunoreactive neurotensin (NTLI). ■ when measured with C-terminally directed antiserum NT 58, ● when measured with N-terminally directed antiserum 07709. The elution positions of void volume (Vo), total volume (Vt), synthetic neurotensin (NT, and NT 1-8 were as indicated.

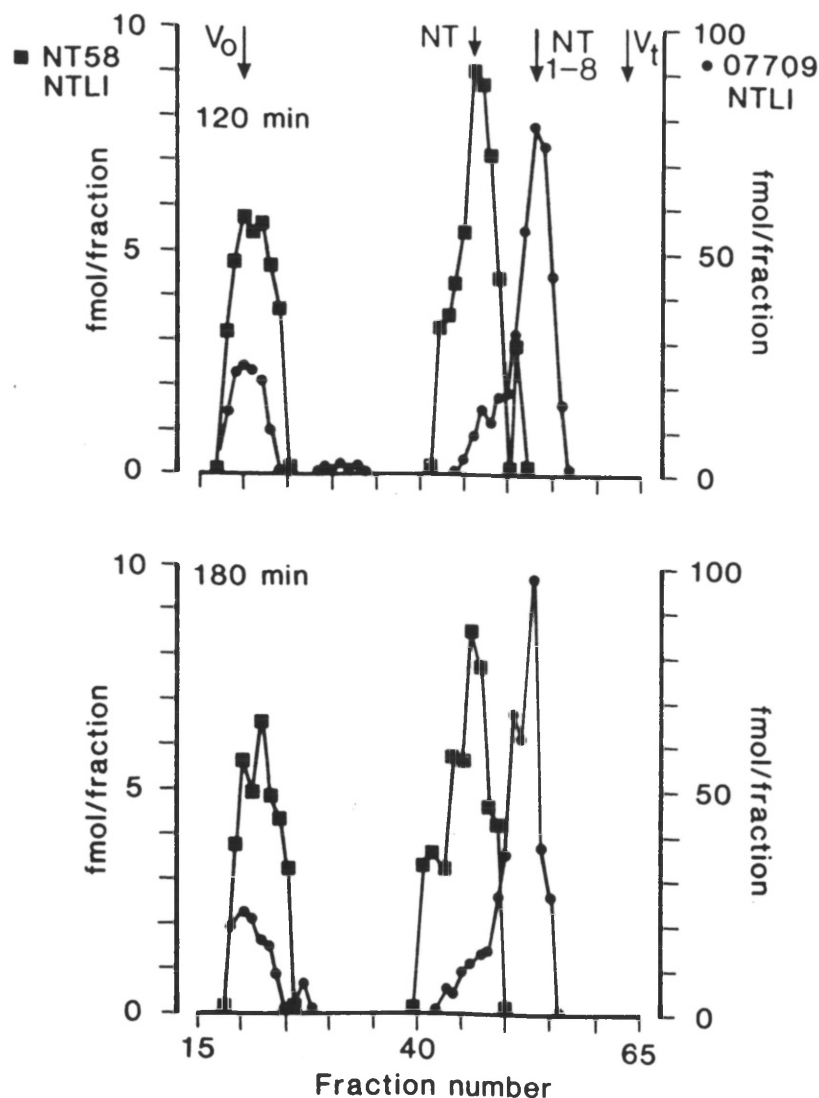


Figure 7:4. Gel filtration profiles of pooled 120-min (upper) and pooled 180-min (lower) plasma samples from five subjects. The G-50 Sephadex superfine column (1.4 X 90 cm) was eluted with 0.06 M phosphate buffer, containing 0.3 % BSA and 0.2 M sodium chloride at 4 ^{0}C. Fractions of 2.2 ml were collected for RIA of immunoreactive neurotensin (NTLI). ■ when measured with C-terminally directed antiserum NT 58, ● when measured with N-terminally directed antiserum 07709. The elution positions of void volume (Vo), total volume (Vt), synthetic neurotensin (NT), and NT 1-8 were as indicated.

Discussion

The present study has shown a marked increase in plasma immunoreactive neurotensin in response to a test meal in man. This is consistent with reports in the literature (Rosell and Rokeau 1979, Blackburn and Bloom 1979, Hammer et al 1982). However, this is the only report to date in which the plasma NTLI of the meal-stimulated samples in unextracted human plasma was measured using both the C- and N-terminally directed neurotensin antisera. Other reports using regional specific neurotensin antisera used extracted plasma samples (Hammer et al 1982, Shaw et al 1983). The elevation of plasma NTLI persisted for the duration of the experimental period (180 minutes). Gel filtration analysis of the timed samples in this study indicated that the elevated immunoreactive neurotensin components have the chromatographic characteristics of both intact neurotensin and its N-terminal fragment neurotensin 1-8. However, no intact neurotensin peak was observed in basal plasma. The presence of another possible neurotensin fragment, neurotensin 1-11, as reported by Hammer et al (1982) cannot be confirmed. Hammer et al (1982) using extracted human plasma reported that the main elevated components of NTLI from samples one hour after a meal were primarily

neurotensin 1-8 and neurotensin 1-11 but not neurotensin itself. A smaller test meal was consumed in their study and no intact neurotensin was detected for the 60 min-sample. We failed to detect neurotensin 1-11 in human plasma in the present study. This may in part be explained by the characteristics of the N-terminally directed antiserum used, namely 07709, which only cross-reacts 37 % with [Gln^4]-neurotensin 1-11 (Theodorsson-Norheim et al 1983).

Chromatographic analysis of pooled meal-stimulated samples has shown a high ratio of neurotensin 1-8 to neurotensin. The data presented in this chapter are in agreement with results obtained in the previous chapter (chapter 6) that neurotensin recovered from the plasma of subjects infused with intact neurotensin constituted 13-15 % of the immunoreactivity that co-eluted with neurotensin 1-8. Exogenously infused neurotensin ($t_{1/2}$, 1.4 min) is rapidly metabolised and neurotensin 1-8 ($t_{1/2}$, 30 min) is much more stable in the circulation. The metabolism of endogenous neurotensin may be similar to that of exogenously infused neurotensin. In this experiment, the long fasting period resulted in undetectable basal levels of intact neurotensin in the chromatogram, the plasma C-terminal "immunoreactivity" consisted

of void volume material. The concentration of void volume "immunoreactivity" detected by both antisera NT 58 and 07709 was unchanged in plasma at all time points examined, including basal plasma. Although the nature of the void volume material remains unclear, treatment with 8 M urea does not generate lower molecular forms of NTLI (Blackburn et al 1979, Theodorsson-Norheim 1983c). It may be of relevance that peptides related to neurotensin have been generated from mammalian albumin by pepsin treatment (Carraway et al 1986, 1987b) and it has been reported that serum albumin contains a substance structurally related to neurotensin, namely kinetensin, (Mogard et al 1986).

The present study did not show a biphasic response of plasma neurotensin after a meal. The data are consistent with secretion of neurotensin into the circulation within 20 min of the start of a meal, followed by a fairly steady rate of secretion persisting for at least 180 min. This results in a plateau concentration of neurotensin in the plasma. Neurotensin 1-8 is generated from the breakdown of neurotensin in the plasma and is more slowly cleared. Neurotensin 1-8 reaches a plateau concentration much lower than would be predicted from the ratio of half life of neurotensin 1-8 compared to that of neurotensin (chapter 6). This

may imply, either that a significant proportion of intact neurotensin is being removed from the circulation by an alternative mechanism which does not generate neurotensin 1-8, or that neurotensin 1-8 has a much larger apparent distribution volume than neurotensin.

Chapter 8

DEGRADATION OF NEUROTENSIN IN HUMAN PLASMA IN VITRO

Introduction

Inactivation of neurotensin has been reported by enzymes such as enkaphalinase (endopeptidase, EC 24:11), angiotensin converting enzyme and metalloendopeptidase in tissue preparations (Checler et al 1983, Checler et al 1984, Emson et al 1985b, Checler et al 1987). However, the mechanism responsible for the degradation of neurotensin in the circulation is at present unknown. In this present chapter the degradation of neurotensin is investigated in fresh human plasma in vitro in the presence and absence of various enzyme inhibitors. The neurotensin-like immunoreactivity is characterised by gel filtration and reverse phase high pressure liquid chromatography (HPLC).

Materials and Methods

Fasting blood samples, 100 ml each from six healthy human subjects were collected into lithium heparin tubes and centrifuged immediately. The separated plasma was used with minimum delay.

Experimental design

For each subject, six sets of 7 X 1.0 ml plasma aliquots were prepared. Set 1-4 consisted of plasma aliquots to which peptidase inhibitors (50 ul of each) were added. The fifth set received 0.9 % saline (50 ul to each plasma aliquot). Plasma aliquots in these five series of samples each received synthetic neurotensin, 200 fmol in 50 ul 0.9 % saline. The sixth set of plasma aliquots had neither neurotensin or enzyme inhibitor added and 0.9 % saline (100 ul) was added to each aliquot in this series of samples. Seven aliquots in each set were required as the reaction was allowed to proceed for the experimental period of 480 minutes. The reaction was terminated for one tube in each series at seven time points, 0, 15, 30, 60, 120, 240 and 480 minutes.

All plasma aliquots were placed into a water-bath, which was maintained at $37^{0}C$ throughout the experimental period of 480 minutes, for 5 minutes prior to the addition of peptidase inhibitors, neurotensin and 0.9 % saline. Four peptidase inhibitors were added separately to set 1-4 plasma aliquots from each volunteers; each plasma sample received 50 ul of the chosen inhibitor solution.

The four chosen enzyme inhibitors were Pepstatin A, EDTA, Phenylmethylsulphonylfluoride (PMSF), and Aprotinin (Trasylol). Disodium EDTA was dissolved in 0.9 % saline to give 10 umol in 50 ul; pepstatin A was dissolved in ethanol and diluted with an equal volume of saline to give 0.1 umol in 50 ul; PMSF was dissolved in acetone to give 2 umol in 50 ul; aprotinin was added as an aqueous solution of 100 kallikrein inhibitory units in 50 ul.

The reaction was stopped for one tube in each series by the addition of absolute ethanol (2 ml) at time points 0, 15, 30, 60, 120, 240 and 480 min. Precipitated protein was removed by centrifugation (20 min at 2000 X g) and the supernatants were evaporated to dryness in a rotary evaporator (Savant, Mass, U S A). Samples were subsequently reconstituted with 1 ml of 0.06 M phosphate buffer, pH 7.4, containing 10 mmol/l EDTA, 0.05 % sodium azide (wt/vol) and 0.25 % gelatin (wt/vol), and their neurotensin concentrations were measured by radioimmunoassay using the C-terminally directed antiserum NT 58.

Chromatographic characterisation

A total of eight chromatographic analyses were performed, four with gel filtration and four with

reverse phase high pressure liquid chromatography (HPLC). Two of the four runs with the respective chromatographic procedures were samples in the absence of peptidase inhibitors at time 0 and 480 min respectively, the other two runs were samples in the presence of EDTA at time 0 and 480 min.

For gel filtration, 50 ul aliquots of reconstituted sample for time points 0 and 480 min from each subject were pooled and applied directly to a column of Sephadex G-50 superfine (1.4 X 90 cm) in separate runs. The column was eluted and calibrated as previously described (chapter 6). The flow rate of elution was 8 ml/hr. The column was also calibrated previously with synthetic neurotensin and neurotensin 1-8 and neurotensin 1-9 to determine their respective elution positions under identical conditions. Fractions of 2 ml were collected. Aliquots (700 ul) of each fraction were assayed for immunoreactive neurotensin with the C-terminally directed antiserum NT 58, and the N-terminally directed antiserum 07709. The recovery of the added neurotensin and its related fragments in identical chromatographic runs ranged from 79 - 85 %.

For reverse phase HPLC, 0.2 ml aliquots of reconstituted sample from each subject were pooled

and applied to octadecylsilylsilica cartridges (Sep-pak C-18) which were prepared by washing with acetonitrile followed by water. The cartridges were washed with 10 ml of water and retained peptides were eluted with 1 ml of 60 % aqueous acetonitrile (vol/vol). The eluates were diluted to a total volume of 4 ml with water; 2 ml of this solution were injected into the HPLC system. HPLC was performed on a Waters system as previously described (chapter 6) with a Techsil C 18 column (5 X 250 mm), eluted with a linear gradient from 15 % to 50 % (vol/vol) acetonitrile in water containing 0.05 % (vol/vol) trifluoroacetic acid over 35 min. The flow rate was 1 ml/min and 1 ml fractions were collected. Synthetic neurotensin and its fragments were run on separate occasions using identical conditions to determine their elution positions. In addition, the elution positions of neurotensin and neurotensin 1-11 were also monitored using the ultra violet spectrum. Aliquots (100 ul) of each fraction were assayed with the antisera NT 58 and 07709. Radioimmunoassay of immunoreactive neurotensin was carried out as previously described (chapter 2).

Results

The time course of the degradation of C-terminally (NT 58) immunoreactive neurotensin by human plasma in vitro at 37^{0}C is shown in Figure 8:1. The half-time of disappearance of immunoreactive neurotensin detected by antiserum NT 58 was calculated to be 226 min. No significant inhibition of degradation of neurotensin was observed when aprotinin or PMSF was added to the plasma. Pepstatin A at 0.1 mmol/l marginally inhibited the degradation of the added neurotensin, giving a calculated half time disappearance of 290 min. EDTA at 10 mmol/l however, markedly inhibited the degradation of neurotensin with respect to C-terminal immunoreactivity, producing a calculated half time of disappearance of 72 hours. The recovery of neurotensin immunoreactivity by the ethanol extraction procedure was estimated from the time 0 values as 61 $\pm$ 1 % (mean $\pm$ sem).

Gel filtration analysis of plasma samples without inhibitor at time 0 and 480 minutes, when fractions were assayed using the NT 58 antiserum, showed the

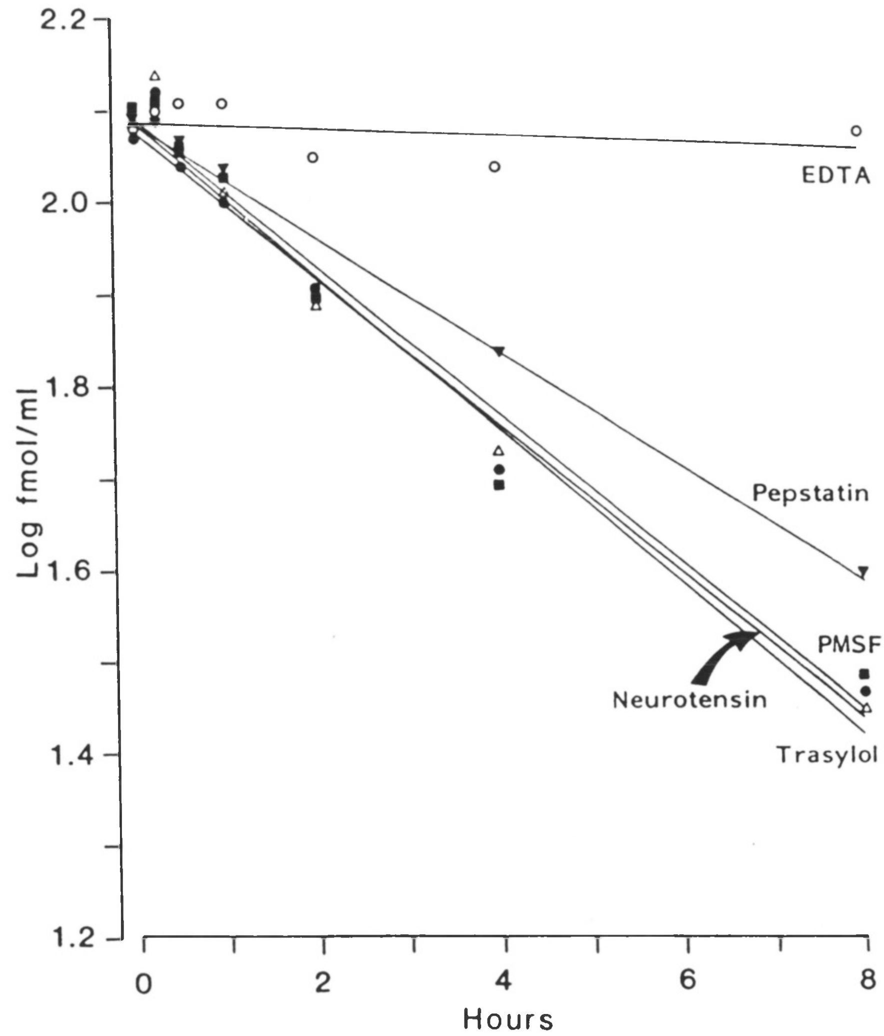


Figure 8:1. Time course of degradation of C-terminal (NT 58) neurotensin-like immunoreactivity by human plasma in vitro at 37^0C. (●) without inhibitor, (△) with Trasylol (Aprotinin) 91 kallikrein inhibitory units per ml, (■) with PMSF 1.8 mM, (▼) with pepstatin A 0.09 mM and (O) with EDTA 9.1 mM.

presence of an immunoreactive peak in the position of neurotensin 1-13 at time 0. The size of this peak was profoundly reduced at 480 min (Figure 8: 2, upper panel). Analysis of the same zero time point chromatographic run with the N-terminally reactive antiserum 07709 showed a similar peak in the position of neurotensin 1-13 as well as a small peak of immunoreactivity attributed to an N-terminally immunoreactive fragment. At 480 minutes the peak in the neurotensin 1-13 position was greatly reduced, and a large peak in the position of neurotensin 1-8 appeared (Figure 8: 2, lower panel).

In the presence of EDTA the rate of disappearance of the NT 58 immunoreactive peak in the position of neurotensin 1-13 was greatly reduced and a slight diminution in size of the peak at 480 min was observed with a one-fraction shift to the right (Figure 8: 3, upper panel). When analysed with the 07709 antiserum the profiles for 0 and 480 min samples were similar except for a one-fraction shift to the left of the 480 min profile (Figure 8: 3, lower panel).

HPLC of the plasma samples without inhibitor confirmed a reduction in the NT 58 immunoreactive peak in the position of neurotensin 1-13 at 480 min

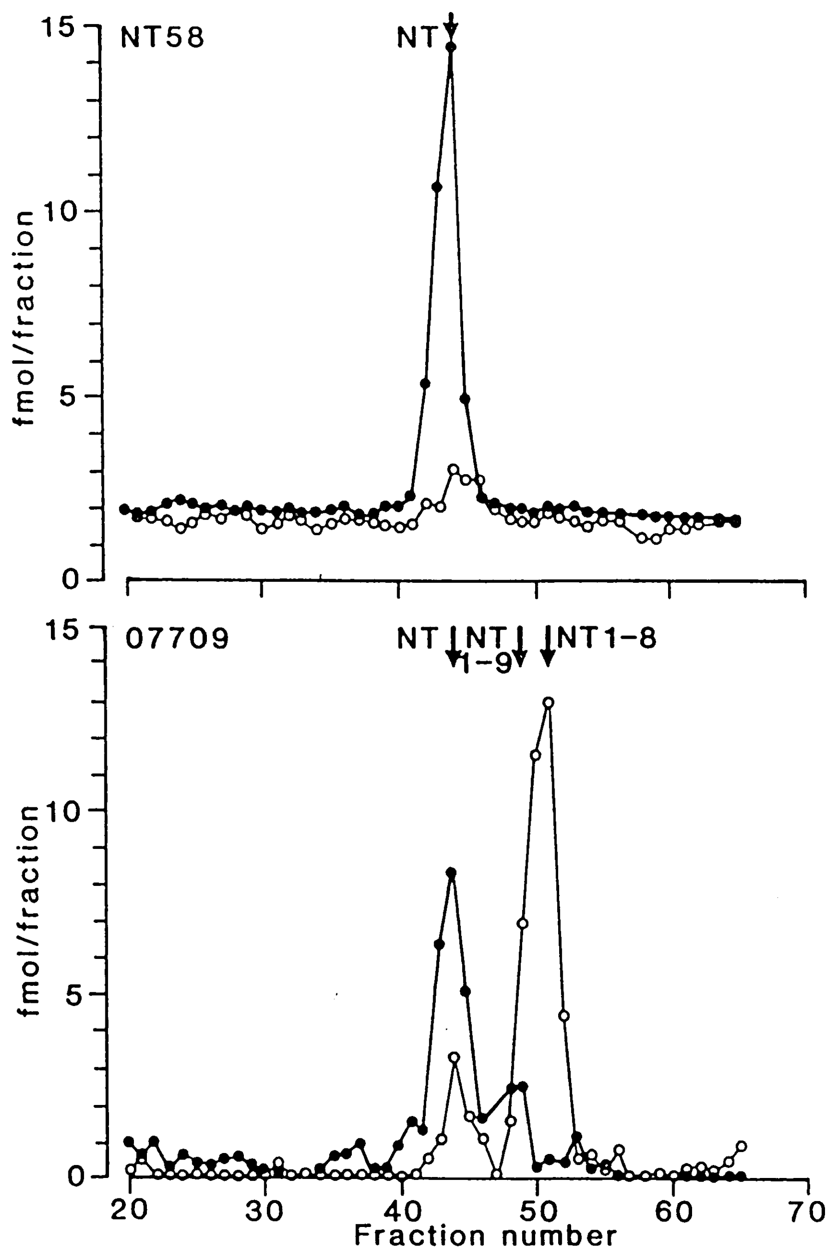


Figure 8:2. Gel filtration profiles of pooled plasma extracts on Sephadex G-50 superfine (1.4 X 90 cm). (●) 0 min after the addition of neurotensin and (○) after 480 min incubation in human plasma at 37^{0}C without enzyme inhibitors. Upper panel: neurotensin immunoreactivity measured with antiserum NT 58; lower panel: neurotensin immunoreactivity measured with antiserum 07709. Elution positions of neurotensin (NT), neurotensin 1-9 (NT 1-9), neurotensin 1-8 (NT 1-8), void volume (Vo) and total volume (Vt) are as indicated.

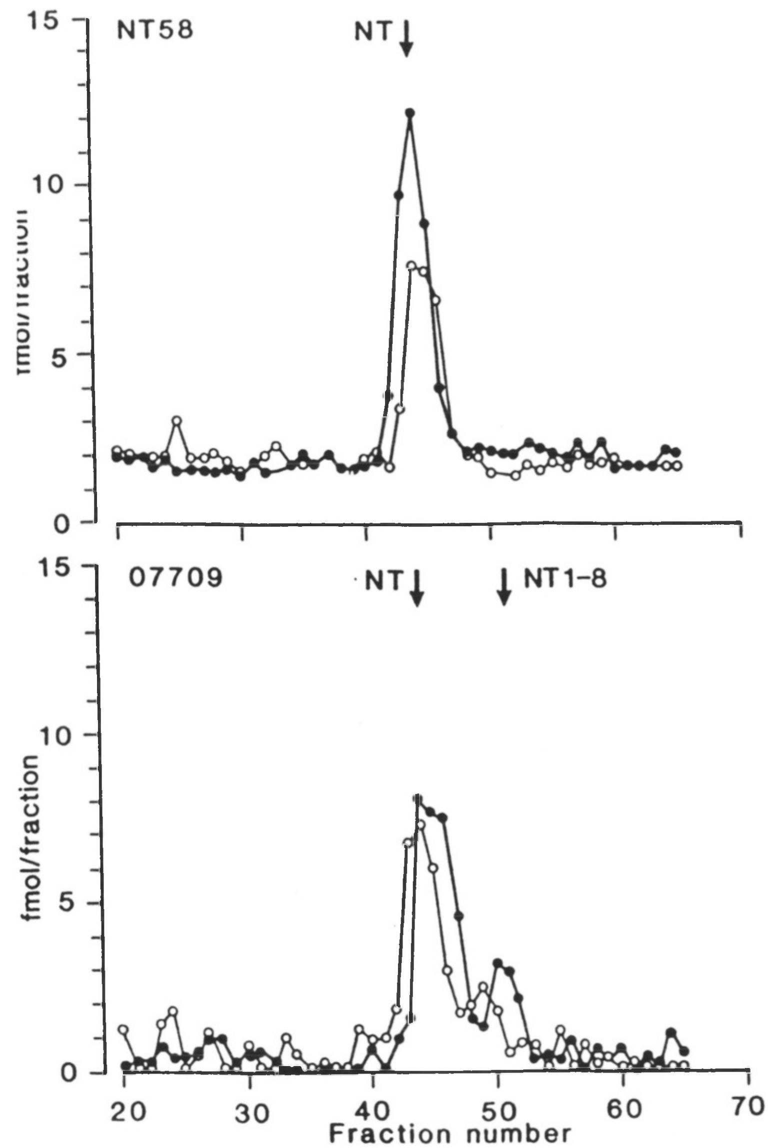


Figure 8: 3. Gel filtration profiles of pooled plasma extracts on Sephadex G-50 column. (●) 0 min after the addition of neurotensin and (O) after 480 min incubation in human plasma at 37 ^{0}C in the presence of 9.1 m M EDTA. Upper panel: neurotensin immunoreactivity assayed with antiserum NT 58; lower panel: neurotensin immunoreactivity measured with antiserum 07709. Elution positions of neurotensin, its fragments and column markers are as indicated

(Figure 8:4, upper panel). Analysis with the 07709 antiserum showed complete disappearance of neurotensin 1-13 with the appearance of immunoreactivity in the neurotensin 1-8 position, plus a further peak in the position of neurotensin 1-11 (Figure 8:4, lower panel).

In the presence of EDTA, comparision of time 0 and 480 min plasma samples showed little apparent change in NT 58 immunoreactivity in the neurotensin 1-13 position (Figure 8:5, upper panel), but there was a diminution of 07709 immunoreactivity without change of elution position (Figure 8:5, lower panel).

Discussion

The present study shows that neurotensin is degraded by human plasma in vitro. However, the disappearance half time at 37^0C is 226 min in vitro whereas it is 1.4 min in vivo with respect to the C-terminal immunoreactivity measured by antiserum NT 58 (chapter 6). This suggests that the vast majority of neurotensin metabolism in vivo is due to uptake and degradation in tissue rather than to degradation by enzyme present in the plasma. Ferris et al suggested that in rat the gastrointestinal tract might also be responsible

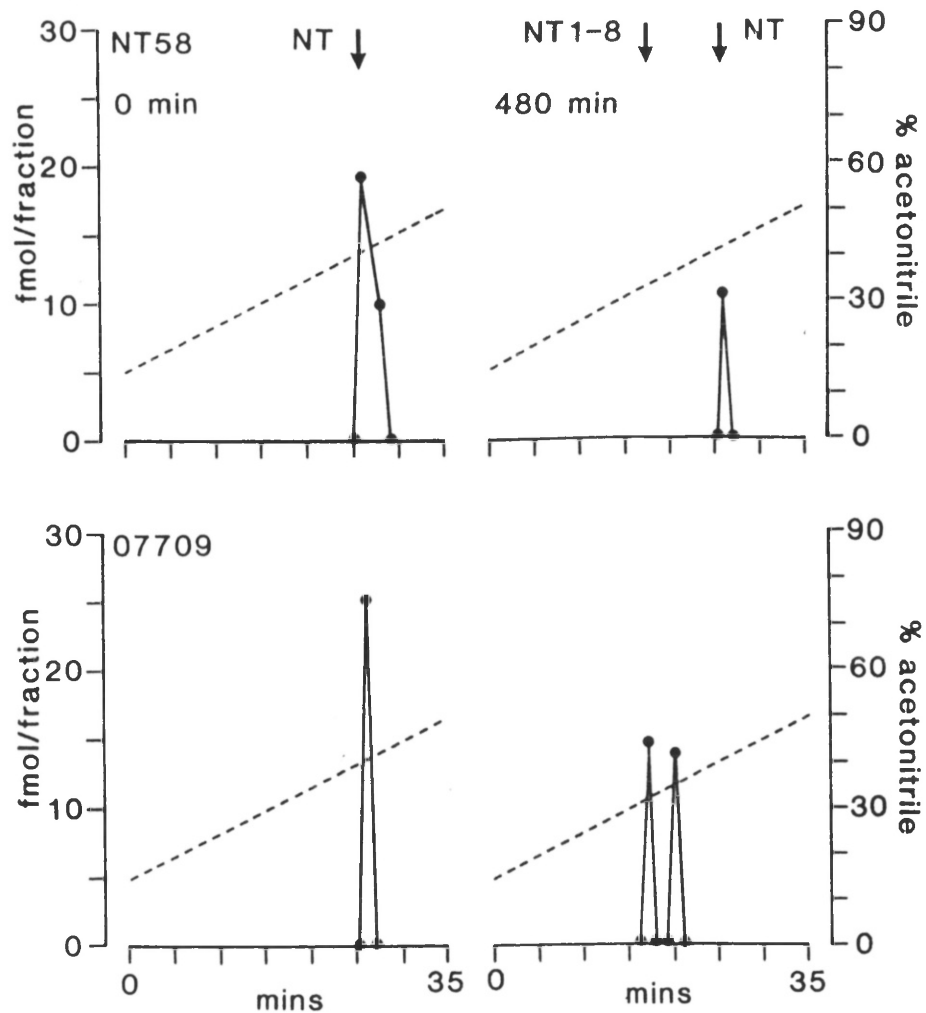


Figure 8:4. HPLC profiles from pooled extracts at time 0 and after 480 min incubation of neurotensin in human plasma at 37 ^{0}C without enzyme inhibitors. Upper panel: neurotensin immunoreactivity measured with antiserum NT 58; lower panel: neurotensin immunoreactivity measured with antiserum 07709. Arrows indicate the elution positions of neurotensin (NT), neurotensin 1-8 (NT 1-8) and neurotensin 1-11 (NT 1-11).

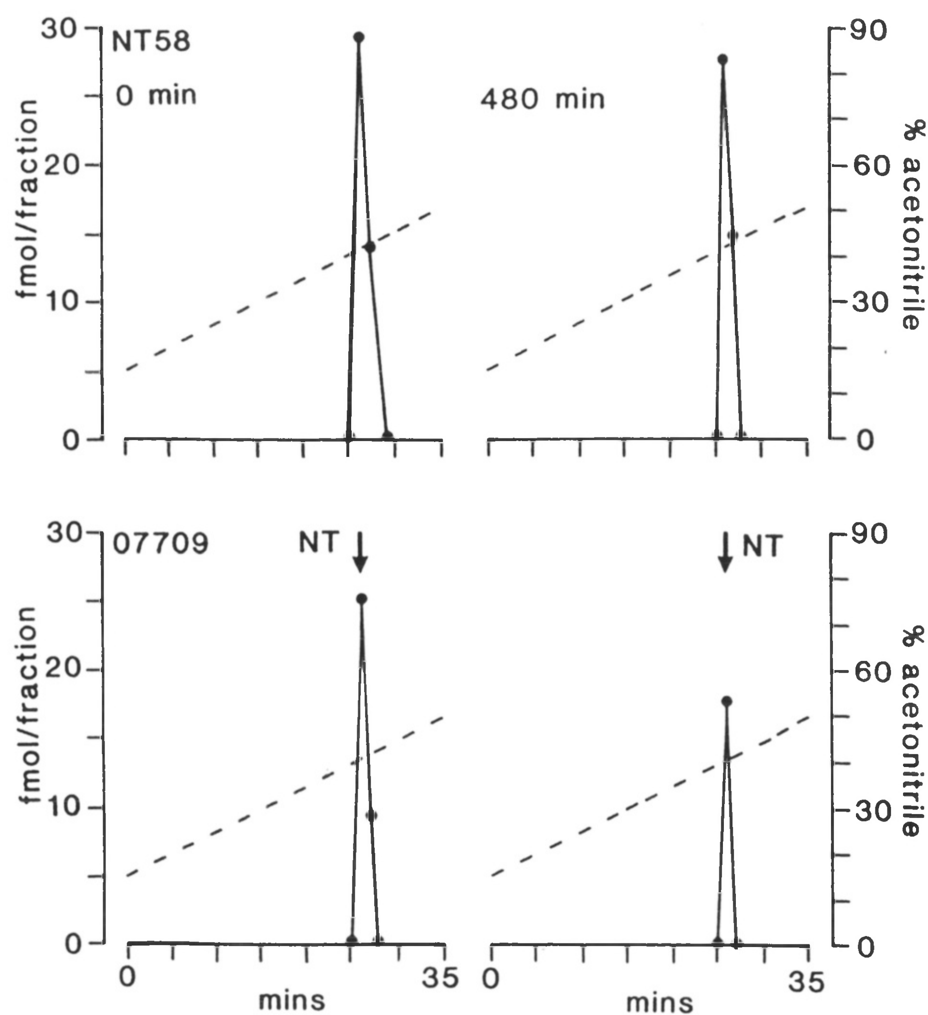


Figure 8:5. HPLC profiles from pooled extracts at time 0 and after 480 min incubation of neurotensin in human plasma at 37°C in the presence of 9.1 mM EDTA. Upper panel: neurotensin immunoreactivity measured with antiserum NT 58; lower panel: neurotensin immunoreactivity measured with antiserum 07709. Arrows indicate the elution positions of neurotensin (NT).

for neurotensin degradation (Ferris et at 1985b). Gel filtration of a human ileal extract demonstrated the presence of N-terminal immunoreactivity in addition to that of intact neurotensin (Lee et al 1985).

The degradation of C-terminally immunoreactive neurotensin in plasma is potently inhibited by EDTA. The chromatographic analysis of the residual immunoreactivity after incubation in the presence of EDTA suggests that there is a significant breakdown of neurotensin 1-13, leading to a fragment which is still appreciably reactive with NT 58 antiserum, but much less or not reactive with the N-terminally directed 07709 antiserum. This product has an identical elution position to that of neurotensin 1-13 on the HPLC system used in this study, but a slightly smaller molecular size on gel filtration, leading to a slight shift in NT 58 immunoreactive peak to the right. The immunological evidence suggests that it may be a large C-terminal fragment of neurotensin. As the presence of this product is inferred from relatively small losses of immunoreactivity, this interpretation requires confirmation by the positive identification of this fragment in a chromatographic system that can clearly separate it from neurotensin 1-13.

In the absence of EDTA, plasma degradation of neurotensin appears to result in fragments chromatographically and immunologically identical to neurotensin 1-8 and neurotensin 1-11. Neurotensin 1-9 and neurotensin 1-10 immunoreactivity cannot be detected in plasma with the antisera used in the present study.

The results in this study are compatible with a complex pattern of neurotensin degradation in plasma in vitro. Breakdown to neurotensin 1-8 and neurotensin 1-11 is brought about by enzymatic activity inhibited by EDTA, but there may be a further enzyme that cleaves the N-terminal region of neurotensin and which is not inhibited by EDTA. Shulkes and coworkers (1983) studied the loss of C-terminal and N-terminal immunoreactivity when neurotensin was incubated with heparinised sheep plasma and found a more rapid loss of C-terminal than N-terminal neurotensin immunoreactivity, and much faster rates of degradation than has been found in human plasma in the present study. In another study by Shulkes et al (1984)a, degradation of C- and N-terminal neurotensin immunoreactivity was measured in normal human plasma and plasma from patients with end-stage chronic renal failure. Loss of C-terminal immunoreactivity was again more rapid than loss of N-terminal immunoreactivity and the

rates of degradation were reduced in chronic renal failure plasma. Neurotensin 1-8 and 1-11 were detected as products of incubation of neurotensin with normal plasma.

The data presented in this chapter show that the plasma enzymatic activity responsible for C-terminal cleavage of neurotensin is powerfully inhibited by EDTA. At present this represents the only available characterisation of the enzyme responsible for the degradation of neurotensin in human plasma. Inhibition by EDTA differentiates the possible plasma enzyme from the neurotensin-degrading peptidases present in brain homogenates (McDermott et al 1982, Checler et al 1983). Enzymes which may be involved with neurotensin breakdown in tissues include angiotensin converting enzyme (Checler et al 1983, Skidgel et 1984), and endopeptidase 24:11 (enkephalinase) (Checler et al 1983, Checler et al 1984, Emsom et al 1985). These are membrane-bound, their role in plasma degradation of neurotensin in vitro is uncertain. As the biological activities of neurotensin depend on the preservation of an intact C-terminal sequence of the neurotensin molecule (Leeman and Carraway 1982, Quirion et al 1980), a practical implication of the present finding is that physiologically relevant immunoassay data on plasma

neurotensin concentrations are more likely to be obtained if EDTA is used as the anticoagulant or preservative.

Chapter 9

Conclusion

Over the past twenty years, there has been an explosion in the discovery and isolation of the biologically active peptides. Some of these were first purified from the central nervous system where they may function as neurotransmitters or neuromodulators and subsequently have also been found in the gastrointestinal tract where they may serve an endocrine or paracrine role (Krieger 1983). Neurotensin is one such brain-gut peptide. Since the discovery of neurotensin fifteen years ago, neurotensin-like immunoreactivity has been found in species as diverse as Escherichia coli and man (Forssmann 1980, Bhatnagar and Carraway 1981, Hammer et al 1980, Reinecke 1986). The C-terminal region of the molecule appears to be conserved through evolution (Carraway et al 1982).

The pattern of distribution of neurotensin has been shown to be discrete in the central nervous system and the peripheral tissues. Highest concentration of immununoreactivity was present in the gastrointestinal tract even though neurotensin was originally isolated from the hypothalamus. A structurally related peptide to neurotensin, namely

neuromedin N, was isolated from the porcine spinal cord when the work for this thesis began. Little was known of the distribution of neuromedin N. With the radioimmunoassays described in the present study, the pattern of distribution of neurotensin was confirmed and found to be similar to that of neuromedin N in the rat gastrointestinal tract. However, neurotensin was absent in the rat kidney whereas a considerable amount of neuromedin N was detected in extracts of this tissue. It is of relevance that the precursor protein sequence for neurotensin has been predicted by nucleotide sequence analysis (Dobner et al 1987) and the 170 amino acid precursor contained the structural sequence of neurotensin as well as that of neuromedin N. The finding that neurotensin and neuromedin N were similarily distributed in the digestive tract suggest that both peptides may be derived from the same precursor; while the absence of neurotensin and the presence of neuromedin N in the kidney suggest that tissue-specific postranslational processing of the precursor may occur.

Possible neural and peptidergic influences of neurotensin release were examined using a rat isolated ileal preparation. It was shown that in addition to the luminal factor, 10% intralipid,

which was specific in stimulating the release of neurotensin from the digestive tract, a significant release of neurotensin was also observed when carbachol or the amphipian peptide, bombesin, were infused. The effect of carbachol with respect to neurotensin release appeared to be secondary to that of bombesin. Experimental data including those reported here suggest that in addition to the peptidergic effect, a local neural reflex may contribute to the release of neurotensin from the rat isolated ileum, and the regulation of neurotensin release is complex. The interaction between the peptidergic and the luminal factors in the release of neurotensin was demonstrated by the finding that the infusion of a somatostatin analogue (SMS 201-995) suppressed the basal and abolished the meal-stimulated release of neurotensin in man.

The synthesis of neurotensin was studied using the PC 12 cell line. The neurotensin content of the PC 12 cell was found to be affected synergistically by nerve growth factor, dexamethasone, forskolin and cholera toxin. The synthesis of neurotensin may thus be cAMP mediated and involving the protein kinase A in this system. However, the participation of other second messenger systems in the synthesis of neurotensin may not be ruled out. The effects of

dexamethasone and forskolin on neurotensin synthesis have since been confirmed by using the clonal, rat C cell-derived 44-2C cell line (Zeytin and Delellis 1987).

The study of the metabolism of neurotensin in human circulation was undertaken. First, the pharmacokinetics and the metabolic fate of the exogenously infused neurotensin were investigated, in addition to that of its fragment, neurotensin 1-8. The infusion of neurotensin 1-8 represented the first investigation of the possible action of this fragment in man. Secondly, the metabolic fate of meal-stimulated neurotensin was studied in man. Region specific antisera were used, which allowed the measurements of intact neurotensin as well as neurotensin fragments in unextracted human plasma. The gel-filtration and high pressure liquid chromatography analyses of the timed samples were performed, which ensured the adequate interpretation of the results. The data presented demonstrated that there was a rapid degradation of both infused and meal-stimulated neurotensin in the human circulation. The disappearance half-life of neurotensin was relatively short, calculated to be 1.4 minutes and 6.0 minutes when plasma immunoreactivity was measured with the C- and N-terminally directed antisera respectively. In

comparison, neurotensin 1-8 was relatively stable, with a half-life of 30 minutes. Neurotensin when infused caused a significant rise of the plasma concentrations of pancreatic polypeptide, whereas no such a effect of neurotensin 1-8 was noted. The result thus confirmed the importance of the carboxy-terminal of the molecule in the biological activity of neurotensin previously reported in other experimental models (Quirion et al 1980). More importantly, the long half-life of neurotensin 1-8 in the circulation in part explained the higher plasma concentrations of immunoreactive neurotensin measured when using a N-terminally directed antiserum.

The data in the meal-stimulated neurotensin study showed that the concentrations of plasma immunoreactive neurotensin was markedly elevated within 30 minutes following the ingestion of a fat-rich meal in man. The plasma immunoreactivity remained elevated up to the end of the study at 180 minutes. This is in contrast to the biphasic response observed by Rosell and Rokaeus (1979) and Fletcher et al (1985) who used the N-terminally directed antisera, but in agreement with that reported by Mogard and colleagues (1987) who used an C-terminally directed antiserum. In accordance with the pharmacokinetic data of the infused

neurotensin and neurotensin 1-8, chromatographic fractionation of the timed samples demonstrated that the main component of the circulating plasma immunoreactive neurotensin was chromatographically indentical to that of neurotensin 1-8. Plasma immunoreactivity co-eluting with authentic neurotensin constituted only about 13-15 % of the immunoreactivity co-eluting with neurotensin 1-8.

Several earlier studies have indicated that neurotensin exhibited gastrointestinal effects such as inhibition of gastric acid secretion, delaying of gastric emptying and stimulation of pancreatic secretion. The interpretation of this data must be put into the context of whether physiological doses were used in these studies. This in turn depends on the plasma concentration of neurotensin after physiological stimulation. In view of the plasma concentrations of neurotensin found following a meal, the rapid degradation of neurotensin in human circulation and the absence of biological effects of its relatively stable fragment, neurotensin 1-8, in man, it is likely that the gastrointestinal effects attributed to neurotensin in those earlier studies may not occur under normal physiological conditions. It is of interest that most recently Mogard et al (1987) reported that neurotensin did not inhibit the peptone meal-stimulated gastric

acid secretion and did not affect plasma gastrin in man.

An attempt was made to examine enzymes responsible for the degradation of neurotensin in the human plasma by using peptidase inhibitors in vitro. The data indicated that EDTA was most effective in the preservation of plasma neurotensin, suggesting metalloendopeptidase(s) which have yet to be identified may play a role in the metabolism of neurotensin. Although several reports have implicated numerous endopeptidases including endopeptidase E C 24.11 (enkaphalinase) and angiotensin converting enzyme in the degradation of neurotensin (Skidgel et al 1984, Emson et al 1985a, Checler et al 1987), these studies were performed on membrane preparations. The specificity of these enzymatic activities is still open to question (Turner et al 1985). There is also the possibility of tissue uptake or tissue degradation of neurotensin. On the basis of the experimental data, including those presented in the present work, neurotensin is likely to serve a paracrine role.

In the past few years, a host of peptides structurally related to neurotensin have been isolated. In addition, generation of neurotensin-like substances from plasma, which are

also recognised by the antisera raised against neurotensin, was reported. Some of the gastrointestinal effects ascribed to neurotensin in early studies may be due to these structural related peptides.

Although in some clinical situations such as endocrine apudomas, hepatomas and neurological diseases, alternations in plasma or tissue concentrations of immunoreactive neurotensin have been reported (Wood et al 1981, Blackburn et al 1981b, Bloom et al 1983, Theodorsson-Norheim et al 1983, Collier et al 1984, Shulkes et al 1984b, Nemeroff et al 1984, Lee et al 1985, Emson et al 1985a, Bissette et al 1985), the exact physiological role of neurotensin is still to be elucidated. The precursor polyprotein for neurotensin has only recently been predicted by nucleotide sequence analysis. The precise relationship of neurotensin with its structurally related peptides and other substances, whether these are neurotransimiters or neuromodulators remains unclear. Further understanding from these studies may unravel the functional role of neurotensin.

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Appendix A Formulae for Calculations

1. Percent Bound (% B)

$$\% B = \frac{B}{B + F} \times 100 \%$$

where B = count per second in the bound fraction
F = count per second in the free fraction

2. Coefficient of Variation (CV)

$$C V = \frac{S. D.}{\bar{X}} \times 100 \%$$

where S. D. = standard deviation

$\bar{X}$ = mean

3. Scatchard Plots (Scatchard 1949)

The typical standard curve is recalculated to obtain the bound/free ratio, i.e. percent bound over free (B/F). The B/F is plotted on the Y-axis against the total peptide bound plotted on the X-axis. The total peptide bound is calculated by taking the percent bound and multipleing by the total amount of cold peptide (as standard) plus the amount of label. The intercept on the Y-axis gives K X N, where K is the affinity constant of the antibody and N the total number of binding sites. The intercept on the X-axis provides the value of N. Dividing K X N by N gives the affinity constant in l/mol. Theoretically, Scatchard plot gives a straight line. However, in practice, straight line plots are rarely obtained. The reason for this is thought to be due either to antibody heterogeneity or to co-operativity between binding sites.

4. Sensiivity

Assay sensitivity is the smallest change in peptide (hormone) concentration that can be detected, which may be calculated as the value obtained in the standard curve which is 2 standard deviation from the zero standard (Feldman and Rodbard 1971, Sarson 1982).

Appendix B Buffers and solutions

(1). Phosphate buffer (0.06 M), pH 7.2, with gelatin:-

Pre-boil 5 litres distilled water, when boilded, dissolve 12.5 g of gelatine in this by leaving to stir for 15 minutes. When cool, add and dissolve the following constituents:

di-Sodium hydrogen orthophosphate dihydrate ($Na_2HPO_4.2H_2O$), 48.0 g; Potassium dihydrogen phosphate (KH_2PO_4), 4.13 g; Ethylenediamine tetra-acetic acid (disodium salt) ($Na_2H_2[EDTA].2H_2O$), 18.61 g; Sodium azide (NaN_2), 2.5 g.

The buffer is stored at 4^0C.

(2). Phosphate buffer (0.4 M), pH 7.4

To make 100 ml:-

KH_2PO_4, 5.44 g in 100 ml distilled water; $Na_2HPO_4.2H_2O$, 7.12 g in 100 ml distilled water;

19.7 ml of KH_2PO_4 + 80.3 ml $Na_2HPO_4.2H_2O$ and pH adjusted to 7.40

(3). Chloramine T (0.4 %), 40 mg in 10 ml 0.04 M phosphate buffer.

(4). Sodium metabisulphite ($Na_2S_2O_5$) (0.24 %), 24 mg in 10 ml 0.04 M phosphate buffer.

(5). Potassium iodide (KI) (1 %), 100 mg in 10 ml 0.04 M phosphate buffer.

(6). Tris (HCL) buffer, pH 8.5.

Tris 9.7 g in one litre of distilled water, NaCl 4.68 g in one litre of distilled water, adjust to pH 8.5 by the addition of 5 M HCL.

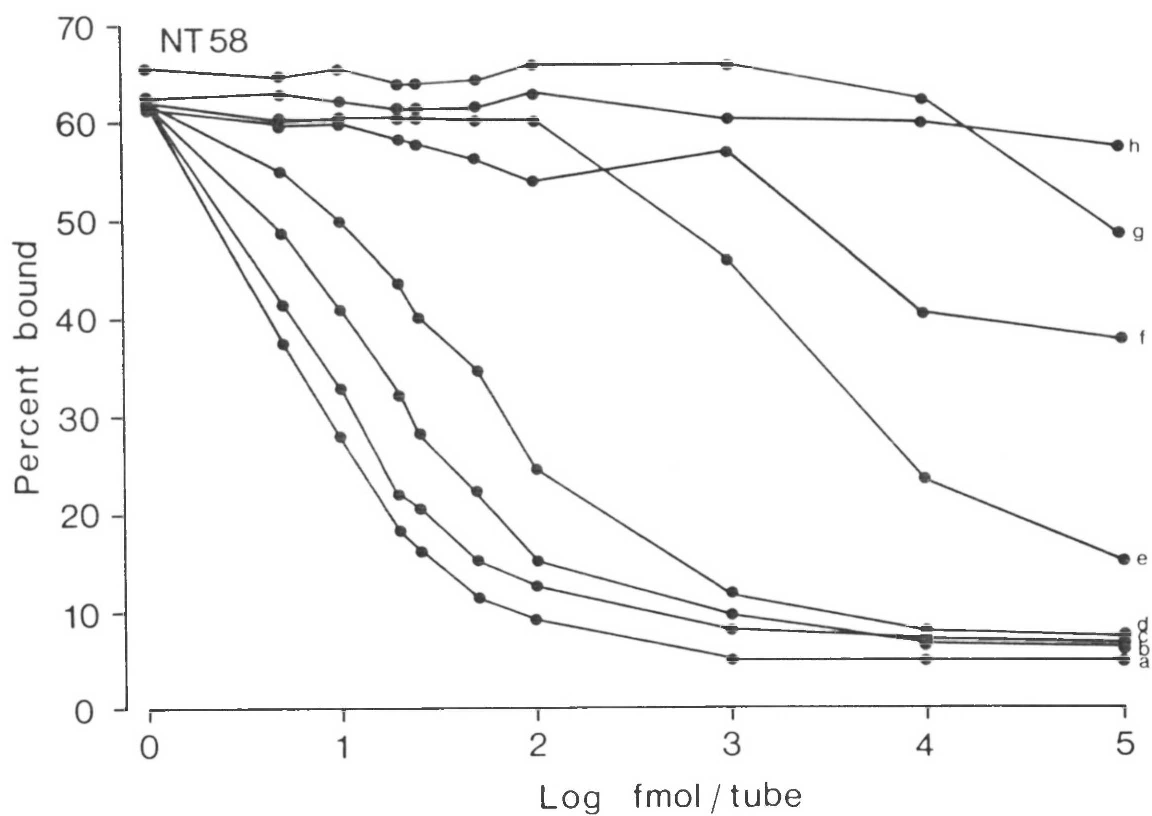
(7). Freeze Drying solution.

Lactose, 10 g; BSA, 5 g; citric acid, 0.42 g; L-cysteine hydrochloride, 0.2 g; Aprotinin (5 ml, 200,000 KIU); made up to 200 ml with formic acid or acetic acid.

Appendix C

Peptides or fragments	Cross-reactivity with antiserum		
	NT 58	07709	Moody
Neurotesin	100 %	100 %	100 %
Neurotensin 6-13	58 %	< 0.01 %	< 0.01 %
Neurotensin 7-13	68 %	< 0.01 %	< 0.01 %
Neurotensin 1-8	< 0.1 %	152 %	30 %
Neurotensin 1-9	< 0.1 %	162 %	- %
Neurotensin 1-11	< 0.1 %	-	39 %

Appendix D: Binding curves of NT 58 with neurotensin and its fragments.



(a) Neurotensin, (b) NT 6-13, (c) NT 7-13,
(d) NT 8-13, (e) NT 9-13, (f) NT 1-8,
(g) Neuromedin N, (h) NT 1-11.

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