Biochemical and Pharmacological Studies of the Metabolism of Dynorphin 1-8

by

Diane M. Dixon

A Doctoral thesis submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of Loughborough University of Technology

November 1990

Acknowledgements

I am indebted to the following:

John Traynor for his guidance and help

Stuart, Tim, Andy and David for making work a pleasure and such fun

Stu and Viv for their prayers

John Brennan and Jenni Caine for their expert technical assistance

Joan for the constant supply of tea

Kevin Fone for his patience

Sue Aspley for her moral support

I also wish to thank my parents and sister for a lifetime of support and encouragement.

And finally Medhat for radically altering my perception of the world

To Lebanon

- may she soon find peace

Sweet is the music of Arabia
In my heart, when out of dreams
I still in the thin clear mirk of dawn
Descry her gliding streams;
Hear her strange lutes on the green banks
Ring loud with the grief and delight
Of the dim-silked, dark-haired Musicians
In the brooding silence of night.

They haunt me - her lutes and her forests;
No beauty on earth I see
But shadowed with that dream recalls
Her loveliness to me:
Still eyes look coldly upon me,
Cold voices whisper and say --
'He is crazed with the spell of far Arabia,
They have stolen his wits away.'

Walter de la Mare

ABSTRACT

Biochemical and pharmacological studies of the metabolism of dynorphin 1-8

Keywords : Spinal cord, opioid peptides, dynorphin 1-8, [Leu]enkephalin, opioid receptors, metabolism, peptidase.

The metabolism of the opioid peptide [^{3}H]dynorphin 1-8 by slices of central nervous system (c.n.s.) and peripheral tissues, from the rat and guinea-pig has been studied. Rat spinal cord rapidly degraded [^{3}H]dynorphin 1-8, the N-terminal tyrosine residue being most susceptible to hydrolysis and therefore forming the major metabolite. Pretreatment of the metabolizing tissue with a standard cocktail of enzyme inhibitors decreased the degradation of [^{3}H]dynorphin 1-8 at both the N- and C-termini. However, inclusion of this enzyme inhibitor cocktail revealed the activity of a further enzyme, an endopeptidase, capable of cleaving the leucine^5-arginine^6 bond within the octapeptide liberating the opioid pentapeptide [Leu]enkephalin. This pattern of metabolism was observed across all rat brain regions and periphery.

The endopeptidase so revealed was resistant to serine protease inhibitors and metal chelating agents, but was inhibited potently by thiol protease inhibitors and two active site directed inhibitors of the endopeptidase E.C. 3.4.24.15. A variety of other opioid peptides were able to act as competitive substrates, the optimum substrate length falling between 8 and 13 amino acid residues.

In addition, the effect of this metabolism on the receptor selectivity of dynorphin 1-8 was assessed using the guinea-pig myenteric plexus longitudinal muscle preparation. A range of opioid antagonists were used to construct Schild plots against dynorphin 1-8, under a variety of inhibitory conditions, in order to derive Ke values which were then compared to values obtained using standard agonists at kappa and mu opioid receptors.

Using the nonselective antagonist naloxone, dynorphin 1-8, in the absence of inhibitors afforded a Ke within the range associated with an interaction with a kappa receptor population. Addition of the standard cocktail of inhibitors lowered the Ke to a value indicative of an action at a mu receptor population. Metabolism studies using the guinea-pig myenteric plexus longitudinal muscle as metabolizing tissue revealed the production of [^{3}H][Leu]enkephalin from [^{3}H]dynorphin 1-8 in the presence of the cocktail of enzyme inhibitors. The mu Ke value thus obtained would appear to be the result of the conversion of the kappa preferring octapeptide to the mu preferring pentapeptide [Leu]enkephalin. In Schild plots constructed using dynorphin 1-8 stabilised with the standard inhibitory cocktail and an enzyme inhibitor capable of blocking the endopeptidase (E.C. 3.4.24.15) activity, the Ke rose significantly to a value comparable to that displayed by the most selective kappa agonists currently available.

An ontogenic study revealed rat c.n.s. was able to liberate [^{3}H][Leu]enkephalinfrom [^{3}H]dynorphin 1-8 as early as post-natal day 1. In addition degradation of the N-termini was also evident even in the presence of the aminopeptidase inhibitor bestatin (10μM). The peak in enzyme activity that liberatees the pentapeptide from dynorphin 1-8 is seen in all c.n.s. areas tested at around post natal day 10. This corresponds to the appearence of the first detectable levels of delta opioid receptors in the rat c.n.s.

ABBREVIATIONS

The following abbreviations have been used throughout this thesis:

ACTH	adrenocorticotropic hormone
BAM	bovine adrenal medulla
Beta-FNA	beta-funaltrexamine
c.n.s.	central nervous system
DADLE	[D-Ala^2,D-Leu^5]enkephalin
DAMGO	[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin
DTT	dithiothreitol
EDTA	ethylenediethylamine
HEPES	N-2-hydroxyethanepiperazine-N-ethanesulphonic acid
HPLC	high performance liquid chromatography
i.c.v.	intracerebroventricular
LH	luetinizing hormone
M8008	16-methylcyprenorphine
MPLM	myenteric plexus longitudinal muscle
MSH	melanocyte stimulating hormone
NEM	N-ethylmalemide
nor-BNI	nor-binaltorphimine
p-HMB	p-hydroxymercuribenzoate
o-phen	o-phenanthroline
PMSF	phenylmethylsulphonylfluoride
POMC	proopioimelanocortin
TRIS	Tris[hydroxymethyl]aminomethane hydrochloride

CONTENTS

LIST OF TABLES AND FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 1

Introduction

GENERAL

Morphine has been used for many centuries to relieve pain. However very little progress was made to answer the question of how morphine was able to elicit pain relief, or its myriad of other pharmacological effects, until the early 1970's.

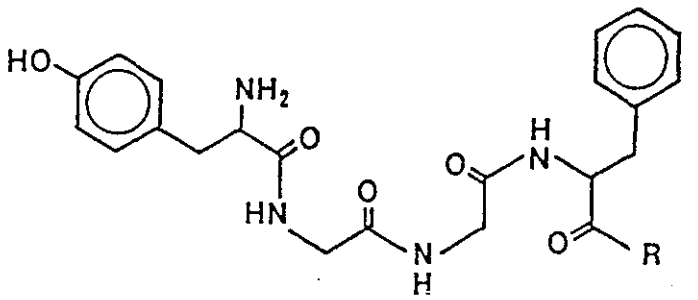
In 1973 several groups, working independently, published results that revealed a saturable binding site for radiolabelled morphine derivatives in central nervous system tissue [125, 142, 153, 169]. The specific binding of [^{3}H]-opiates to this site was highest in the synaptosomal fraction of brain homogenates and was decreased in the presence of proteolytic enzymes [125, 126, 142] indicating the binding site was on, or near to, nerve terminals and was proteinaceous in nature. The binding site was also found to be sensitive to temperature, pH and sulphydryl reagents. The ability of agonists, but not antagonists, to displace bound tritiated antagonist was decreased in the presence of cations, especially sodium [125, 126, 142]. In addition the opiate levorphanol was able to displace [^{3}H]dihydromorphine bound to membranes of rat brain whereas its stereoisomer dextrorphan was not [170]. This stereoselective binding provided further strong evidence to support the existence of an opioid specific receptor within the nervous system and this evidence gave impetus to the search for an endogenous ligand for this newly discovered receptor.

In 1975 John Hughes [77] working with Hans Kosterlitz in Aberdeen was successful in isolating a substance from porcine brain that mimicked the action of morphine in *in vitro* pharmacological assays, in particular the electrically stimulated mouse vas-deferens. Earlier work had shown this tissue was responsive to opiates which depressed electrically stimulated

contractions in a naloxone reversible manner, a finding later extended to include the rat, rabbit and hamster vas deferens Naloxone, a pure opiate selective antagonist, is able to completely reverse the actions of morphine. Consequently antagonism by naloxone is the reference point for definition of all opioid activity. The "morphine-like factor", isolated by Hughes and Kosterlitz, could also displace the opioid antagonist [^{3}H]-naloxone bound to rat brain membranes and thus displayed an affinity for the receptor recognised by this tritiated ligand [121].

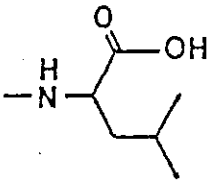
The "morphine-like factor" had the properties of a peptide as both its ability to displace [^{3}H]-naloxone from specific binding sites in rat brain homogenates and its potency in pharmacological assays decreased after treatment with proteolytic enzymes [79, 121]. Indeed towards the end of 1975 the structure of the "morphine-like factor" was elucidated and found to consist of two related pentapeptides [79, 80]. These two peptides were named leucine-enkephalin and methionine-enkephalin [Fig. 1.1] (hereafter written [Leu]enkephalin and [Met]enkephalin respectively). As can be seen from Figure 1.1 the two peptides differ only in their C-terminal amino-acid residue, from which their respective names are derived. Peptides such as [Leu] and [Met]enkephalin and many more recently discovered peptides are known as opioid peptides.

To differentiate the terms opiate and opioid the following definition will be used throughout this thesis. The term opiate describes any molecule that is derived directly from an alkaloid present in the opium poppy and is active at opioid receptors. Whereas the term opioid denotes any molecule active at opioid receptors that is not derived from the opium poppy.



Tyr —— Gly —— Gly —— Phe — R

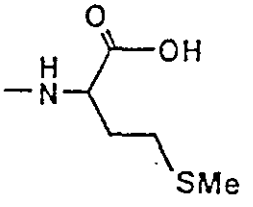
when R=



Leucine

Leucine-enkephalin

([Leu]enkephalin)



Methionine

Methionine-enkephalin

([Met]enkephalin)

Figure 1.1 : The Enkephalins

At approximately the same time indications that the receptor to which opioids and opiates specifically bound was not of a single type were suggested. A decade earlier Martin and co-workers [100, 101, 104] observed that two synthetic opioid antagonists nalorphine and cyclazocine, in addition to their antagonist effects, produced analgesia and euphoric effects in man. However the subjective effects produced by the chronic administration of cylazocine or nalorphine were qualitatively different to those produced by morphine. Futhermore cyclazocine was unable to suppress abstinence syndrome in subjects physically dependent on morphine. This evidence lead Martin to propose that morphine and nalorphine were acting via different receptors and consequently that the opioid receptor population was not homogeneous. Following further work in the mid 1970's Martin and colleagues proposed three distinct classes of opioid receptor based on the observed pharmacological properties of different opiates in the non-dependent and morphine-dependent chronic spinal dog preparation [47, 102]. In this preparation Martin observed differences in behaviour caused by the intravenous administration of morphine, ketocylazocine and N-allylnormetazocine (SKF-10,047). All three drugs are agonists as their actions can be antagonised by a pure antagonist, in this case, naltrexone. Ketocylazocine failed to suppress abstinence syndrome in morphine-dependent animals. Whereas buprenorphine, a partial agonist at the morphine receptor, both precipitated and suppressed abstinence in the morphine dependent dog. In addition morphine and cylazocine displayed differences in their sensitivity to the opioid antagonists naloxone and naltrexone. Both antagonists are 20 to 6 times less potent in precipitating abstinence in cyclazocine dependent dogs [103]. From these observations Martin described three receptor types namely the mu receptor, the kappa receptor and the sigma receptor at which morphine, ketocyclazocine and N-allylnormetazocine respectively were the putative ligands.

The presence of mu and kappa receptors was generally confirmed using isolated tissue preparations, in particular the mouse vas-deferens and the myenteric plexus longitudinal muscle of the guinea-pig ileum [82]. However these and other investigations produced results that were difficult to explain in terms of the three opioid receptors, namely mu, kappa and sigma. For example [Met]enkephalin was 20 times more potent than normorphine at inhibiting the electrically invoked contractions of the mouse vas-deferens whereas the two drugs were equipotent in the guinea-pig ileum assay [80]. Additionally [Met]enkephalin was at least 3 times more potent than morphine in displacing [^{3}H]naloxone bound to guinea-pig brain homogenate [80]. Futhermore the rank order of potency of a variety of opioid agonists at inhibiting the electrically evoked contractions in the mouse vas-deferens and the guinea-pig myenteric plexus longitudinal muscle were widely different [96]. In the mouse vas-deferens the relative potency, referred to normorphine as standard, of the ligands for the kappa receptor, namely the ketocyclazocines, is only 25% of that found in the guinea-pig ileum [82]. Indeed in both preparations the ketocylazocines require 3-6 times more naloxone or naltrexone to reverse their agonist action compared with the dose of antagonist necessary to reverse the action of morphine [82]. These varied observations were reinforced by the actions of the opioid peptides on these two preparations. Beta-endorphin, a 31 amino acid opioid peptide, discovered following the isolation of the enkephalins, as a C-terminal fragment of beta-LPH, is the only peptide to display similar potency in both the mouse vas-deferens and the guinea-pig ileum. Both [Met] and [Leu]enkephalin are more potent in the mouse vas-deferens than in the guinea-pig ileum [96]. These discrepancies are borne out by the action of antagonists. In the guinea-pig ileum the enkephalins and normorphine are antagonised equally well by naloxone [96]. Whilst in the mouse vas-

deferens 10 times more naloxone is required for the antagonism of the pentapeptides than for that of normorphine. In addition the agonist activity of opioids in the guinea-pig ileum was better correlated to their inhibition of [^{3}H]naloxone binding than to inhibition of [^{3}H][Leu]enkephalin binding. In contrast, opioid agonist activity in the mouse vas-deferens is not correlated to inhibition of [^{3}H]naloxone binding but has a similarity to the pattern of inhibition of [^{3}H][Leu]enkephalin binding [96].

All the above evidence supports the presence of a further receptor type. Kosterlitz and colleagues therefore proposed the existence of a receptor type, that they named the delta receptor, which displayed a high affinity for [Leu] and [Met]enkephalin [95]. Subsequent studies have shown that the actions of agonists at the sigma receptor are not reversed by naloxone [151]. It is therefore, common practice to refer to only three types of opioid receptor, namely the kappa-receptor, the mu-receptor and the delta-receptor.

[Met] and [Leu]enkephalin and beta-endorphin are not the only endogenous peptides capable of acting at opioid receptors. By 1982 a further family of opioid peptides had been isolated, namely the dynorphins, and many products related to [Leu] and [Met]enkephalin had been identified [Table 1.1]. These form three distinct families of peptides and are classified according to the prohormones from which they are derived. These prohormones, once fully sequenced, were named proopiomelanocortin (POMC), proenkephalin A and prodynorphin (also termed proenkephalin B).

Table 1.1 Opioid Peptides

Peptide	Alternative Name	Amino Acid Sequencnce
Proopiomelanocortin products		
β-endorphin	C-fragment, β-LPH$_{61-91}$	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-^{76}Thr^{77}Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-^{87}His-Lys-Lys-Gly-^{91}Gln
C'-fragment	δ-endorphin, β-LPH$_{61-87}$	
γ-endorphin	β-LPH$_{61-77}$	
α-endorphin	β-LPH$_{61-76}$	
Proenkephalin A products		
[Met]enkephalin	Methionine-enkephalin, Met-enk	Tyr-Gly-Gly-Phe-Met
[Met]enkephalyl-Arg^6	pro-methionine-enkephalin	.....-Arg
[Met]enkephalyl-Lys^6		.....-Lys
[Met]enkephalyl-Arg^6-Phe^7	MEAP, MERF	.....-Arg-Phe
[Met]enkephalyl-Arg^6-Arg^7		.....-Arg-Arg
[Met]enkephalyl-Arg^6-Gly^7-Leu^8	MERGL	.....-Arg-Gly-Leu
[Leu]enkephalin	Leucine-enkephalin, Leu-enk	Tyr-Gly-Gly-Phe-Leu
Peptide E		Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-^{12}Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-^{20}Arg-Tyr-^{22}Gly-Gly-Phe-^{25}Leu

Table 1.1 Opioid Peptides (cont)

Peptide	Alternative Name	Amino Acid Sequence
Proenkephalin A products (cont)		
BAM-12P		Peptide E (1-12)
BAM-20P		Peptide E (1-20)
BAM-22P		Peptide E (1-22)
Metorphamide	Adrenorphin	Peptide E (1-8) NH2
Peptide F		Tyr-Gly-Gly-Phe-Met-Lys-Lys-Met-Asp-Glu-Leu-Tyr-Pro-Leu-Glu-Val-Glu-Glu-Glu-Ala-Asn-Gly-Gly-Leu-Val-Leu-Gly-Lys-Arg-Tyr-Gly-Gly-Phe-Met
Prodynorphin products		
[Leu]enkephalin		Tyr-Gly-Gly-Phe-Leu
β-neo-endorphin		.....-Arg-Lys-Tyr-Pro
α-neo-endorphin		.....-Arg-Lys-Tyr-Pro-Lys
Dynorphin 32		^{1}Tyr-Gly-Gly-Phe-Leu-Arg-Arg-^{8}Ile-Arg-Pro-Lys-Leu-^{13}Lys-Trp-Asp-Asn-^{17}Gln-Lys-Arg-^{20}Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-^{32}Thr
Dynorphin A	Dynorphin 1-17	Dynorphin 32 sequence (17)
Dynorphin B	Rimorphin	Dynorphin 32 sequence (20-32)
[Leu]enkephalin-Arg^{6}	Dynorphin 1-6	Tyr-Gly-Gly-Phe-Leu -Arg
[Leu]enkephalin-Arg^{6}-Arg^{7}	Dynorphin 1-7	Tyr-Gly-Gly-Phe-Leu-Arg-Arg
Dynorphin 1-8	PH-8P	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile

Taken with kind permission from Kitchen, I. 1984. Prog Neurobiol., 22 345-358

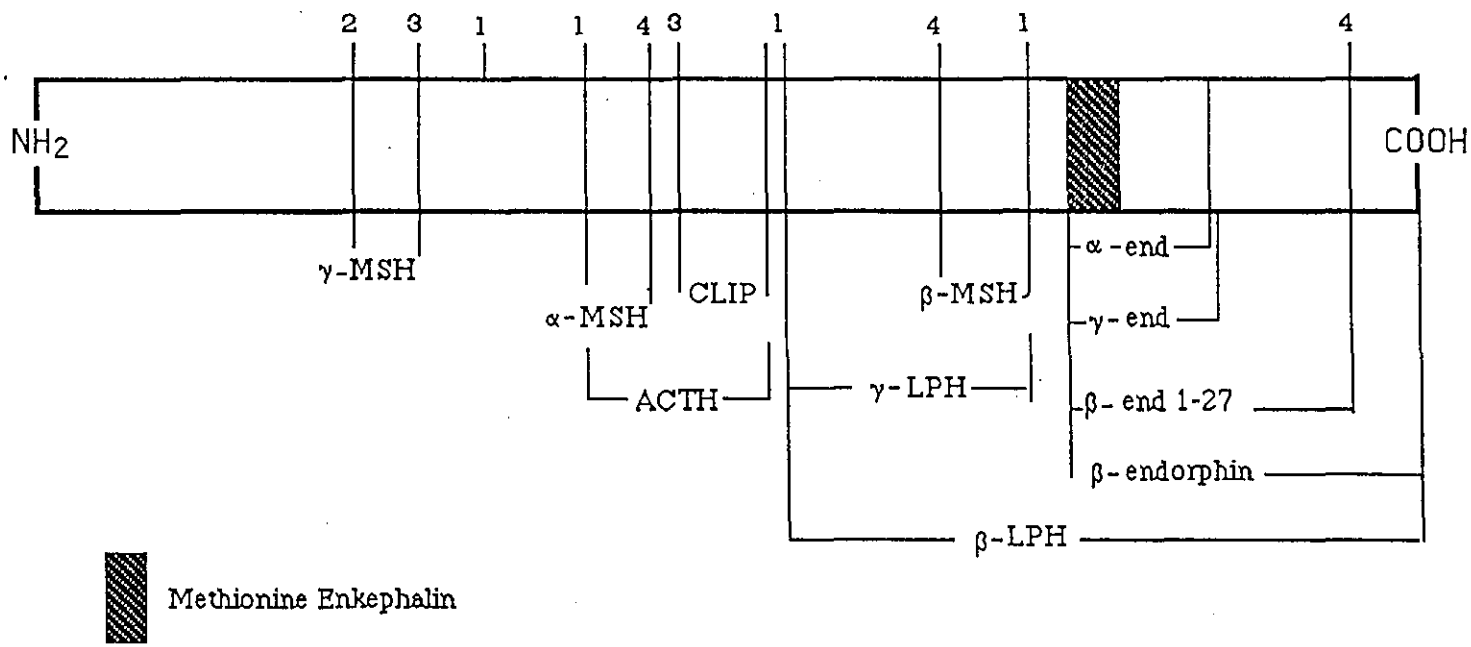


Figure 1.2 : Schematic representation of proopioimelanocortin (POMC)

1 = lysine - arginine

2 = arginine - lysine

3 = arginine - arginine

4 = lysine - lysine

MSH = melanocyte stimulating hormone

ACTH = adrenocorticotropic hormone

CLIP = corticotropin like intermediate lobe peptide

LPH = lipotropin

PROOPIOMELANOCORTIN

Proopiomelanocortin (POMC) is the precursor for the opioid peptide beta-endorphin, and also adrenocorticotropic hormone (ACTH) and various melanocyte stimulating hormone (MSH) containing peptides [Fig. 1.2; Table 1.1]. All metabolic products of POMC are bound by pairs of basic amino acids which are common sites for peptidase attack [111].

The major source of POMC is the pituitary gland and both anterior and intermediate lobes contain beta-endorphin. The intermediate lobe produces a greater amount of post-translational modification of the released hormones, such as alpha-N-acetylation leading to the loss of opioid activity and shortening of the C-terminus [73]. The processing of POMC in brain parallels that found in the intermediate lobe. Although POMC contains one sequence of [Met]enkephalin, located at the N-terminal of beta-endorphin, it is not the primary source of [Met]enkephalin found in central nervous system (c.n.s.) tissue [165]. POMC products are not found in adult spinal cord, although beta-endorphin is present in the embryonic spinal cord of the rat [61].

In radioligand binding experiments beta-endorphin shows a slight selectivity for mu over delta-receptors with negligible affinity for the kappa-receptor [88]. However in a variety of isolated tissue studies beta-endorphin has not been seen to display selectivity between the mu and delta-receptor [73]. In the rat vas-deferens beta-endorphin appears to act via a receptor that is not of the mu, delta or kappa type [141]. Although this has been termed the epsilon receptor its nature remains to be confirmed. Indeed its actual existence is still a matter of debate as some have suggested a low receptor

reserve is responsible for the low potency of various agonists in this tissue and that the epsilon receptor is in fact a mu receptor [49, 145].

Beta-endorphin is resistant to enzymatic attack, a feature that increases its potency in pharmacological assays [107]. Consequently intracerebroventricular (i.c.v.) injections of beta-endorphin produce profound analgesia [74]. Furthermore beta-endorphin administered i.c.v. releases [Met]enkephalin from the spinal cord of anaesthetised rats [159], which may suggest that beta-endorphin elicits its analgesic effect by stimulating a descending opioid pathway [149]. However caution should be used when interpreting results as i.c.v. injections of beta-endorphin also cause profound catalepsy. In addition to analgesia, beta-endorphin affects several hormonal systems, being, for example, involved in the release of prolactin from the pituitary [72]. Beta-endorphin therefore serves a variety of roles, many of which tend to be neurohormonal rather than those of a classical neurotransmitter.

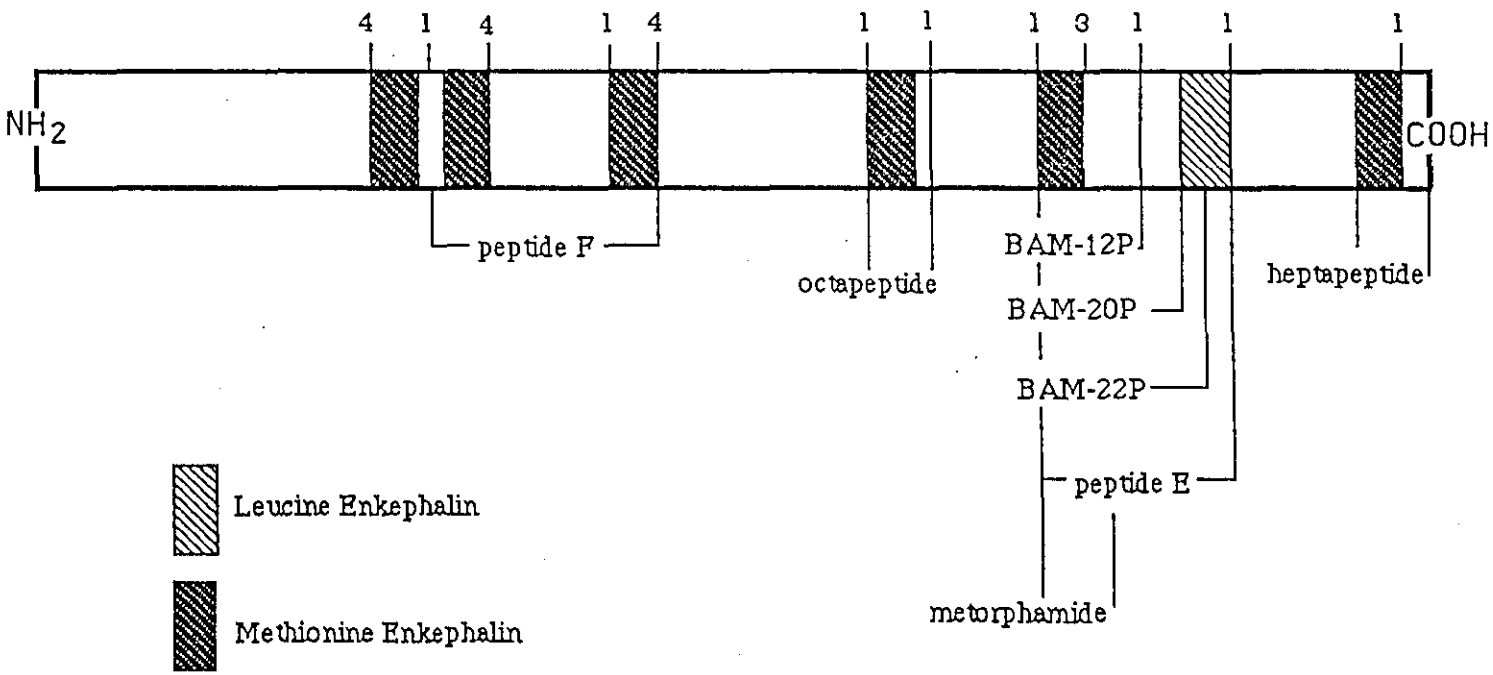


Figure 1.3 : Schematic representation of proenkephalin A

1 = lysine - arginine

3 = arginine - arginine

4 = lysine - lysine

BAM = bovine adrenal medulla (peptide)

PROENKEPHALIN A

Once sequenced, proenkephalin A was seen to contain four copies of [Met]enkephalin, one copy of [Leu]enkephalin, a single copy of [Met]enkephalyl-Arg^6-Phe^7 and one copy of [Met]enkephalyl-Arg^6-Gly^7-Leu^8, each bound by basic pairs of amino acids [Fig. 1.3] [5, 113]. However several other extended forms of [Met]enkephalin have been isolated, for example the BAM (Bovine Adrenal Medullary) peptides purified from bovine adrenal medulla [Table 1.1].

The processing products of proenkephalin A are found throughout the brain, especially in areas associated with nociception, for example the periaquaductal gray and basal ganglia [39]. The smaller fragments namely, [Met]enkephalin, [Met]enkephalyl-Arg^6-Phe^7, [Met]enkephalyl-Arg^6-Gly^7-Leu^8 and [Leu]enkephalin are all found in the dorsal horn of the spinal cord, especially in lamina I (marginal zone), lamina II (the substantia gelatinosa) and lamina V of Rexed [50, 83]. In general the processing in brain and spinal cord appears more complete than that in the adrenal medulla [160].

The various processing products of proenkephalin A display a broad spectrum of opioid receptor selectivity. [Met]enkephalin and [Leu]enkephalin display a 10 and 17 fold preference respectively for the delta receptor over the mu receptor, but do act as agonists at the mu receptor. However neither peptide is likely to interact with the kappa receptor [123, 174]. [Met]enkephalyl-Arg^6-Gly^7-Leu^8 is non-selective between mu and delta and displays some kappa affinity. The ability of this peptide and [Met]enkephalyl-Arg^6-Phe^7 to act as agonists at kappa receptors is illustrated in isolated tissue experiments. These peptides are able to inhibit the electrically evoked contractions of the isolated vas-deferens of

the rabbit [75] a tissue that contains an homogenous kappa receptor population [115, 130]. However, the amidated peptide [Met]enkephalyl-Arg^6-Arg^7-Val^8-NH_2 is the only member of the shorter processed products that shows high affinity for the kappa receptor [88]. More extended forms of [Met]enkephalin do display an increased affinity for the kappa receptor. Thus both BAM-12P and BAM-22P are especially active in the rabbit isolated vas-deferens assay [75]. Both peptides also interact with the mu receptor as illustrated by their effectiveness in the rat vas-deferens and the guinea-pig myenteric plexus longitudinal muscle preparations [75]. Though the potency of BAM-12P is relatively low in the guinea-pig myenteric plexus longitudinal muscle preparation, this however is an indication of its susceptibility to enzymatic degradation causing an apparent lack of potency in this particular assay system [17, 73].

The longer peptides, E and F are related only in so much as they both contain enkephalin sequences at their N- and C-termini. Peptide E contains one [Met]enkephalin sequence at its N-terminal and one [Leu]enkephalin sequence at its C-terminal, and displays a non-selective profile in isolated tissue assays, acting at both mu and kappa receptors [73]. Peptide F contains two [Met]enkephalin sequences, one at each terminal, and has negligible activity in the guinea-pig myenteric plexus longitudinal muscle preparation, mouse vas-deferens and rabbit vas-deferens bioassay systems. This lack of activity cannot be attributed to enzymatic degradation as the potency of peptide F is not substantially increased by the addition of peptidase inhibitors to the assay systems. Peptide F is, however, active in the rat vas-deferens assay, this coupled with its low affinity in the guinea-pig myenteric plexus longitudinal muscle preparation may indicate an ability to act at the proposed non-mu, non-delta (epsilon) receptor in the rat vas-deferens [73].

The involvement of the mu opioid receptor in supraspinal analgesia is well documented [171]. Thus all the proenkephalin A products that display affinity and efficacy at the mu receptor have the potential to elicit analgesia at a supraspinal level. Peptides E and BAM-22P induce analgesia when injected intracerebroventricularly into mice [74]. However intraventricular injection of [Leu] or [Met]enkephalin in mice cause only weak inhibitory effects in behavioural analgesic tests. This is probably due to their rapid metabolism since the half life in rat plasma of both peptides is less than 2.5 minutes [57]. Intraventricular injections of the enzyme inhibitor thiorphan, which is able to prevent the peptidase activity responsible for the hydrolysis of the Gly^4-Phe^5 bond within opioid peptides, both increases the analgesic potency of exogenously applied opioid peptides and when given alone produces a naloxone reversible analgesia, suggesting a stabilization of endogenous peptides [131a].

All three types of opioid receptor have been found in spinal cord [155] and when occupied by appropriate opioid agonists analgesia can result [102, 171]. This is difficult to demonstrate following intrathecal injections of the shorter proenkephalin A products as these are again fraught with the problems of enzymatic inactivation. However, the intrathecal injection of the pentapeptide analogue namely [D-Ala^2,D-Leu^5]enkephalin, synthesized to be resistant to enzymatic attack, has been shown to elicit naloxone reversible analgesia at the spinal level in hot plate tests carried out in rats as has the more selective delta ligand [D-Pen^2,D-Pen^5]enkephalin and the mu ligand [D-Ala^2,$MePhe^4$, $Glyol^5$]enkephalin [40, 135, 136, 158].

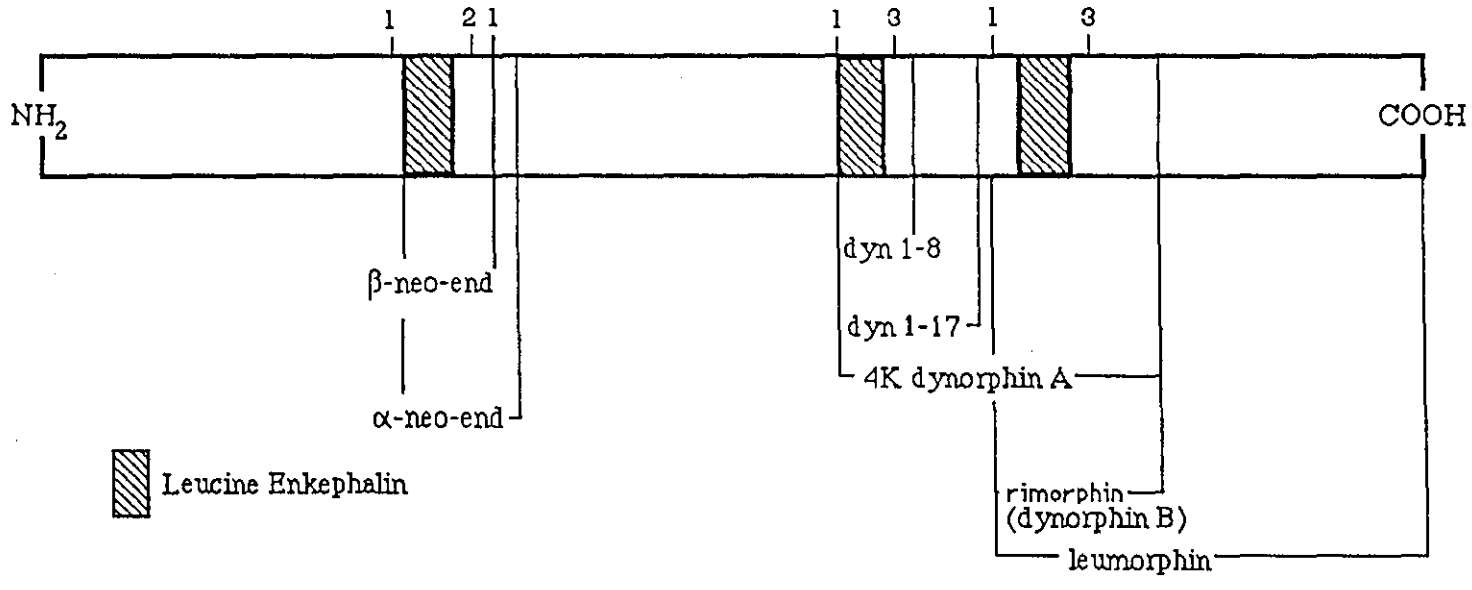


Figure 1.4 : Schematic representation of prodynorphin (proenkephalin B)

1 = lysine - arginine

2 = arginine - lysine

3 = arginine - arginine

PRODYNORPHIN

Prodynorphin, the last of the opioid prohormones to be sequenced [87], contains three copies of [Leu]enkephalin each flanked by basic amino acid pairs [Fig. 1.4]. Processing of this prohormone yields extended forms of the three [Leu]enkephalin copies. Two of these namely, beta-neo-endorphin and dynorphin 1-17 are both contained within pairs of basic amino acids. However cleavage at single arginine residues also occurs yielding alpha-neo-endorphin; dynorphin B, from cleavage of the threonine^{13}-arginine^{14} bond of leumorphin; and dynorphin 1-8 via the hydrolysis of the isoleucine^{8}-arginine^{9} bond within the dynorphin 1-17 sequence. Dynorphin 1-13 is liberated via the cleavage of the leucine^{13}-lysine^{14} bond within dynorphin 1-17. As with proenkephalin A the smaller processing products of prodynorphin appear to be most abundant in brain tissue and spinal cord [138]. However the processing is not homologous throughout the c.n.s. and regional variations do occur.

Dynorphin 1-8 is found throughout the brain, its concentration being especially high in the substantia nigra. In all brain areas alpha-neo-endorphin is more abundant than beta-neo-endorphin [138], this is probably due to the higher rate of hydrolysis of the beta-neo-endorphin by certain endopeptidases (see below). Dynorphin B formed via thiol protease activity from dynorphin B-29, is also widely distributed throughout the brain, the highest concentration being located in the substantia nigra [175, 178]. The posterior pituitary is rich in all prodynorphin products, whereas the anterior pituitary contains only one species of prodynorphin product, a 6000 Da high molecular weight species [138, 140]. Immunoreactive dynorphin is found in rat spinal cord, the dorsal horn containing the highest level, probably in short axoned neurons [14]. Dynorphin A and alpha-neo-

endorphin in human spinal cord are again located in the dorsal horn, especially in the substantia gelatinosa [128].

Zamir and colleagues have also shown that [Leu]enkephalin in the substantia nigra is synthesized via a dynorphinergic pathway [176]. This same group had previously observed the concentrations of alpha- and beta-neo-endorphin to be considerably higher than the concentrations of dynorphin A (plus its metabolic fragment dynorphin 1-8) or dynorphin B. This was especially striking in the substantia nigra, where the level of [Leu]enkephalin is more than twice that of [Met]enkephalyl-Arg^6-Gly^7-Leu^8, a peptide derived exclusively from the proenkephalin precursor where like [Leu]enkephalin it is found as a single copy [176]. These observations led to a further series of experiments designed to determine the source of [Leu]enkephalin in the substantia nigra of rat brain. Unilateral lesions just rostral to the mesencephalon that transected descending fibres from the forebrain resulted in a marked depletion of nigral alpha-neo-endorphin, dynorphin B and [Leu]enkephalin but did not alter the levels of [Met]enkephalyl-Arg^6-Gly^7-Leu^8. This suggested that [Met]enkephalyl-Arg^6-Gly^7-Leu^8 is not contained in descending inputs into the substantia nigra whereas the dynorphin related peptides and [Leu]enkephalin must be in axons of neurons rostral to the substantia nigra. In a second set of animals the globus pallidus was lesioned from the caudate putamen resulting in a decrease in alpha-neo-endorphin, dynorphin B and [Leu]enkephalin in the ipsilateral substantia nigra without altering the level of [Met]enkephalyl-Arg^6-Gly^7-Leu^8. This lesion also resulted in a decrease in alpha-neo-endorphin, [Leu]enkephalin and [Met]enkephalyl-Arg^6-Gly^7-Leu^8 in the globus pallidus confirming earlier observations of a proenkephalinergic striatopallidal pathway [31]. In addition these observations provide evidence for the existence of a dynorphinergic striatonigral and a striato (or cortico)-

pallidal pathway. The most appropriate explanation for the parallel decreases in alpha-neo-endorphin, dynorphin B and [Leu]enkephalin whilst the level of [Met]enkephalyl-Arg^6-Gly^7-Leu^8 remains unaltered is that in the striatonigral pathway [Leu]enkephalin is produced in dynorphinergic neurons [176]. The production of [Leu]enkephalin from a dynorphinergic neuron may have important physiological implications, as it allows the neuron to produce both a kappa (dynorphin) and a delta ([Leu]enkephalin) ligand from the same precursor. This may occur by direct processing or could conceivably occur at a later stage via the hydrolysis of dynorphin A and thus provides a possible explanation of why the concentration of dynorphin B is higher than that of dynorphin A in most brain areas if the latter is converted to smaller fragments especially in the substantia nigra.

In addition the co-localization of prodynorphin products is not uncommon. Immunohistochemical methods have shown alpha-neo-endorphin and dynorphin 1-17 staining in the same fibres in numerous areas of the rat brain including substantia nigra, hypothalamus, nucleus accumbens, hippocampus and medulla oblongata [166, 167]. There is also evidence to suggest that co-localization of proenkephalin A and prodynorphin derived peptides occurs in both brain and spinal cord [56, 167]. This is seen in the spinal cord of arthritic rats where neurons in Laminae IV and V stain for four opioid peptides namely [Met]enkephalyl-Arg^6-Phe^7, [Met]enkephalyl-Arg^6-Gly^7-Leu^8, dynorphin 1-17 and alpha-neo-endorphin [168].

All the fragments of prodynorphin greater than seven amino acids in length exhibit varying degrees of selectivity towards the kappa receptor, as illustrated by their ability to inhibit the electrically evoked contractions of the rabbit vas-deferens, a tissue known to contain exclusively kappa receptors [28, 88]. Increasing the length of the peptide above seven amino acids

progressively increases their affinity for the kappa site up to dynorphin 1-13 which has the highest affinity for the kappa receptor. However dynorphin 1-13 also displays the highest relative affinities at all three opioid binding sites [28]. [Leu]enkephalin is the only prodynorphin product to show any appreciable selective affinity for the delta receptor. As peptide length decreases towards the pentapeptide an increase in the delta affinity over kappa affinity is observed, the affinity towards the mu receptor is not affected to the same degree [28, 88].

The dynorphin peptides when injected into cerebral ventricles show a distinct lack of effect in analgesic tests [23, 58] though evidence exists to suggest that the dynorphins are able to elicit their analgesic effect via an activation of spinal opioid receptors. Thus dynorphin 1-17 induces profound analgesia in the tail flick test following i.c.v. administration [58]. However this effect is only partially reversed by intrathecal injections of naloxone and is completely unaffected by subcutaneous application of naloxone. Dynorphin B also increases tail flick latency, but again this effect is only partially reversed by naloxone [59]. Unfortunately neither of the above studies involved the assessment of motor function following the intrathecal injection of the dynorphin peptides. This is an important omission as Stevens and Yaksh [147] have shown that severe motor dysfunction occurs following the intrathecal injection of dynorphin 1-17 and 1-13 at levels well below those used in the above analgesic tests. This effect appears to be non-opioid in nature as the motor dysfunction is not reversed by naloxone. In addition dynorphin 3-13, a peptide fragment inactive at opioid receptors, due to its lack of N-terminal tyrosine also induces motor dysfunction [22]. The shorter dynorphins 1-7, 1-8, 1-9 and 1-10 did not appear to impair motor function, however when co-administered with peptidase inhibitors these peptides also caused hindlimb dysfunction [94].

Dynorphin 1-17 and 1-13 were ineffective at eliciting analgesia in the hot plate, tail flick and writhing tests when injected intrathecally at doses just below those shown to elicit impairment of motor function [147]. None of the shorter dynorphin peptides were able to elicit analgesia in any of the tests listed above. Similarly intrathecal application of the kappa preferring synthetic non-peptide agonist U50 488H (trans,3,4-dichloro-N-methyl-N[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide) which did not affect motor function was ineffective in the hot plate and tail flick tests. However this latter compound elicited a dose dependent inhibition of writhing that was naloxone reversible [147]. It is possible therefore to suggest that activation of kappa receptors by dynorphins is unlikely to cause motor dysfunction, but other actions have a powerful influence on motor output. The ability of the dynorphins, especially the shorter fragments, to elicit analgesia remains therefore open to question. In light of their possible metabolism by central nervous system tissue [28, 48, 173] it is difficult to ascertain whether the effects observed following the administration of dynorphin peptides are due to the peptide or unknown metabolic products.

OPIOID PEPTIDES IN THE SPINAL CORD

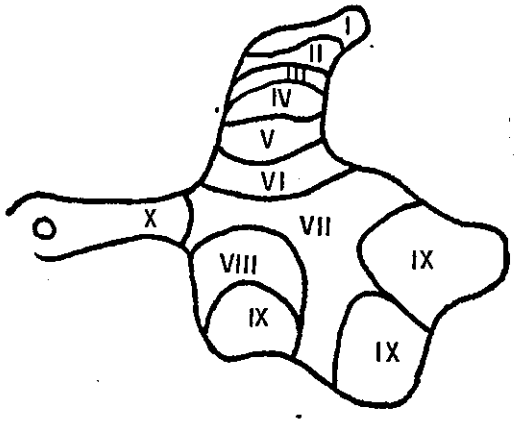
The dorsal horn of the spinal cord is an important site for the control and modulation of nociceptive information [41, 172]. High levels of opioid receptors are found in the dorsal horn of the spinal cord and are distributed throughout the rostral-caudal axis [6]. The ratio of kappa/mu/delta receptors is approximately the same along the rostral-caudal axis with kappa binding accounting for the majority of bound radioactivity [155]. Autoradiographic studies have shown opiate receptors to be concentrated in specific laminae within the dorsal horn of the spinal cord. The superficial layers namely laminae I and II (the substantia gelatinosa) [Fig. 1.5] are especially rich in opioid binding sites [6, 143]. The presence of opioid receptors within the superficial layers of the dorsal horn is indicative of their importance in the modulation of nociceptive inputs into the spinal cord. The substantia gelatinosa receives inputs from and sends inputs to the periaquiductal grey and adjacent reticular formation nuclei within the brainstem all of which are major nociceptive processing centres [41, 54, 172]. Dorsal root section or neonatal capsaicin, which causes a selective destruction of unmyelinated and fine myelinated primary afferent fibres, significantly reduces opioid receptor density in the dorsal horn [7, 42, 110, 112] suggesting a proportion of opioid receptors within the dorsal horn are located on presynaptic primary afferent nerve terminals.

The dorsal-ventral distribution of opioid receptors is broadly paralleled by the distribution of opioid peptides. The concentrations of proenkephalin A and prodynorphin products are higher in the dorsal rather then ventral horn of the spinal cord throughout the rostral-caudal axis [128, 175]. Along the rostral-caudal axis the levels of prodynorphin derived peptides in the ventral portion of the spinal cord are low, however [Met]enkephalin levels in the

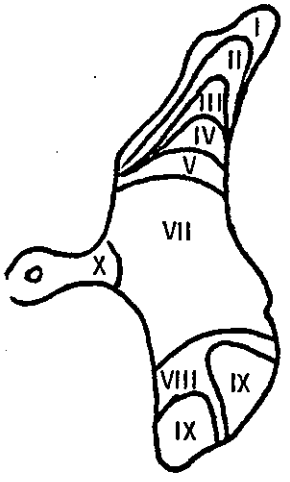
ventral horn can reach 60% of those found in the dorsal horn [128]. Unlike the dorsal-ventral distribution, the levels of opioid peptides along the rostral-caudal axis of the spinal cord do not parallel the homogenous distribution of opioid binding sites. The majority of studies have revealed the highest levels of immunoreactive peptide to be located in the sacral region of the cord with the concentration decreasing in a rostral direction. For example the level of [Met]enkephalyl-Arg^6-Phe^7 in the sacral region of the rat spinal cord is over twice that found in the cervical region [97]. This distribution pattern is seen more acutely in the dorsal horn where the level of sacral [Met]enkephalyl-Arg^6-Gly^7-Leu^8 is three times that found in the cervical spinal cord [83]. However discrepancies are reported, Zamir and colleagues have described cervical levels of immunoreactive dynorphin B that are over twice as high as those found in lumbar spinal cord [175]. In human spinal cord the distribution of the prodynorphin products dynorphin 1-17 and alpha-neoendorphin and the proenkephalin A product [Met]enkephalin closely parallels that of [Met]enkephalyl-Arg^6-Phe^7 and [Met]enkephalyl-Arg^6-Gly^7-Leu^8 [128] derived from proenkephalin A.

Taken together these results show a distribution of prodynorphin and proenkephalin A products along the rostral-caudal axis of the spinal cord. Prodynorphin products appear to be concentrated within the dorsal horn and are especially high in the substantia gelatinosa [128, 175]. Proenkephalin A products are also high in the dorsal horn but the distribution of [Met] and [Leu]enkephalin is not as precise and both pentapeptides are also found in substantial concentrations in the ventral horn [50, 175]. It would appear therefore that both prodynorphin and proenkephalin A products are found in areas of the spinal cord where they are able to modulate sensory information [70]. The role of proenkephalin A products located in the ventral spinal cord, where they have been shown to be present in serotonergic neurones

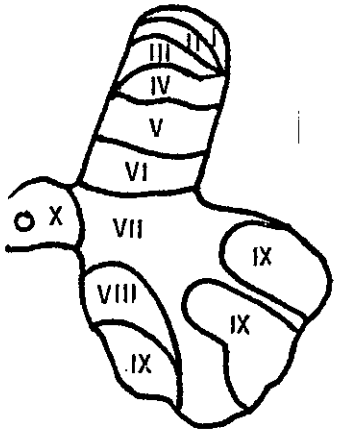
making synaptic contact with alpha-motoneurones, may be able to modulate spinal motor function [71].



CERVICAL



THORACIC



LUMBAR

Figure 1.5 : Positions of cytoarchitectonic laminae of Rexed in the grey matter of the human spinal cord at 3 levels, the cervical enlargement, the thoracic region and the lumbosacral enlargement. Note : lamina VI is only present in limb enlargemants.

METABOLISM OF OPIOID PEPTIDES

It became apparent shortly after the isolation of [Met] and [Leu]enkephalin that the pentapeptides were susceptible to enzymatic attack [10, 57, 77, 91, 173]. Since an intact tyrosine residue at the N-terminus of an opioid peptide is necessary for binding to opioid receptors [95] cleavage of the tyrosine^1-glycine^2 bond by aminopeptidases is, therefore, especially important. Deactivation of both pentapeptides and the dynorphins has been demonstrated to occur via cleavage of this bond [36, 48, 57] in a variety of tissues, including rat and mouse brain homogenates [36, 48, 99] and guinea-pig ileum [78].

Cleavage of the Tyr^1-Gly^2 bond is not confined to a single aminopeptidase. A variety of aminopeptidases, varying in their susceptibility to a broad spectrum of inhibitors, appear to be responsible for the release of the N-terminal tyrosine. However most are metalloenzymes and are inhibited by a wide variety of chelating agents [66]. Much of the enzyme activity is contained within the soluble fraction of brain homogenates and is found throughout the brain rather than in discrete areas specifically associated with opioid peptide content [137]. An exception to this rule is a membrane bound aminopeptidase, designated aminopeptidase M II, that has been purified from rat brain. This enzyme displays a regional distribution parallel to that displayed by opioid receptors [68] and is inhibited by bestatin but is distinguished from other membrane aminopeptidases by its sensitivity to puromycin [65, 68].

Immunohistochemical studies have revealed a further aminopeptidase, namely aminopeptidase M, which unlike aminopeptidase M II is insensitive

to puromycin and is located on the walls of cerebral blood vessels [69].and would therefore be unable to hydrolyse synaptic enkephalin but could maintain a concentration gradient between synaptic and interstitial enkephalin levels thereby ensuring efficient diffusion of enkephalin from the synapse. In addition Hui and co-workers have isolated a opioid receptor associated aminopeptidase from rat brain that is able to hydrolyse the pentapeptides [81], however peptide binding to the opioid receptor is not coupled to biodegradation [109].

The importance of aminopeptidase M II in enkephalin metabolism is questionable. When administered i.c.v. into mice the aminopeptidase M II inhibitor puromycin failed to prevent the hydrolysis of co-administered [^{3}H][Leu]enkephalin. In addition the latency time in the hot plate test was unaffected by puromycin administered alone i.c.v. indicating the inability of puromycin to prevent the hydrolysis of endogenous enkephalin. In contrast the general aminopeptidase inhibitor bestatin did increase the latency time when administered i.c.v. [20]. However in another study puromycin produced a naloxone reversible, dose related analgesia in rats accompanied by an increase in striatal enkephalin levels [64].

In addition to the aminopeptidases cleaving the N-terminal tyrosine residue, a dipeptidylaminopeptidase, releasing Tyr^1-Gly^2 fragments is also found in brain [24, 53]. This enzyme is also a metallopeptidase [148] thus far found to be present in brain tissue of pig [24], monkey [62], calf and rat [161]. The three enzymes are probably closely related as they display the same type of substrate specificity, namely the requirement of three hydrophobic amino acid residues, as the minimal sequence to be cleaved [24]. However an active site directed inhibitor of the dipeptidylaminopeptidase purified from porcine brain, administered with [Leu]enkephalin into the cerebral ventricles

of mice, failed to affect the jump latency time in the hot plate test as compared with [Leu]enkephalin given alone [24]. It would appear therefore that the dipeptidylaminopeptidase does not play a major role in enkephalin metabolism at supraspinal levels.

Degradative attack of the C-terminus of the pentapeptides and dynorphins is carried out by a dipeptidylcarboxypeptidase. As early as 1978 cleavage of the Gly^3-Phe^4 bond within [Leu]enkephalin, by an enzyme associated with the membrane fraction of mouse brain was observed [98]. This enzyme appeared to have a higher specificity towards the enkephalin molecule than that displayed by any of the aminopeptidases. Neurochemical and neuropharmacological studies suggest a close relationship between the dipeptidylcarboxypeptidase and the opioid systems within the c.n.s. [98]. Indeed, an association between opioid pathways and the high affinity dipeptidylcarboxypeptidase is probable considering the activity of the enzyme is increased after chronic treatment with morphine [98].

This dipeptidylaminopeptidase has been named "enkephalinase" and is distinct from angiotensin converting enzyme [137]. The term "enkephalinase" is rather an unfortunate choice as it implies a degree of specificity towards the pentapeptides that is not to displayed by the enzyme, which is capable of hydrolysing a variety of neuropeptides [3]. For example the enzyme hydrolyses angiotensin I and II [44], neurotensin, bradykinin, oxytocin [4], substance P [105], beta-lipotropin 61-69 [67] and gamma-endorphin [67]. The enzyme also displays a wide tissue distribution being found in the kidney brush border [13] and in the microvillar membrane of pig intestine [32]. Indeed the enzyme content of these peripheral tissues is higher than that found in the brain [45, 92]. More fully the enzyme is now classified as E.C. 3.4.24.11. Several inhibitors of opioid degrading

enzymes appear to show activity *in vivo* and *in vitro*. Thus thiorphan, a synthetic, highly potent site directed inhibitor of E.C. 3.4.24.11 produces analgesia in the tail flick test when administered to mice, in addition to increasing the potency of opioid peptides in bioassays [27, 35, 107, 131a].

A relatively new inhibitor of enkephalin degradation, kelatorphan, is able to inhibit all three enzymes responsible for the degradation of the opioid peptides [43]. Kelatorphan is more potent at inhibiting the degradation of endogenous and exogenous enkephalin than bestatin or thiorphan or a combination of both [35, 43, 163].

ONTOGENY OF OPIOID PEPTIDES

The ratios of the various opioid peptides differ not only across brain regions, they also vary with age. The levels of all opioid peptides, within the c.n.s. increase from birth to adult animals, for example the beta-neo-endorphin content of rat neurointermediate pituitary increasing about 1000 fold over this period [139]. An exception to this rule is beta-endorphin, which is not present in the spinal cord of adult animals but can be detected in embryonic spinal cord tissue [61]. The levels of rat striatal [Leu] and [Met]enkephalin increase 11 fold from birth to 21 days, however the level of striatal [Leu]enkephalin develops more rapidly than that of [Met]enkephalin. Moreover the ratio of [Leu] to [Met]enkephalin is not constant throughout post-natal development and in addition a variation in the ratio occurs between brain regions [122]. In the rat neurointermediate pituitary the ratio of dynorphin 1-8 to dynorphin 1-17 at birth is 1:3 whereas in the adult the ratio falls to 1:0.8. A similar pattern is seen in the ratio of beta-neo-endorphin to alpha-neo-endorphin, in newborn rats compared to

adult rats [139]. It would appear, therefore, that the enzymes responsible for cleaving single basic amino acid residues are not fully developed at birth.

There is little change in aminopeptidase activity from birth to adulthood in either rat cortex or striatum [122], adult levels are only twice those recorded at birth, the maximum level being reached within 1-2 weeks. In contrast the level of dipeptidylcarboxypeptidase ("enkephalinase" E.C. 3.4.24.11) displays a 6 fold increase during development, with a time course parallel to changes in both [Leu] and [Met]enkephalin levels and total opioid receptor binding capacity [122].

AIMS

In various brain regions the ratio of prodynorphin and proenkephalin A derived products differ from the ratios found within the prohormones. Of special interest are [Leu]enkephalin and [Met]enkephalyl-Arg^6-Gly^7-Leu^8 which have the predicted ratio of 1:1, as both peptides occur in single copies within the proenkephalin A molecule. However the actual ratio varies from 0.5 to 2.1, being especially high in the substantia nigra [178]. The levels of prodynorphin products are also high in the substantia nigra, in particular products of low molecular weight, for example dynorphin 1-8. This allows for the possibility that the high ratio of [Leu]enkephalin to [Met]enkephalyl-Arg^6-Gly^7-Leu^8 is as a result of [Leu]enkephalin being processed from prodynorphin [138]. Indeed, as cited earlier, lesion studies have shown that in the striatonigral pathway [Leu]enkephalin is produced by dynorphinergic neurons [176].

It is the aim of the present study to investigate the nature and distribution of the possible enzymic production of [Leu]enkephalin from dynorphin 1-8. This metabolism will be studied in various regions of the rat c.n.s. but attention will be focused on the spinal cord as this is an important site for the modulation of nociceptive information and contains high levels of dynorphin 1-8 The effect of this enzymatic metabolism on the receptor selectivity of dynorphin 1-8 will be investigated in the isolated myenteric plexus longitudinal muscle of the guinea-pig. The physiological consequences of such a metabolism will be discussed.

CHAPTER 2

Materials and Methods

MATERIALS AND EQUPIMENT

Peptides

Tyrosine; tyrosyl-glycine; tyrosyl-glycyl-glycine; leucyl-leucine.

Sigma Chemical Company, England

Tyrosyl-glycyl-glycyl-phenylalanine; Leucine-enkephalin ([Leu]enkephalin); [Leu]enkephalyl-Arg^6; [Leu]enkephalyl-Arg^6-Arg^7; dynorphin 1-8; DAGOL ([D-Ala^2,$MePhe^4$,Gly-ol^5]enkephalin); DADLE ([D-Ala^2,D-Leu^5]enkephalin); Beta-Endorphin; metorphamide, dynorphin 1-17; dynorphin 1-13, Methionine-enkephalin ([Met]enkephalin) and [Met]enkephalyl-Arg^6-Gly^7-Leu^8.

Cambridge Research Biochemicals, England

Drugs

Naloxone

Sigma Chemical Company, England

Thiorphan; bestatin

Cambridge Research Biochemicals, England

The following drugs were kindly donated:

Morphine

McFarlane Smithand Co., Edinburgh, Scotland

Captopril

Merck, Sharp and Dohme, England

Beta-Funaltrexamine (Beta-FNA) and nor-Binaltorphamine (nor-BNI)

Dr. A. G. Hayes, Glaxo Pharmaceuticals, England

M8008 (16-methylcyprenorphine)

Dr. C. F. C. Smith, Reckitt and Colman Pharmaceutical Division

N-[-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-p-aminobenzoate

N-[-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-p-aminobenzoate

Dr. M. Orlowski, Mt. Sinai School of Medicine, New York, U.S.A.

Radiochemicals

[tyrosyl-3,5-^{3}H(N)]-Dynorphin 1-8 (27.6 Ci/mmol)

Du Pont, NEN Research Products, England

Chemicals

Trizma base (Tris[hydroxymethyl]aminomethane hydrochloride) and HEPES (N-[2-hydroxyethane]piperazine-N'-[2-ethanesulphonic acid]) buffer

Sigma Chemical Company, England

Ecoscint, scintillation fluid

National Diagnostics, England

Acetonitrile, trifluroacetic acid, triethylamine all HPLC grade

Fisons Chemical Company, England

All other chemicals were of analytical grade

Physiological Solutions

Formula for Krebs solution

NaCl, 118; $NaHCO_3$, 29; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 4.0; KH_2PO_4, 1.2; Glucose, 11.1 (mM)

Formula for Krebs-HEPES buffer

NaCl, 118; $NaHCO_3$, 29; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 4.0; KH_2PO_4, 1.2; Glucose, 11.1; HEPES, 25 (mM)

Animals were supplied by the following:

Male Wistar rats (250-300g), pregnant female rats

Animal Unit, University of Nottingham, Sutton Bonnington

Male Dunkin-Hartley guinea-pigs (300-400g)

David Hall, Newchurch, Burton-upon-Trent

Animals were fed on a standard laboratory diet and kept on a 12hr light/dark cycle at a temperature of 20°C.

Equipment

The following items of equipment were used throughout the project;

McIlwain tissue chopper

Gilson HPLC system including:

Gilson Holochrome UV variable wavelength detector

Altex ODS C_{18} reverse phase column

SRI square wave stimulators (harvard)

Washington 400 MD2R chart recorder

Isotonic transducers (Hanod bioscience)

Phillips PW4700 liquid scintillation counter

METHODS

1. *PURITY OF [^{3}H]DYNORPHIN 1-8*

A sample of [^{3}H] dynorphin 1-8 was analysed for purity using HPLC. A 1µl sample of the radiolabelled dynorphin 1-8 was mixed with 10µl of a 100µM solution of unlabelled dynorphin 1-8. The mixture was then applied to an Altex ODS C_{18} reverse phase column and was eluted using the following gradient system:

Time (min)	%B in A	Flow Rate (ml/min)
0	50	2
20	90	2

Solvent A: 26mM trifluroacetic acid, to pH 3 with triethylamine

Solvent B: 50% acetonitrile in 13mM trifluroacetic acid, to pH 3 by triethylamine

Fractions were collected every 30 seconds. 3ml of Ecoscint scintillation fluid were added to each fraction which were then counted for radioactivity. Three 1µl samples of [^{3}H]dynorphin 1-8 were also counted for radioactivity, in order to calculate the percentage recovery off the column. Radioactivity that co-eluted with the marker peptide, monitored at 280nm, was assumed to represent [^{3}H]dynorphin 1-8.

The percentage recovery off the column was greater than 98%. Each batch of [^{3}H]dynorphin 1-8 used was greater than 97% pure by this method.

Area	Weight (mg)
Medulla	233 ± 9
Cortex	812 ± 35
Striatum	103 ± 6
Hippocampus	120 ± 6
Hypothalamus	106 ± 7
Cerebellum	236 ± 6

Table 2.1 : Weights of various regions from the rat c.n.s.

Values represent mean ± standard error of mean

2. *METABOLISM STUDIES*

a. *Brain*

Male Wistar rats (250-300g) were killed by decapitation between 9.30 and 10am, and the brains immediately removed and placed on ice. The brains were dissected according to the method of Glowinski and Iverson (51). Each area was weighed [Table 2.1] and then chopped into 0.5mm cubes, using a McIlwain tissue chopper. The tissue was placed in 4ml polypropylene test tubes and washed a minimum of three times with Krebs solution buffered with 25mM HEPES at 37°C. Each area was then suspended in buffer to give a final tissue concentration of 200mg/ml. To a 50µl sample (10mg of tissue) of the final tissue suspension was added, where appropriate, enzyme inhibitors (diluted from distilled water stock solutions in Krebs/HEPES in a volume no greater than 30µl) and the final volume of the reaction mixture was adjusted to 1ml with Krebs/HEPES. Enzyme inhibitors, where added, were preincubated with the tissue for various times [Table 2.2], then [^{3}H]Dynorphin 1-8 was added to give a final concentration of 12nM. After varying incubation times, at 37°C, the reaction was terminated by the addition of phosphoric acid to give a final concentration of 50mM. The reaction tubes were immediately placed on ice. A 500µl sample of supernatant from each tube was filtered through cellulose nitrate filters (pore size 0.45µm) and frozen at -20°C prior to separation by HPLC. Separations were carried out within 48hrs of freezing.

Time course studies were linearised and t1/2 values calculated.

b. *Lumbar-Sacral Spinal Cord*

Male Wistar rats were killed by decapitation between 9.30 and 10am. The spinal cord contained within vertebrae below the second rib was removed and placed

on ice. The cord was chopped coronally into slices 1mm thick. The slices were weighed into 10mg units, each unit being chopped further into 0.5mm cubes. The tissue was then treated as described above for brain tissue.

c. *Guinea-pig Cerebellum*

Male Dunkin-Hartley guinea-pigs were killed by cervical dislocation. The cerebella were removed and placed on ice then chopped coronally into slices 1mm thick. The slices were weighed into 10mg units, each unit being chopped further into 0.5mm cubes.
The tissue was then treated as described above for rat brain tissue.

d. *Myenteric Plexus Longitudinal Muscle*

Male Dunkin-Hartley guinea-pigs were killed by cervical dislocation. The ilea were removed, after discarding 10cm lengths immediately after the pyloric sphincter and immediately prior to the ileal-caecal junction the remaining ilea were flushed of their contents and placed in Krebs solution aerated with 5% CO_2 in 95% O_2 at 37°C. The myenteric plexus longitudinal muscle was removed from each ileum by placing the ileum over a 1ml glass pipette and gently wiping the longitudinal muscle with cotton wool soaked in Krebs buffer. The myenteric plexus longitudinal muscle was cut into 3 inch strips (approximately 100mg) and chopped into 0.5mm slices. The slices were then treated as for brain tissue described above.

Inhibitor	Pre-Incubation Time (min)
All endogenous peptides	N.P.
Dynorphin 1-9	N.P.
DAGOL	15
DADLE	15
Morphine	15
PMSF	15
DTT	15
NEM	15
pCMB	15
o-Phen	15
EDTA	15

Table 2.2 : Pre-incubation times for various inhibitors used to prevent the metabolism of dynorphin 1-8.
N.P. = not pre-incubated

e. *Rat Ventricular Muscle*

Male Wistar rats were decapitated between 9.30-10am and the hearts removed and placed in Krebs aerated with 5% CO_2 in 95% O_2 at 37°C. The ventricle tissue was dissected from the atria and chopped as for brain tissue.

3. *SEPARATION OF METABOLIC PRODUCTS BY HPLC*

An 80µl sample of reaction mixture was added to 20µl of a marker peptide solution containing the following, all at a concentration of 100µg/ml: tyrosine; tyrosyl-glycine; tyrosyl-glycyl-glycine; tyrosyl-glycyl-glycyl-phenylalanine; [Leu]enkephalin; [Leu]enkephalyl-Arg^6; [Leu]enkephalyl-Arg^6-Arg^7 and dynorphin 1-8. The resulting mixture was applied to an Altex ODS C_{18} reverse phase column and was eluted using the following gradient at a flow rate of 1ml/min:

Time (min)	%B in A
0	15
15	30
45	65
50	75

Solvent A: 26mM trifluroacetic acid, to pH 3 with triethylamine.
Solvent B: 50% acetonitrile in 13mM trifluroacetic acid, to pH 3 with triethylamine.

This gradient was not adequate for the accurate separation of the tyrosine, tyrosyl-glycine and tyrosyl-glycyl-glycine fractions. These three N-terminal

fragments were therefore collected together and separated further using the following gradient at a flow rate of 0.5ml/min:

Time (min)	%B in A
0	0
20	5

Solvents A and B as above.

Peaks were monitored at 280nm and fractions co-eluting with the marker peptides were collected in 0.5ml aliquots in scintillation minivials. Ecoscint scintillation fluid (3ml) was added to each of the fractions which were then counted for radioactivity. Three 80µl samples of incubation mixture were also counted for radioactivity, in order to calculate the percentage recovery off the column, which was 94.1 ± 0.8% [n=25]. Metabolite formation was calculated as percentage of total radioactivity recovered from the column.

4. *ISOLATED TISSUE STUDIES*

a. *Preparation of Myenteric Plexus Longitudinal Muscle for Electrical Stimulation*

Male Dunkin-Hartley guinea-pigs (300-400g) were killed by cervical dislocation. The ileum was immediately removed, flushed of its contents, and placed in Krebs solution at 37°C aerated with 5% CO_2 in 95% O_2. Strips of myenteric plexus longitudinal muscle were removed and mounted in 3ml organ baths previously coated with silicon to reduce adsorption of peptides onto the glass surface. Tissues were bathed constantly in Krebs at 37°C, aerated with 5% CO_2 in 95%O_2. After allowing a recovery period of one hour, each plexus

was stimulated through platinum ring electrodes using square wave pulses at supramaximal voltage at a frequency of 0.16Hz and a pulse width of 400μs. Contractions were recorded isotonically.

b. *Experimental Procedure*

i. Agonist Potencies

Agonists were added to the tissue baths 30min after electrical stimulation commenced. Agonist inhibition of contraction was allowed to reach a plateau and then washed out. The concentration of agonist required to reduce the twitch height to half its maximum value (IC_{50}) was determined. Where used, the peptidase inhibitors captopril, 10μM; bestatin, 10μM; thiorphan, 0.3μM (hereafter collectively referred to as the peptidase inhibitor cocktail) were incubated with the tissue for 30min prior to the addition of agonist. In some cases varying concentrations of N-[(R,S)-carboxy-2-phenylethyl]Ala-Ala-Phe-pAB or N-[(R,S)-carboxy-3-phenylpropyly]Ala-Ala-Phe-pAB were added in a similar manner. Following each application of agonist, tissues were washed by overflow until maximum contraction was restored to control levels.

ii. Measurement of Antagonist Affinities

Antagonists were preincubated with the guinea-pig myenteric plexus longitudinal muscle for the following times: naloxone, 20min; M8008, 30min; nor-binaltorphamine, 45min, prior to the addition of agonist. Dose-response curves for agonists were obtained before the addition of antagonist and then repeated in the presence of varying concentrations of antagonist. Dose-ratios were calculated at IC_{50} values and Schild [5] plots constructed (as described in section 2.5). Antagonists were removed from the tissue by continuous washing until the response to added agonist was fully recovered. All dose-response curves were cumulative, with 10min between subsequent dose response curves to agonists. Tissues were maintained under 1g resting tension.

iii. Irreversible Inhibition by Beta-Funaltrexamine

Dose-response curves for agonists in the guinea-pig myenteric plexus longitudinal muscle were constructed. After washout of the agonist 100nM beta funaltrexamine (beta-FNA) was added to the bath and the tissue was then incubated with the beta-FNA for 30min. After this time the beta-FNA was removed by continuous washing for 60min or until maximum control contraction was attained. Agonist dose-response curves were then repeated. Dose-ratios before and after beta-FNA were calculated at IC_{50} values.

5. *THE SCHILD PLOT*

In order to define the receptor at which an agonist acts it is necessary to employ an antagonist. From the ability of the antagonist to compete with an agonist at any given receptor type a value ~~known~~ such as the Ke or pA_2 can be determined. This value varies between receptor type and is a measure of the affinity of an antagonist for a particular receptor. In addition the Ke value is also a measure of the ability of any given agonist to displace a particular antagonist from a particular receptor site and consequently can function as an indicator of the receptor selectivity of a given agonist. Of particular importance is the slope of the Schild plot which should be unity if a selective antagonist has been displaced from a single receptor type (but see page 39).

A competitive antagonist can be regarded as a drug that interacts reversibly with a set of receptors to form a complex, but unlike an agonist-receptor complex, it fails to elicit a response (ie the antagonist has no intrinsic activity). The antagonist-receptor complex can therefore be characterised by a dissociation constant. The interaction can be expressed as:

$$\underset{[A]}{\text{Antagonist}} + \underset{[R]}{\text{Receptor}} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{[AR]}{\text{Antagonist-Receptor Complex}}$$

where k_1 and k_2 are the association and dissociation rates for the complex.

According to the law of mass action the rates of the forward and reverse reactions are the same once equilibrium is attained therefore:

$$k_1[A][R] = k_2[AR]$$

therefore:

$$\frac{k_2}{k_1} = K_e = \frac{[A][R]}{[AR]} \qquad \text{(equation 1)}$$

where Ke is the antagonist dissociation constant.

When both an antagonist and an agonist are present, the various interactions with receptors can be expressed thus:

$$[D] + [A] + [R] \rightleftharpoons [AR] + [DR]$$

where [D] is the concentration of agonist and [DR] is the concentration of the agonist-receptor complex.

Now if the total number of receptors is $[R_T]$ then the number of free receptors [R] can be expressed as:

$$[R] = [R_T] - [AR] - [DR]$$

Dividing through by [DR]

$$\frac{[R]}{[DR]} = \frac{[R_T]}{[DR]} - \frac{[AR]}{[DR]} - 1 \qquad \text{(equation 2)}$$

The principles applied to the antagonist are valid for an agonist therefore:

$$K_D = \frac{[D][R]}{[DR]} \quad \text{ie} \quad \frac{[R]}{[DR]} = \frac{K_D}{[D]} \qquad \text{(equation 3)}$$

where K_D is the dissociation constant for the agonist.

Substituting equations 3 and then 1 into equation 2:

$$\frac{K_D}{[D]} = \frac{[R_T]}{[DR]} - \frac{[AR]}{[DR]} - 1$$

and therefore

$$\frac{K_D}{[D]} = \frac{[R_T]}{[DR]} - \frac{[A][R]}{K_e} \div \frac{[D][R]}{K_D} \quad -1$$

which reduces to

$$\frac{K_D}{[D]} = \frac{[R_T]}{[DR]} - \frac{[A]K_D}{K_e[D]} \quad -1$$

multiplying throughout by $K_e[D]$:

$$K_D K_e = \frac{[R_T]K_e[D] - [A]K_D - K_e[D]}{[DR]}$$

rearranging

$$K_D K_e + [A]K_D + K_e[D] = \frac{[R_T]K_e[D]}{[DR]}$$

therefore

$$\frac{[R_T]}{[DR]} = \frac{K_D K_e + K_D[A] + K_e[D]}{K_e[D]} \qquad \text{(equation 4)}$$

The reciprocal of this equation gives the fraction of receptors occupied by an agonist in terms of concentrations and dissociation constants of agonist and antagonist. Assuming that $[DR]/[R_T]$ (ie. the proportion of receptors occupied by the agonist) is equal to E/E_{max} , the ratio of effect produced by a given dose of agonist to the maximal possible effect, then the reciprocal of the equation expresses any given response to an agonist as a fraction of the maximum possible response. Indeed when the concentration of agonist is zero then equation 4 simplifies to:

$$\frac{[DR]}{[R_T]} = \frac{[D]}{[D]+K_D} \qquad \text{(equation 5)}$$

Equation 4 also predicts that the linear portion of the agonist concentration response curves carried out in the presence and absence of a competitive antagonist will be parallel, but displaced to the right in the presence of antagonist. The most important feature of competitive antagonism is that it may be overcome by increasing concentrations of agonist ie. the maximum response

is not affected by a competitive antagonist. The degree of shift to the right of the agonist logarithmic concentration response curve is proportional to the antagonist concentration and to the affinity of the antagonist for the receptor. The affinity of the antagonist for any given receptor type is inversely proportional to the antagonist-receptor dissociation constant K_e. The value of the K_e can be determined from the concentration of agonist producing equal responses in the absence $[D_O]$ and presence $[D_A]$ of antagonist. Since the response to the agonist is equal, the proportion of receptors occupied is assumed to be the same.

Therefore from equations 4 and 5:

$$\frac{[D_O]}{[D_O]+K_D} = \frac{K_e[D_A]}{K_DK_e+K_D[A]+K_e[D_A]}$$

taking reciprocals

$$\frac{[D_O]+K_D}{[D_O]} = \frac{K_DK_e+K_D[A]+K_e}{K_e[D_A]}$$

dividing by denominators

$$\frac{1+K_D}{[D_O]} = \frac{K_DK_e}{K_e[D_A]} + \frac{K_D[A]}{Ke[D_A]} +1$$

reducing and rearranging

$$\frac{1+ K_D}{[D_O]} = \frac{K_D}{[D_A]} + \frac{K_D[A]}{K_e[D_A]} +1$$

reducing

$$\frac{K_D}{[D_O]} = \frac{K_D[1+[A]]}{[D_A][1+K_e]}$$

$$\frac{[D_A]}{[D_O]} -1 = \frac{[A]}{[K_e]}$$

The value of the K_e is independent of the agonist used provided the antagonist competes with it for the receptor.

When the concentration of antagonist ($[A_2]$) is such that :

$$[D_A] = 2[D_O]$$

then

$$[A_2] = K_e$$

ie. the value of the dissociation constant for the antagonist is the concentration of antagonist with which the ratio of concentrations of agonist producing equal responses in its presence $[D_A]$ and absence $[D_O]$ equals 2.

The negative logarithm of the molar concentration of antagonist with which the ratio of equi-effective concentrations of agonist in the presence and absence of antagonist is two, has been designated by Schild as the pA_2 value thus:

$$pA_2 = -\log[A_2] = \log[1/[A_2]] \qquad \text{(equation 7)}$$

Equation 6 can be converted to a form containing pA_x where pA_x is the negative logarithm of the molar concentration of antagonist in the presence of which the potency of the agonist is decreased x times.

$$x-1 = \frac{[A_X]}{K_e}$$

taking logs

$$\log[x-1] = \log[A_X] - \log K_e \qquad \text{(equation 8)}$$

Equation 8 predicts that the plot of $\log[x-1]$ verses $\log[A_X]$ is a straight line, the intercept on the log[A] axis giving the value of $\log K_e$ or $-pA_2$. The slope of the line is 1 provided the agonist is acting at a single receptor class. Deviation from unit slope is consistent with a system of more than one receptor class. However departures from linearity for the double log relationship for a system of one receptor type have been predicted and observed in a variety of experimental situations.

There are several reasons why this error can occur:

1).Tissue uptake of agonist,

2).The use of insufficient incubation times for the antagonist resulting in a non-equilibrium situation,

The first two problems result in slopes of less than unity.

3).The use of antagonists that are toxic can result in a slope greater than unity. [90].

Results throughout are expressed as mean ± standard error of the mean (sem). Statistical analysis was carried out using ANOVA analysis of variance followed by Dunnett's t-test . Other results were analysed by the Mann Whitney U test where stated.

CHAPTER 3

Distribution of the Metabolism of [^{3}H]Dynorphin 1-8

INTRODUCTION

All peptides when in contact with tissue or body fluids are susceptible to attack by metabolising enzymes. The opioid peptides are not an exception to this rule, for example the hydrolysis of the N-terminal portion of the pentapeptides was described immediately following their isolation and sequencing [57].

Several years later the sequencing of the prohormone for the larger dynorphin molecules revealed the dynorphin 1-17 sequence to be the precursor for dynorphin 1-8 which was in turn an extended form of [Leu]enkephalin. The ratio of these three peptides in the c.n.s. does not parallel that found within the prodynorphin molecule [138]. Also the ratio of [Leu]enkephalin to [Met]enkephalyl-Arg^{6}-Gly^{7}-Leu^{8}, both found in single copies within the prohormone proenkephalin A, varied widely throughout the rat c.n.s.[138]. This led to speculation that [Leu]enkephalin could be produced from two prohormones, namely proenkephalin A and prodynorphin [176]. It was also evident that the enzymatic activity responsible for the production of [Leu]enkephalin was not uniform as the ratios of the peptides derived from prodynorphin differed across various brain regions [138].

The [Leu]enkephalin produced could be formed from the prohormone directly or from larger dynorphin molecules by post-translational processing. Here we examine the extent to which dynorphin 1-8 is metabolised by the rat spinal cord and investigate the distribution of this metabolism within the rat and guinea-pig c.n.s. and the rat periphery.

RESULTS

Metabolism of [^{3}H]Dynorphin 1-8 by Central Nervous System Tissue

1.) *Rat Spinal Cord*

Incubation of [^{3}H]dynorphin 1-8 (12nM) with slices of rat lumbo-sacral spinal cord resulted in the rapid metabolism of the peptide [Fig. 3.1a]. Separation of the metabolites by HPLC [Fig. 3.2] revealed that, following a 20min incubation period 80.4 ± 4.1% of the recovered radioactivity co-eluted with the N-terminal fraction of the peptide (consisting of Tyr, Try-Gly and Tyr-Gly-Gly). Upon further separation this N-terminal fraction was seen to be almost exclusively composed of [^{3}H]tyrosine [Fig. 3.3]. The fraction co-eluting with the [Leu]enkephalin marker contributed 11.0 ± 3.3% to the radioactivity total. Also after 20min only 2.4 ± 1.3% of the radioactivity co-eluted with the parent peptide [Fig 3.1a]. Indeed when in contact with the tissue [^{3}H]dynorphin 1-8 had a half life of just 2.5 ± 0.4 min.

A 30 min incubation of lumbo-sacral spinal cord slices with the enzyme inhibitors captopril (10μM); bestatin (10μM); thiorphan (0.3μM) and

prior to the addition of [^{3}H]dynorphin 1-8 resulted in a stabilization of the [^{3}H]dynorphin 1-8. Under these conditions the half life of the [^{3}H]dynorphin 1-8 increased to 7.3 ± 3.1 min. Although at the end of the 20min incubation period [^{3}H]dynorphin 1-8 still only constituted 16.2 ± 4.2% of the recoverable radioactivity [Fig. 3.1b]. The major metabolite formed from [^{3}H]dynorphin 1-8 by the spinal cord slices following preincubation with the enzyme inhibitors was [^{3}H][leu]enkephalin, accounting for 60.5 ± 2.2% of the recovered radioactivity. The level of recovered N-terminal fraction concomittantly decreased to 9.6 ± 2.2%.

In the absence or presence of the enzyme inhibitors the other possible metabolites of [^{3}H]dynorphin 1-8 namely, [^{3}H]tyrosyl-glycyl-glycyl-phenylalanine, [^{3}H][Leu]enkephalyl-Arg^6 and [^{3}H][Leu]enkephalyl-Arg^6-Arg^7 accounted for 5.1 ± 0.3%, 1.4 ± 0.1% and 0.7 ± 0.2% respectively of the recovered radioactivity following a 20 min incubation period.

2.) *Rat Brain and Guinea-Pig Cerebellum*

In all rat brain areas tested [^{3}H]dynorphin 1-8 (12nM) was readily metabolised giving a variety of products. In the presence of the enzyme inhibitor cocktail (namely, bestatin, 10μM; captopril, 10μM and thiorphan, 0.3μM) [^{3}H][Leu]enkephalin formed the major metabolite following a 10min incubation with c.n.s. tissue [Fig. 3.4]. In all brain regions the amount of recoverable [^{3}H][Leu]enkephalin, formed after a 10min incubation, was greater than that of recoverable [^{3}H]dynorphin 1-8. Indeed as the level of [^{3}H][Leu]enkephalin increased a parallel decrease in [^{3}H]dynorphin 1-8 was observed. Thus the highest level of intact [^{3}H]dynorphin 1-8 was recovered following incubation with the hypothalamus, the area least effective at producing [^{3}H][Leu]enkephalin. In contrast, the cortex produced the greatest amount of [^{3}H][Leu]enkephalin, affording 67.0 ± 3.6% of the recoverable activity, with [^{3}H]dynorphin 1-8 accounting for only 17.8 ± 5.0%.

The pattern of metabolism recorded using guinea-pig cerebellum was similar to that observed using rat c.n.s. tissues [Fig. 3.5]. In the absence of enzyme inhibitors, the N-terminal fraction accounted for the largest portion of the recovered radioactivity, 49.5 ± 3.2% after a 10min incubation period, [^{3}H]dynorphin 1-8 contributing a mere 6.3 ± 2.1% to the total amount of recovered radioactivity. Once again following pre-incubation with the enzyme inhibitor cocktail [^{3}H][Leu]enkephalin emerged as the most copious metabolite,

affording 55.9 ± 2.4% of the total recovered radioactivity following a 10 min incubation period [Fig. 3.5].

Peripheral tissues

1.) *Guinea-Pig Myenteric Plexus Longitudinal Muscle (MPLM)*

The production of [^{3}H][Leu]enkephalin from [^{3}H]dynorphin 1-8 was not confined to central nervous system tissue. Thus [^{3}H]dynorphin 1-8 was also degraded by slices of MPLM with a half life of 2.3 ± 0.5min. Following a contact time of 20min only 1.3 ± 0.4% of the added [^{3}H]dynorphin 1-8 was recovered. On the other hand the N-terminal fraction accounted for 96.3 ± 1.5% of the recovered radioactivity [Fig 3.6a]. On pre-incubation with the enzyme inhibitor cocktail the half life of [^{3}H]dynorphin 1-8 increased to 7.0 ± 1.1min. Following a 20min incubation period the level of recoverable [^{3}H]dynorphin 1-8 rose to 14.0 ± 3.8%, whereas the level of N-terminal metabolites decreased to 31.4 ± 4.9%. Once again [^{3}H][Leu]enkephalin emerged as the major metabolite, contributing 41.7 ± 1.0% to the total amount of recovered activity [Fig. 3.6b].

2.) *Rat Heart Tissue*

Rat ventrical tissue displayed a similar pattern of metabolism of [^{3}H]dynorphin 1-8 as that found using c.n.s. tissue [Fig. 3.7]. In the absence of enzyme inhibitors the major site of hydrolysis was the N-terminus of the [^{3}H]dynorphin 1-8 molecule affording an N-terminal fraction accounting for 64.4 ± 7.4% of the recovered radioactivity following a 10min incubation period. [^{3}H][Leu]enkephalin and intact [^{3}H]dynorphin 1-8 contributing only

15.6 ± 2.9% and 16.8 ± 4.8% respectively to the total amount of recovered radioactivity [Fig. 3.7]. Preincubation of the ventrical tissue with the enzyme inhibitor cocktail increased the amount of recoverable [^{3}H][Leu]enkephalin to 55.2 ± 2.5% and [^{3}H]dynorphin 1-8 to 19.6 ± 2.4%..The N-terminal fraction decreased to represent 13.9 ± 0.9% of recovered activity.

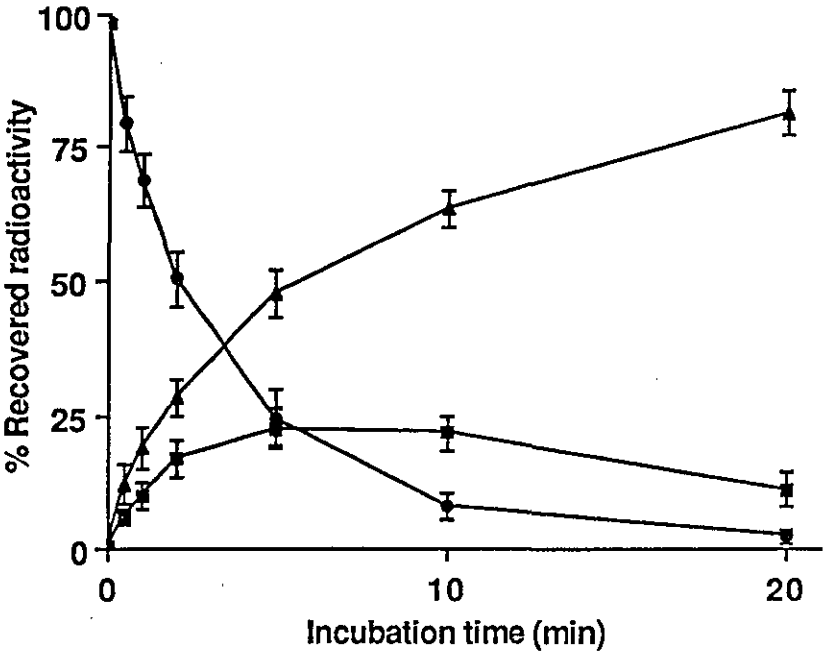


Figure 3.1a : Time course of the metabolism of [^{3}H]dynorphin 1-8 by slices of rat lumbo-sacral spinal cord. [^{3}H]dynorphin 1-8 (circles); [^{3}H][Leu]enkephalin (squares); [^{3}H]N-terminal fraction (triangles) [n=6]

Values represent mean ± standard error of mean

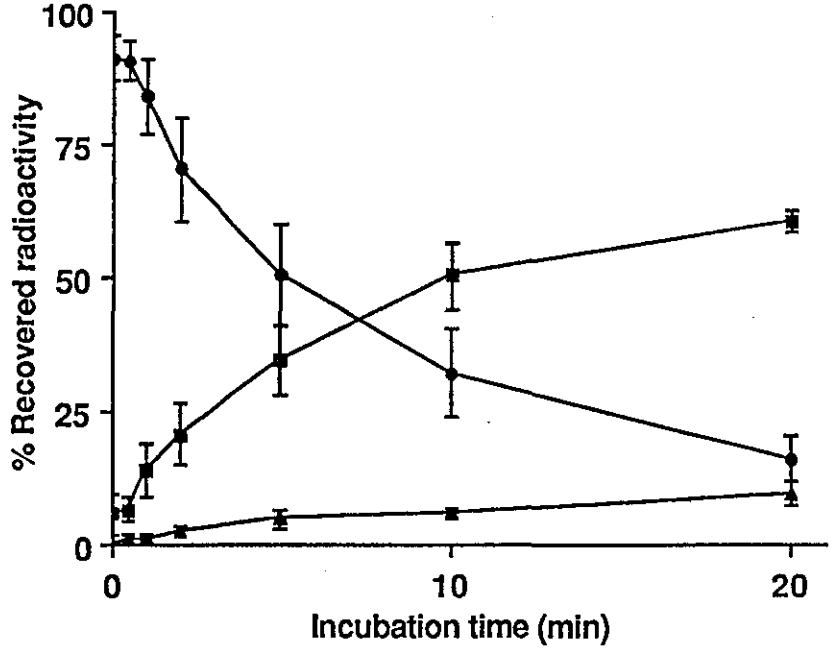


Figure 3.1b : Metabolism of [^{3}H]dynorphin 1-8 by slices of rat lumbo-sacral spinal cord following a 30 min preincubation with the enzyme inhibitory cocktail (bestatin 10μM, captopril 10μM, thiorphan 0.3μM). [^{3}H]dynorphin 1-8 (circles), [^{3}H][Leu]enkephalin (squares), [^{3}H]N-terminal (triangles) [n=5]

Values represent mean ± standard error of mean

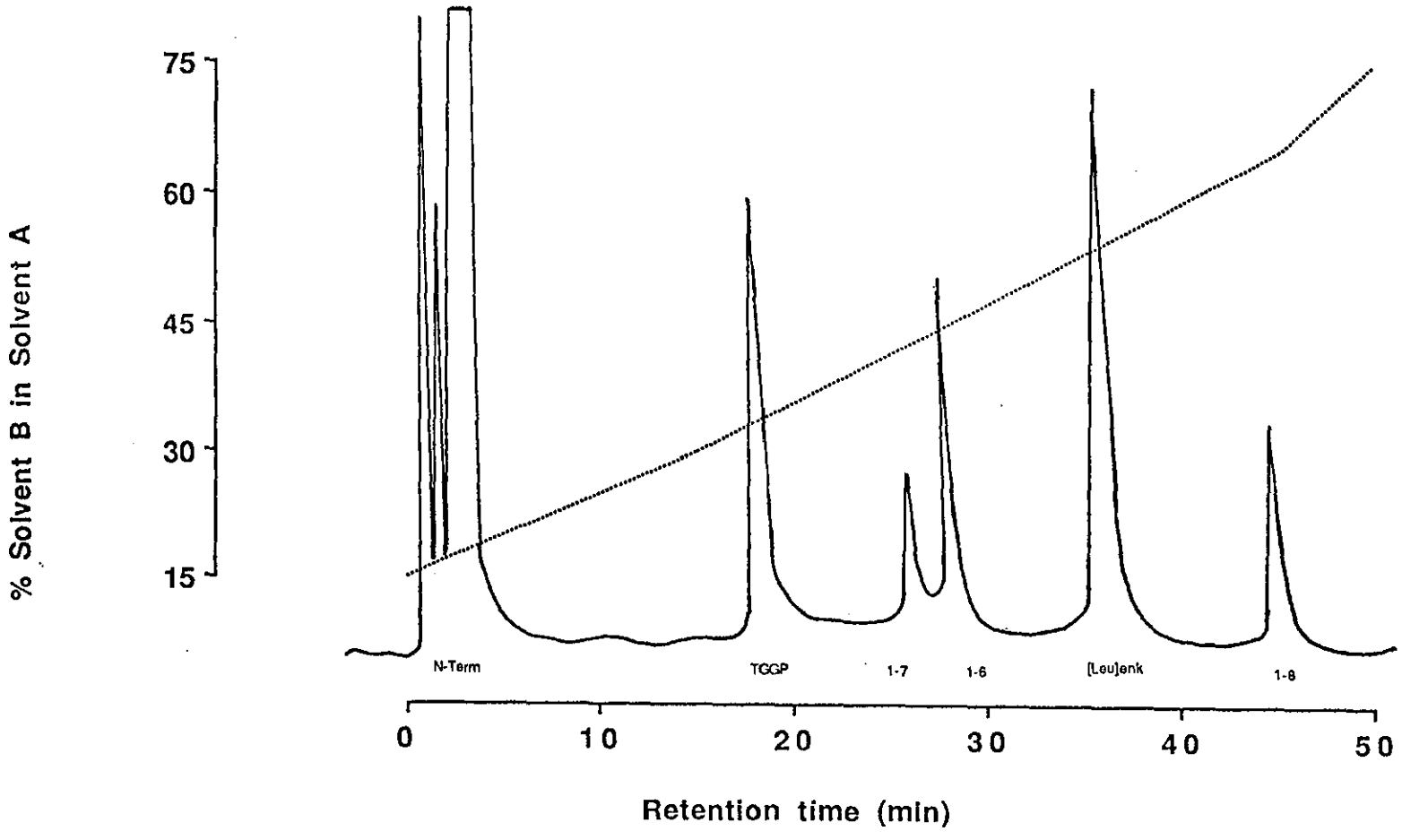


Figure 3.2 : HPLC trace (solid line) showing the separation of the metabolites of dynorphin 1-8. The solvent gradient is shown by the dotted line. N-terminal fraction consists of tyrosine, tyrosyl-glycine, tyrosyl-glycyl-glycine.

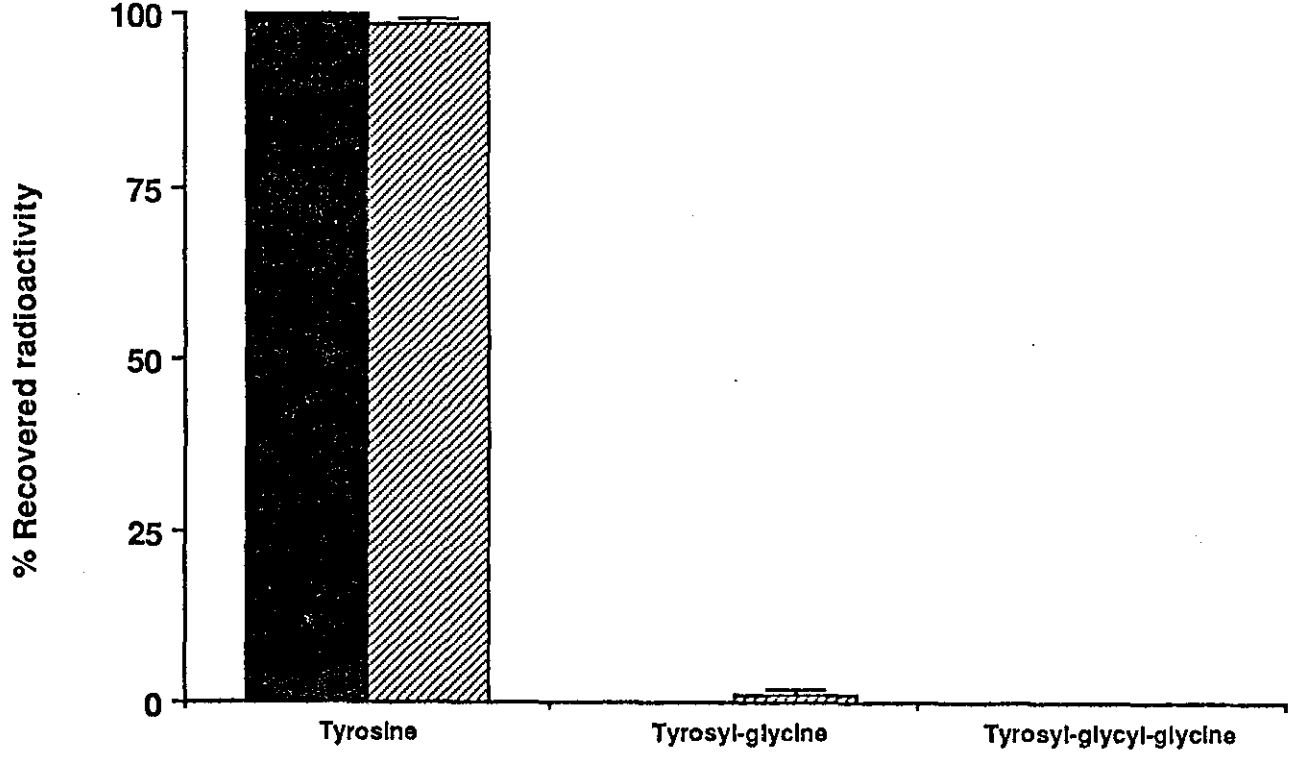


Figure 3.3 : Further separation of the [^{3}H]N-terminal fraction of [^{3}H]dynorphin 1-8 following its metabolism by rat spinal cord [n=3]

Values represent mean ± standard error of mean

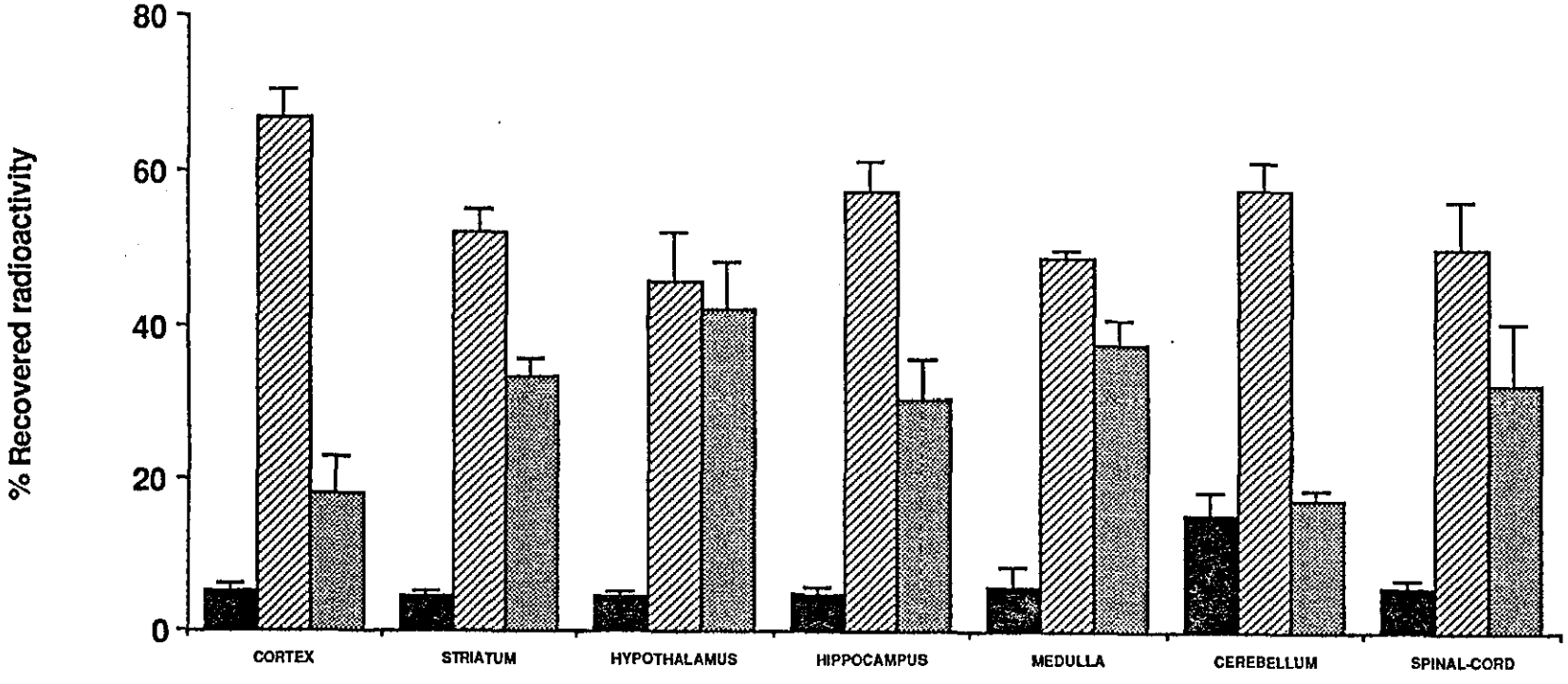


Figure 3.4 : Metabolism of [^{3}H]dynorphin 1-8 following a 10 min incubation with various regions of rat c.n.s.preincubated (30 min) with the enzyme inhibitory cocktail (bestatin 10μM, captopril 10μM and thiorphan 0.3μM). [^{3}H]dynorphin 1-8 (stippled column), [^{3}H][Leu]enkephalin (hatched column), [^{3}H]N-terminal fraction (solid column) [n=4]

Values represent mean ± standard error of mean

For weights of each region see Table 2.1

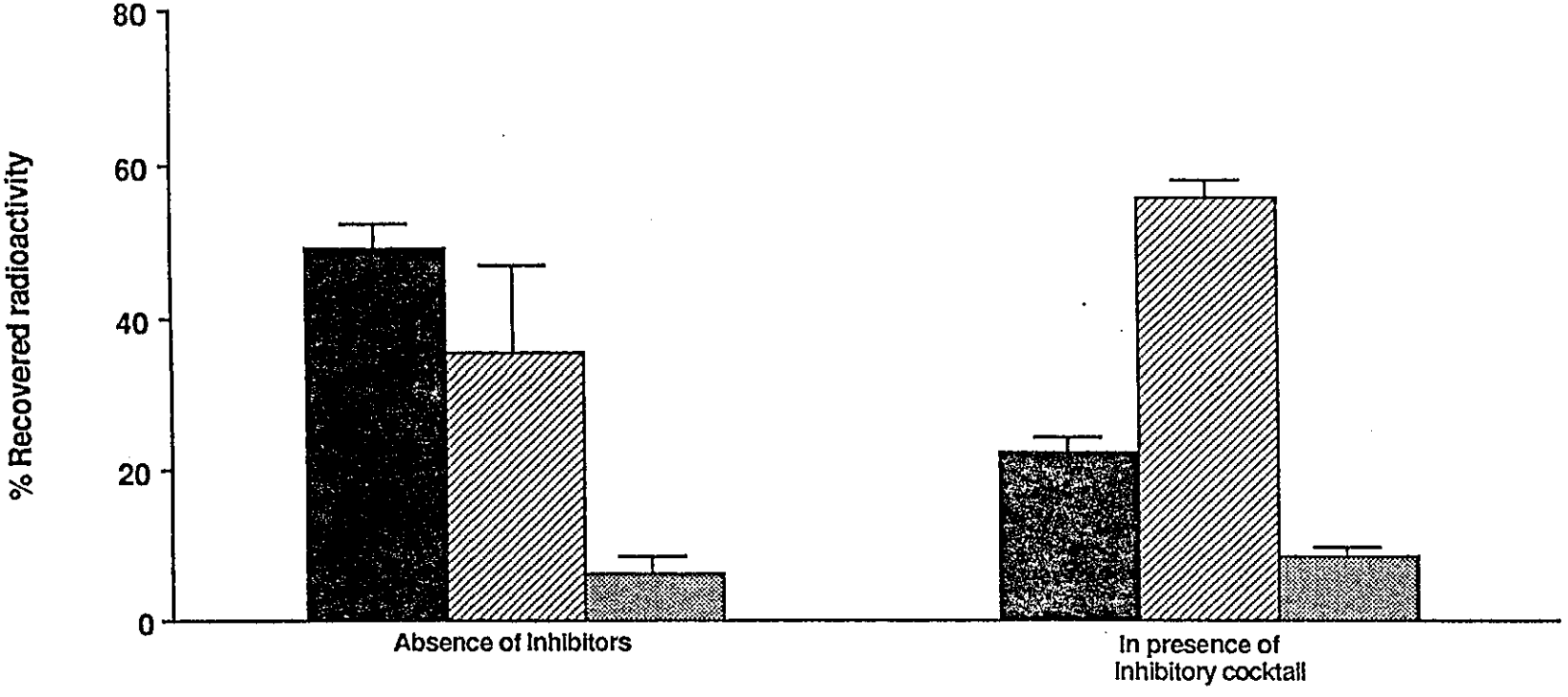


Figure 3.5 : Metabolism of [^{3}H]dynorphin 1-8 following a 10 min incubation with slices of guinea-pig cerebellum in the absence of and presence of the enzyme inhibitory cocktail (bestatin 10μM, captopril 10μM and thiorphan 0.3μM) preincubated with the tissue for 30 min. [^{3}H]dynorphin 1-8 (stippled column), [^{3}H][Leu]enkephalin (hatched column), [^{3}H]N-terminal fraction (solid column) [n=9]

Values represent mean ± standard error of mean

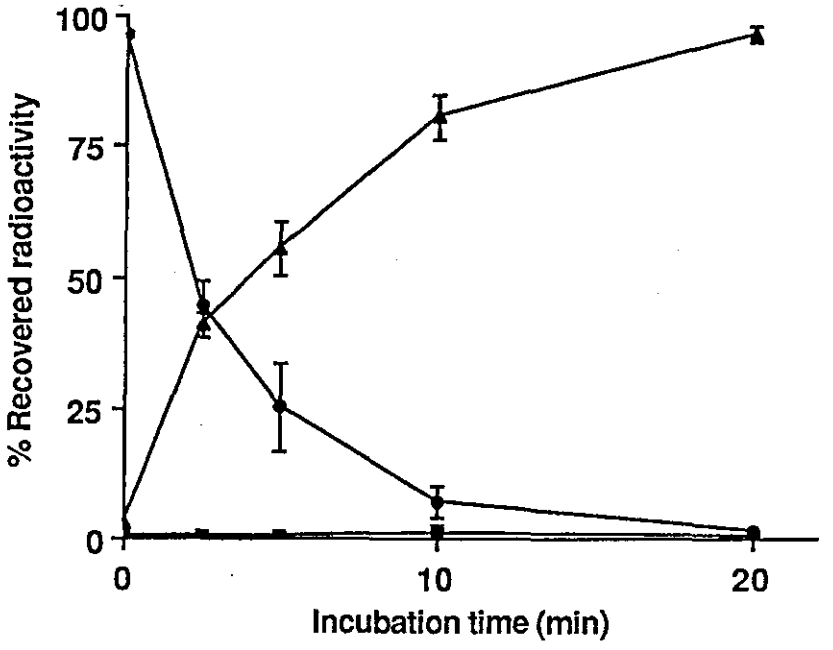


Figure 3.6a : Time course of the metabolism of [^{3}H]dynorphin 1-8 by slices of guinea-pig myenteric plexus longitudinal muscle. [^{3}H]dynorphin 1-8 (circles); [^{3}H][Leu]enkephalin (squares); [^{3}H]N-terminal fraction (triangles) [n=3]

Values represent mean ± standard error of mean

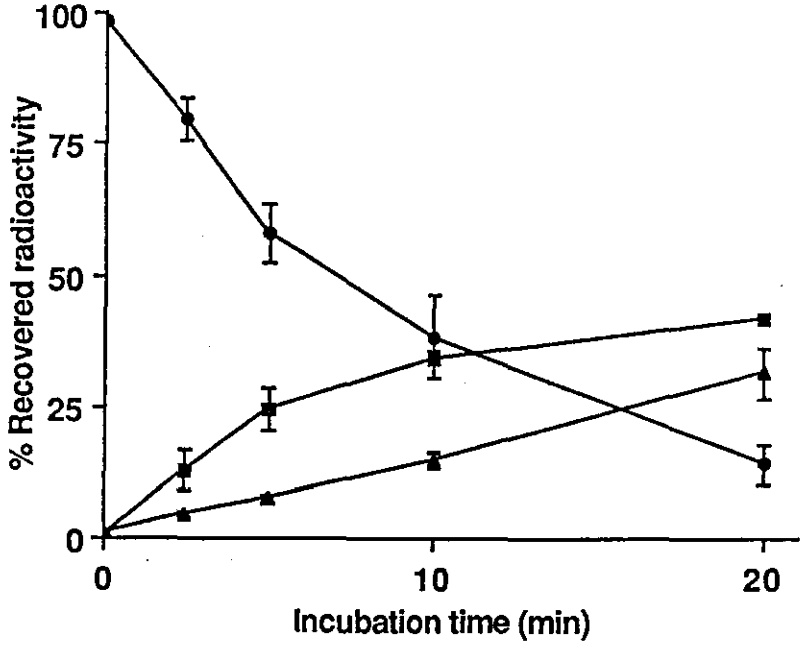


Figure 3.6b : Metabolism of [^{3}H]dynorphin 1-8 by slices of guinea-pig MPLM following a 30 min preincubation with the enzyme inhibitory cocktail (bestatin 10μM, captopril 10μM, thiorphan 0.3μM). [^{3}H]dynorphin 1-8 (circles), [^{3}H][Leu]enkephalin (squares), [^{3}H]N-terminal (triangles) [n=3]

Values represent mean ± standard error of mean

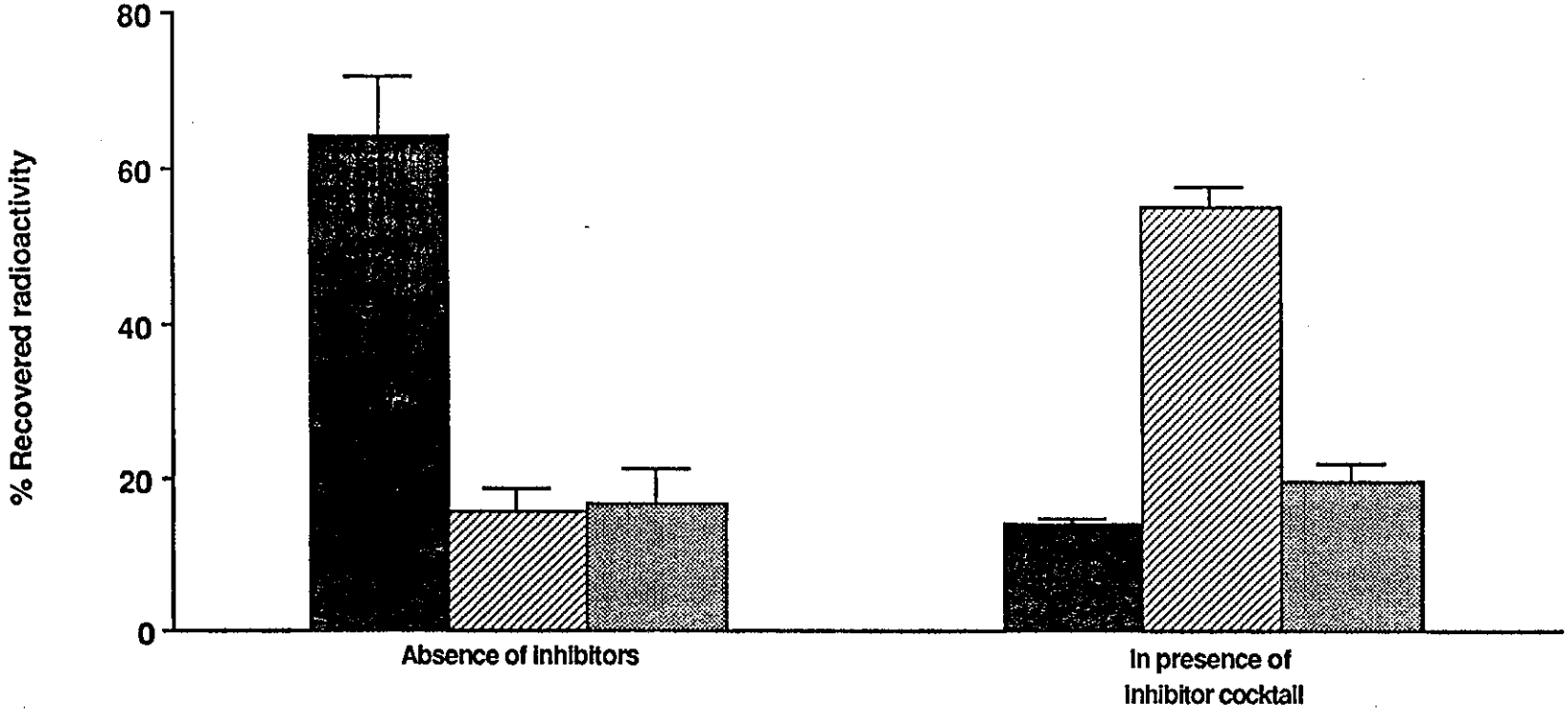


Figure 3.7 : Metabolism of [^{3}H]dynorphin 1-8 following a 10 min incubation with slices of rat ventricle in the absence of and presence of the enzyme inhibitory cocktail (bestatin 10μM, captopril 10μM and thiorphan 0.3μM) preincubated with the tissue for 30 min. [^{3}H]dynorphin 1-8 (stippled column), [^{3}H][Leu]enkephalin (hatched column), [^{3}H]N-terminal fraction (solid column) [n=3]

Values represent mean ± standard error of mean

DISCUSSION

The results presented demonstrate that the peptide dynorphin 1-8 is rapidly broken down by rat spinal cord tissue *in vitro* via hydrolysis of the amide bond between the N-terminal tyrosine and glycine[2]. Previous studies have shown that the hydrolysis of the N-terminus is not limited to the dynorphin 1-8 molecule, indeed a host of opioid peptides appear to be susceptible to attack at this position by aminopeptid .ases [48, 91, 173]. This is an important cleavage site as removal of the N-terminal tyrosine results in a molecule that is unable to bind to any opioid receptor [95].

Previous studies have reported the presence of a dipeptidylaminopeptidase within brain tissue that is capable of releasing Tyr^1-Gly^2 from opioid peptides [25, 53]. However no evidence of such activity was detected under the incubation conditions presented here. This does not, however, discount the possiblity of a rapid turnover of the Tyr^1-Gly^2 fragment to a single tyrosine residue. Similarly no evidence of the hydrolysis of the Gly^3-Phe^4 bond was detected in either the presence or absence of the cocktail of enzyme inhibitors. Previous studies have revealed the activity of a dipeptidylaminopeptidase (E.C 3.4.24.11) that is able to hydrolyse the Gly^4-Phe^5 bond within [Leu] and [Met]enkephalin and similar bonds in a variety of other peptides including substance P, cholesystokinin 8 and somatosatin [11, 33, 98]. However the rapid metabolism, to [^{3}H]tyrosine, of the subsequently released [^{3}H]Tyr^1-Gly^2-Gly^3 fragment again cannot be ruled out.

Removal of the N-terminal portion of dynorphin 1-8 or more extended forms such as dynorphin 1-13 and dynorphin 1-17 results in a molecule that is

incapable of eliciting analgesia [147]. However when administered intrathecally or intracerebroventricularly (i.c.v.) dynorphins display a wide variety of effects in addition to analgesia. Intrathecal application of dynorphin 1-13 or dynorphin 1-17 causes flaccid paralysis of hindlimbs and tail [63, 129], reduced spinal cord blood flow [93], and marked neuropathalogical changes characterised by neuronal loss and necrosis through the central grey matter of the lumbo-sacral spinal cord [94]. Injections of dynorphin 1-17 given i.c.v. produced electroencephalographic changes in the rat [164]. However non of these deletrious actions are the result of an interaction with opioid receptors as high doses of the opioid antagonist naloxone fail to prevent such actions [94, 147]. These non-opioid effects are shared by dynorphin 2-17, dynorphin 2-13 and dynorphin 3-13 [129] indicating that perhaps removal of the N-terminal portion of the molecule is required before the dynorphin is able to elicit such delitrious effects. Smaller dynorphin fragments namely [Leu]enkephalyl-Arg^6-Arg^7 and dynorphin 1-8 do display paralytic effects when administered intrathecally in the presence of peptidase inhibition. However, such paralytic effects appear only when peptidase inhibitors are co-administered, suggesting a rapid metabolism of the molecule is responsible for the lack of effect in the absence of peptidase inhibition. These paralytic effects were not observed following injection of either [Leu]enkephalyl-Arg^6 or [Leu]enkephalin in either the presence or absence of peptidase inhibitors [94], though these compounds, in the presence of peptidase inhibitors, display naloxone reversible antinociceptive properties in the tail flick test. This display of naloxone reversible antinociception by [Leu]enkephalyl-Arg^6 and [Leu]enkephalin indicates that their enzymatic degradation was inhibited and their opioid bioactivities were preserved.

Likewise i.c.v. administration of the peptidase inhibitors bestatin, thiorphan and kelatorphan given alone, or in combination at doses shown to inhibit the degradation of the N-terminal portion of the dynorphin molecule, caused a

naloxone reversible increase in the latency time in hot plate and tail flick tests carried out in mice [27, 35, 43, 163]. In addition direct intrathecal application of kelatorphan, bestatin or thiorphan into halothane anaesthetised rats has the ability to reduce the responsiveness of dorsal horn sensory neurons to noxious stimuli applied to cutaneous receptive fields [43]. The effectiveness of intrathecal application of peptidase inhibitors at eliciting naloxone reversible analgesia suggests they are able to potentiate the effects of endogenously released dynorphins or enkephalin by preventing their enzymatic degradation.

The prevention of enzymatic damage to the dynorphin molecule has important physiological implications. Studies involving spinal cord trauma have revealed the level of dynorphin 1-17 to be elevated at the site of injury in rats [38]. As described previously removal of the N-terminus from the dynorphin molecule has paralytic effects perhaps therefore contributing futher spinal injury. However the opioid antagonist naloxone improves the recovery from spinal cord injury in the cat suggesting that elevated dynorphin levels may also act through opioid receptor occupation to slow down or prevent recovery from such trauma [37].

Inclusion of enzyme inhibitors in the incubation medium to protect against both N- and C-terminal attack results in a partial stabilization of the dynorphin 1-8 molecule. This is illustrated by an increase in the half-life of the octapeptide with a concomittant decrease in the level of recoverable [^{3}H]tyrosine following a 20min incubation period. Importantly, under these inhibitory conditions the pentapeptide [^{3}H][leu]enkephalin is the major metabolite contributing more to the total recovered radioactivity than intact [^{3}H]dynorphin 1-8.

Gillan and co-workers [48] described the production of [Leu]enkephalyl-Arg^6 and [Leu]enkephalyl-Arg^6-Arg^7 from dynorphin 1-8, the two peptides

affording 5-10% of the total recovered radioactivity following a 120min incubation with suspensions of guinea-pig brain membranes at 0°C. However in the present studies the sum of both peptides contributed less than 3% to the total recovered activity. Again the rapid turnover of these peptides could explain the discrepency between the two sets of data, this however seems unlikely as one would expect to observe an increase in the level of such metabolites upon the addition of enzyme inhibitors to the incubation medium.

Tissue from each brain region studied was seen to liberate [Leu]enkephalin from dynorphin 1-8. Indeed, when peptidase inhibitors were included in the incubation medium this pentapeptide became the major metabolite. Metabolism of this type, therefore appears not to be confined to areas containing a specific receptor population. However, the ability of all areas of c.n.s. tissue assayed to produce a delta/mu-preferring ligand in [Leu]enkephalin from the kappa-preferring dynorphin 1-8 confers a high degree of flexibility of response on the dynorphinergic system within the c.n.s. and possibly in the periphery. As described in the introduction, a dynorphinergic system of [Leu]enkephalin production has been located in vivo in the neuronal pathway between the stiatum and the substantia nigra in the rat [176].

The apparent lack of a relationship between metabolism and opioid receptor specificity might seem to be confirmed by studies in guinea-pig cerebellum. The guinea-pig cerebellum contains an opioid receptor population of which 80% is of the kappa type [15]. The production of the delta-preferring [Leu]enkephalin from dynorphin 1-8 by this tissue is perhaps therefore somewhat surprising if a specific role for this metabolism is envisaged. However recent evidence suggests that the guinea-pig cerebellum contains a low population of mu receptors in addition to the kappa receptor population [131]. The ability of this tissue to produce [Leu]enkephalin, which can interact at the mu receptor [95, 96]

may therefore again serve to confer a level of flexibility on dynorphinergic systems within the guinea-pig cerebellum. It may be that if a greater degree of refinement can be attained then a relationship between opioid receptor type and location of the enzyme(s) may be observed.

The metabolism of dynorphin 1-8 is not confined to c.n.s. tissue, both the guinea-pig myenteric plexus longitudinal muscle and rat ventrical muscle were able to produce the pentapeptide from dynorphin 1-8. Previous studies have described the production of [Leu]enkephalin from dynorphin 1-9 by slices of mouse vas deferens in the presence of enzyme inhibitors [108]. In the present study both the myenteric plexus longitudinal muscle of the guinea-pig and rat ventrical muscle are able to degrade dynorphin 1-8 via the cleavage of the N-terminal tyrosine. Again incorporation of the cocktail of enzyme inhibitors into the incubation medium results in the protection of the octapeptide, illustrated in the MPLM by an increase in the half-life of dynorphin 1-8. This protection of the octapeptide is not at the expense of [Leu]enkephalin since, in both tissues, [Leu]enkephalin forms the major metabolite in the presence of peptidase inhibitors.

It would appear, therefore, in all tissue systems investigated that [Leu]enkephalin is formed via a direct cleavage of the Leu^5-Arg^6 bond within the dynorphin 1-8 molecule. The reasons supporting this are twofold:

1.) There is no evidence of a sequential breakdown of dynorphin 1-8 since the metabolic products [Leu]enkephalyl-Arg^6 and [Leu]enkephalyl-Arg^6-Arg^7 represent less than 2% of the total recovered radioactivity under both inhibited and uninhibited conditions, although a rapid turnover of such metabolites cannot be completely ruled out.

2). The production of [Leu]enkephalin is markedly enhanced when the reaction is performed in the presence of enzyme inhibitors designed to prevent

degradation of the N- and C-termini of the octapeptide [107]. These inhibitors in addition to protecting dynorphin 1-8, also appear to stabilize any [Leu]enkephalin formed, thereby substantially increasing the level of recoverable pentapeptide.

In light of these results the production of [Leu]enkephalin from dynorphin 1-8 may be viewed as a conversion process rather than a metabolic inactivation.

CHAPTER 4

Characterisation of the Metabolism of [^{3}H]Dynorphin 1-8

INTRODUCTION

The metabolism of dynorphin 1-8 to [Leu]enkephalin, discussed in Chapter 3, is widespread and apparently the result of a direct hydrolysis of the Leu^5-Arg^6 bond within the octapeptide. A survey of the literature suggests there are two known possible candidate enzymes responsible for this metabolism. The first is a metalloendopeptidase initially isolated from rat brain and classified as EC 3.4.24.15 [119]. The second is a thiol protease designated endo-oligopeptidase A [18]. Both enzymes are able to hydrolyse opioid peptides but are not specific, being able to efficiently hydrolyse a variety of other neuropeptides. As both peptides appear able to liberate [Leu]enkephalin from dynorphin 1-8 it is possible that one or other or a combination of these two enzymes may be responsible for the dynorphinergic production of [Leu]enkephalin.

In the following chapter the nature of the enzyme responsible for the hydrolysis of dynorphin 1-8 to [Leu]enkphalin is examined. Results with a variety of substrates give information on the active site of the enzymic reaction and a series of inhibitors will enable some characterisation of the enzymic reaction. In addition, the ability of two active site directed inhibitors of E.C. 3.4.24.15. to prevent the metabolism of dynorphin 1-8 to [Leu]enkephalin will be studied.

RESULTS

1.) *Peptides as competing substrates*

In order to uncover the optimum substrate length for the enzyme involved in the formation of [Leu]enkephalin the degradation pattern of dynorphin 1-8, in the presence of the enzyme inhibitor cocktail (bestatin 10μM, thiorphan 0.3μM and captopril 10μM) was studied and compared to the pattern of metabolism obtained when, in addition to the above inhibitory cocktail, other opioid peptides were added to the incubation medium as competing substrates. As can be seen from Fig 4.1 the production of [^{3}H][Leu]enkephalin from [^{3}H]dynorphin 1-8 (12nM), by slices of rat lumbar-sacral spinal cord, is inhibited to a varying degree by a variety of opioid peptides added at a concentration of 1μM. The pentapeptides [Leu] and [Met]enkephalin do not significantly inhibit the production of [^{3}H][Leu]enkephalin nor do they increase the percentage of recoverable [^{3}H]dynorphin 1-8. Inhibition of [^{3}H][Leu]enkephalin production however, increases with the length of the opioid peptide added as competitor [Fig. 4.1]. The most potent peptide in this respect is dynorphin 1-13, which inhibits the production of [^{3}H][Leu]enkephalin by 88.8 ± 3.5%. In addition the presence of the peptide dynorphin 1-13 results in a large increase (158.6 ± 14.2%) in the level of recoverable [^{3}H]dynorphin 1-8 compared with the amount of recovered [^{3}H]dynorphin 1-8 in the absence of dynorphin 1-13. In contrast dynorphin 1-17 and the large opioid peptide beta-endorphin have negligible effect on the metabolism of [^{3}H]dynorphin 1-8. Stable analogues of the smaller opioid peptides namely DAGOL (10μM) and DADLE (10μM) also failed to effect the metabolism of [^{3}H]dynorphin 1-8 as did the alkaloid morphine (10μM) and the kappa agonist U50 488H (10μM).

2.) *Other Non-Specific Inhibitors*

A variety of other enzyme inhibitors, preincubated with the spinal cord tissue for 30min at 37°C, were also assayed for their ability to inhibit the production of [^{3}H][Leu]enkephalin from [^{3}H]dynorphin 1-8. These experiments were performed in TRIS buffer to avoid possible interference from metal ions present in Krebs buffer in addition to the usual Krebs/HEPES buffer system. The TRIS buffer did not alter the metabolism of [^{3}H]dynorphin by rat spinal cord.

The metal chelator EDTA (1mM) failed to inhibit the production of [^{3}H][Leu]enkephalin, as did the serine protease inhibitor phenylmethylsulphonylfluoride (PMSF) (1mM). The chelating agent o-phenanthroline (1mM) caused a 28.2 ± 8.6% inhibition of [^{3}H][Leu]enkephalin production [Fig. 4.2]. However this inhibitory effect may not be due to metal chelation because repeating the experiment in Krebs/Hepes buffer also resulted in an inhibition of [^{3}H][Leu]enkephalin production [Fig. 4.2]. The thiol blocking agents N-ethylmalemide (NEM) (1mM) and p-hydroxymercuribenzoate (pHMB) (0.2mM) caused a reduction in [^{3}H][Leu]enkephalin that was equieffective in both TRIS and Krebs/Hepes buffer systems, p CMB being the most effective, 0.2mM resulting in a 93% inhibition of metabolism [Fig. 4.2].

3.) *Site Directed Inhibitors*

Preincubation of spinal cord tissue, at 37°C for 30min, with the site directed inhibitor of EC 3.4.24.15, namely N-[(RS)-carboxy-2-phenylethyl]Ala-Ala-Phe-pAB (N-[(RS)-carboxy-2-phenylethyl]Ala-Ala-Phe-pAB) resulted in a concentration dependent inhibition of dynorphin breakdown [Fig. 4.3a]. A

concentration of 100μM inhibited [^{3}H][Leu]enkephalin production by 96.7%. Preincubation with a more recently synthesized inhibitor of EC 3.4.24.15, namely N-[(RS)-carboxy-3-phenylpropyl]Ala-Ala-Phe-pAB (N-[(RS)-carboxy-3-phenylpropyl]Ala-Ala-Phe-pAB) resulted in a far more potent inhibition of metabolism. Thus at a concentration of 0.3μM a 90.2 ± 2.8% inhibition of [^{3}H][Leu]enkephalin was seen, the amount of recovered [^{3}H]dynorphin 1-8 increasing from 34.3 ± 7.3% to 90.1 ± 3.1% [Fig. 4.3b].

Production of [^{3}H][leu]enkephalin from [^{3}H]dynorphin 1-8 by peripheral tissue was also inhibited by the site directed inhibitor N-[(RS)-carboxy-3-phenylpropyl]Ala-Ala-Phe-pAB. Preincubation of guinea-pig MPLM with the inhibitor at a concentration of 0.3μM caused a 93.5 ± 1.9% inhibition of [^{3}H][Leu]enkephalin production after a 20min incubation period [Fig. 4.4]. Likewise the production of [^{3}H][Leu]enkephalin by rat ventrical muscle was almost completely inhibited by 0.3uM N-[(RS)-carboxy-3-phenylpropyl]Ala-Ala-Phe-pAB [Fig. 4.5]. The percentage of recoverable [^{3}H]dynorphin 1-8 increasing from 19.6 ± 2.4% to 86.7 ± 1.8% on the addition of 0.3μM N-[(RS)-carboxy-3-phenylpropyl]Ala-Ala-Phe-pAB to the incubation medium.

A large overall protection of [^{3}H]dynorphin 1-8 can thus be obtained when the protease enzymes within the metabolising tissue are inhibited with the site directed inhibitor N-[(RS)-carboxy-3-phenylpropyl]Ala-Ala-Phe-pAB in addition to the standard enzyme inhibitor cocktail of bestatin, captopril and thiorphan [Fig. 4.6].

Figure 4.1 : Key to abbreviations

[Leu]enk.....	[leu]enkephalin
[Met]enk.....	[met]enkephalin
1-6.....	[leu]enkephalyl-Arg^{6}
1-7.....	[leu]enkephalyl-Arg^{6}-Arg^{7}
1-8.....	dynorphin 1-8
1-9.....	dynorphin 1-9 (dynorphin 1-8-Iso^{9})
1-13.....	dynorphin 1-13
1-17.....	dynorphin 1-17
[Met]enkAGL.....	[met]enkephalyl-Arg^{6}-Gly^{7}-Leu^{7}
B-end.....	beta-endorphin.

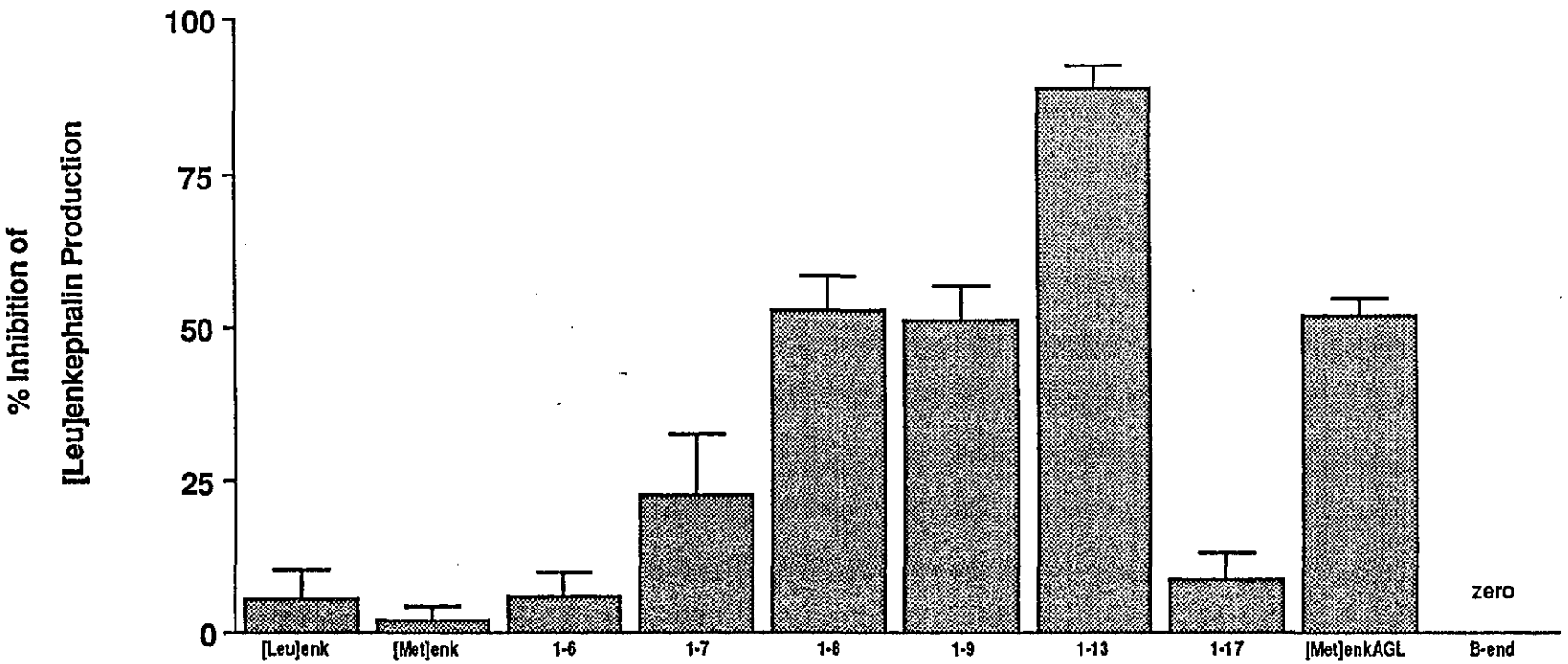


Figure 4.1 : The ability of various opioid peptides to inhibit the production of [^{3}H][Leu]enkephalin from [^{3}H]dynorphin 1-8 following a 10 min incubation with rat spinal cord in the presence of bestatin 10μM, captopril 10μM and thiorphan 0.3μM.

[n=4] Values represent mean ± standard error of mean All peptides were incubated at a concentration of 1μM

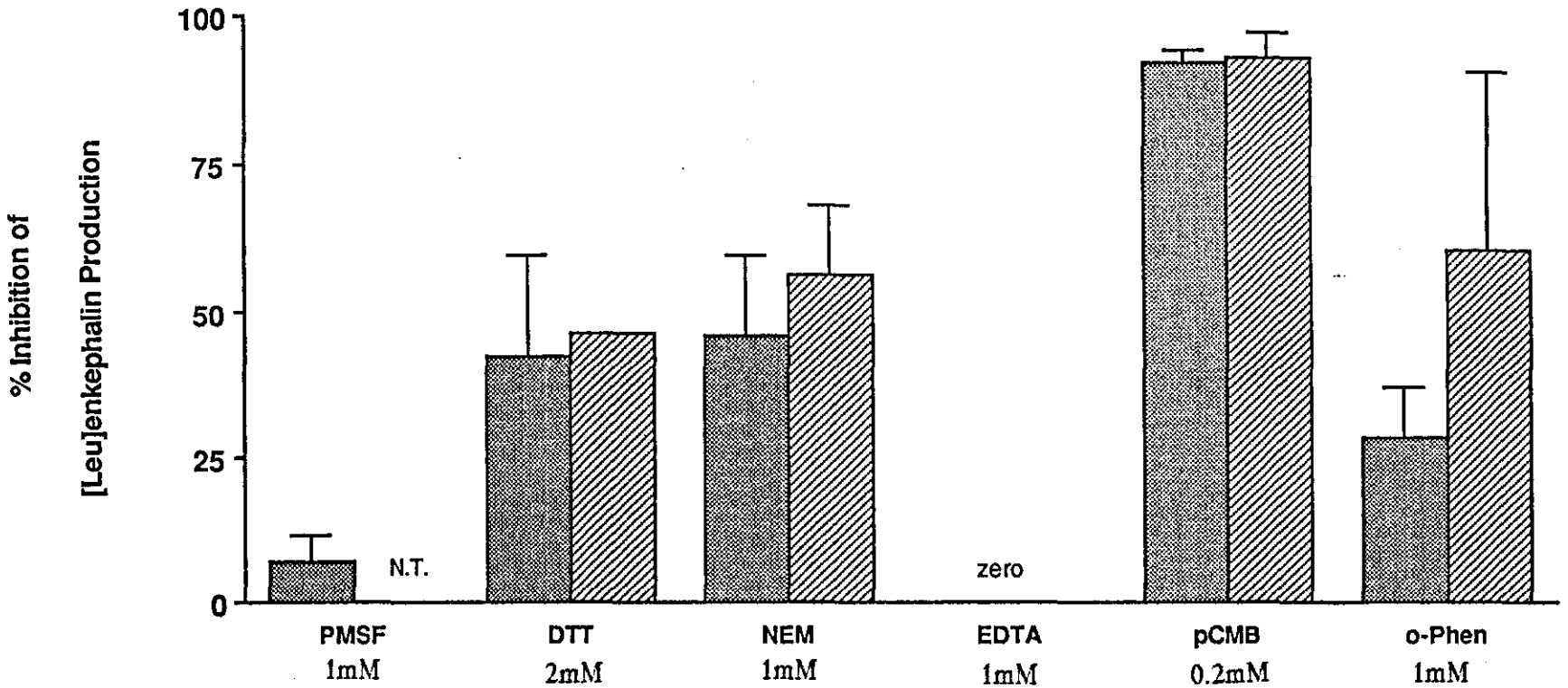


Figure 4.2 : The ability of various chemical enzyme inhibitors to prevent the production of [^{3}H][Leu]enkephalin from [^{3}H]dynorphin 1-8 following a 10 min incubation with slices of rat spinal cord. Experiments were carried out in TRIS buffer (stippled column) and in Krebs buffer (hatched column)

[n=4] Values represent mean ± standard error of mean

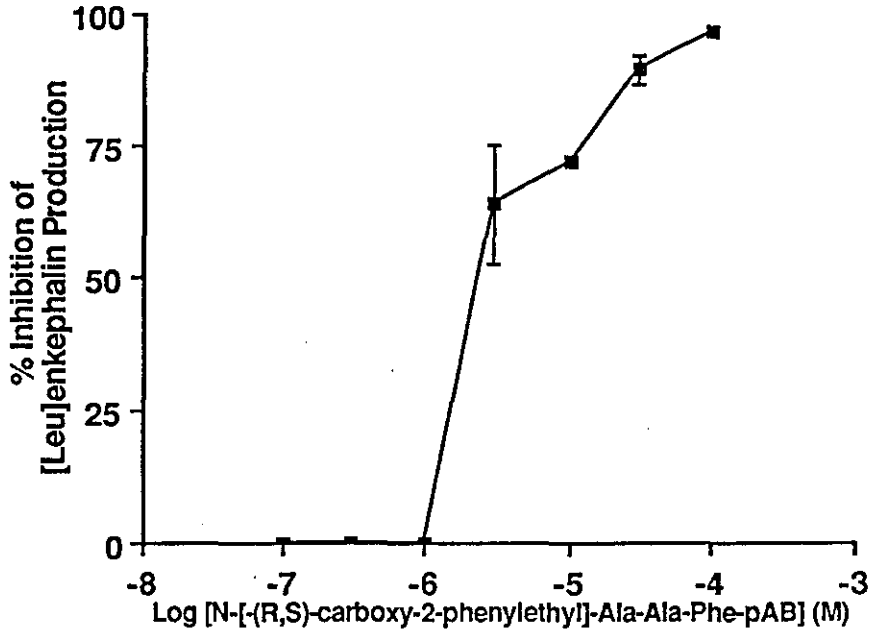


Figure 4.3a : Dose-response study of the ability of the EC 3.4.24.15 inhibitor N-[(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB to inhibit the production of [^{3}H][Leu]enkephalin from [^{3}H]dynorphin 1-8 following a 10 min incubation with rat spinal cord in the presence of bestatin 10μM, captopril 10μM and thiorphan 0.3μM. [n=3 except where error bars are absent when n=2]

Values represent mean ± standard error of mean

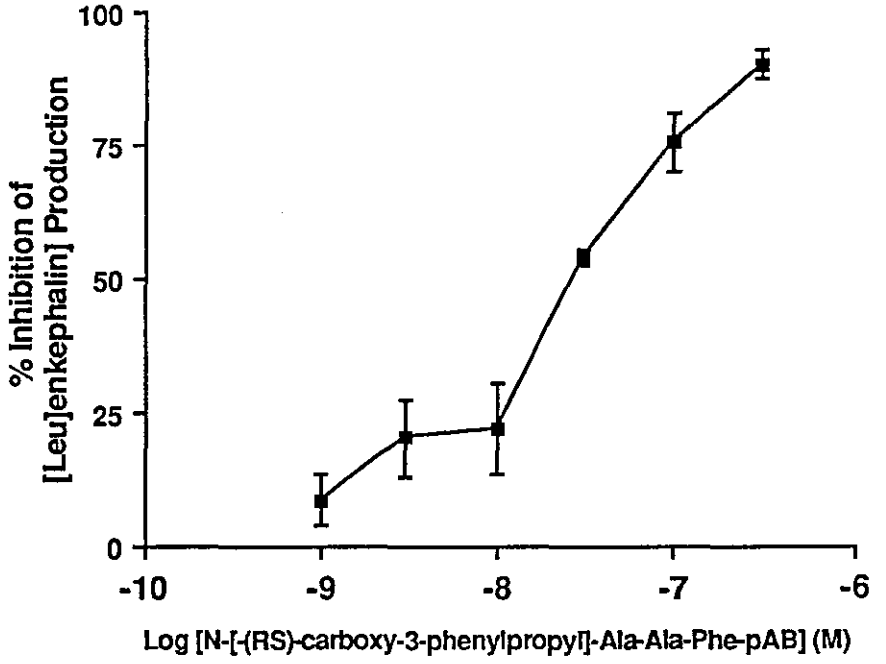


Figure 4.3b : Dose-response study of the ability of the EC 3.4.24.15 inhibitor N-[(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB to inhibit the production of [^{3}H][Leu]enkephalin from [^{3}H]dynorphin 1-8 following a 10 min incubation with rat spinal cord in the presence of bestatin 10μM, captopril 10μM and thiorphan 0.3μM. [n=3]

Values represent mean ± standard error of mean

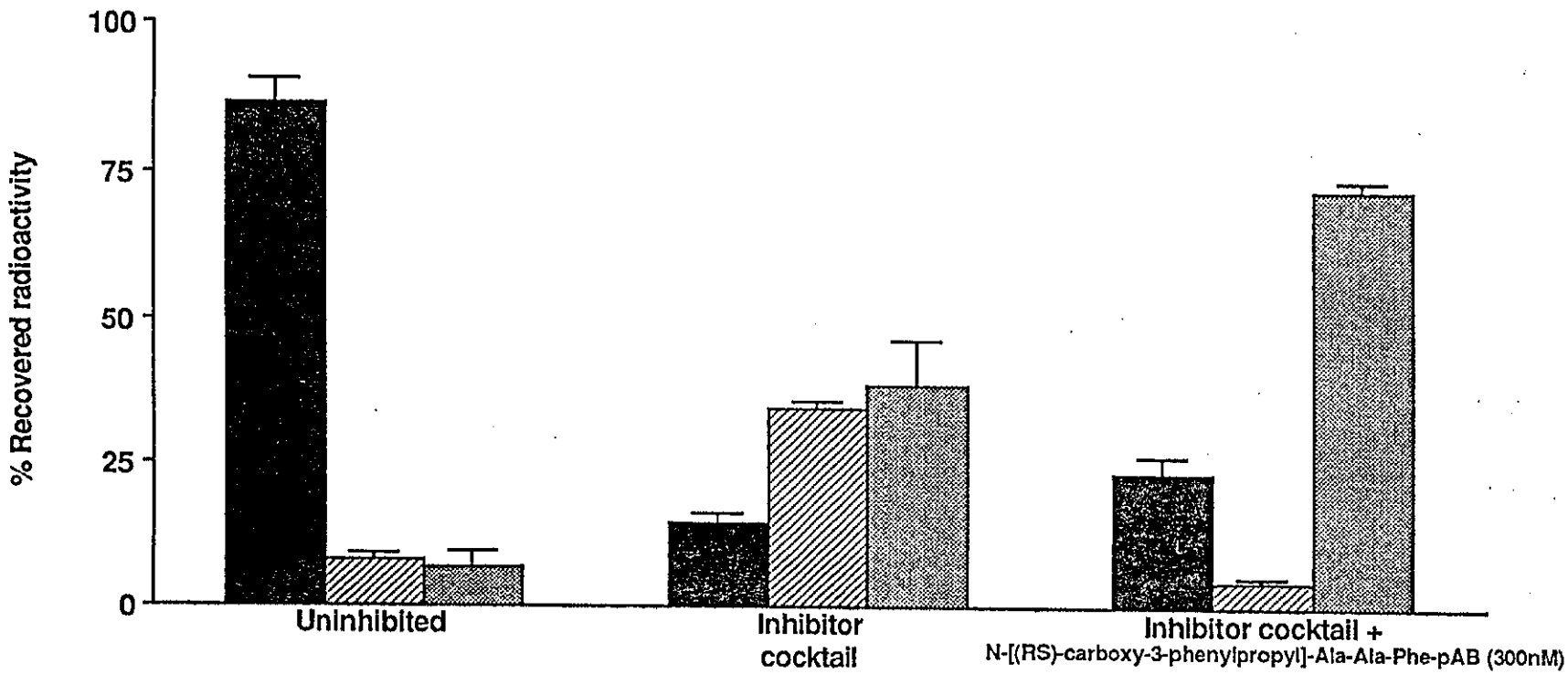


Figure 4.4: The effect of various incubation conditions on the metabolism of [^{3}H]dynorphin 1-8 by guinea-pig MPLM. Inhibitor cocktail consists of bestatin 10μM, captopril 10μM and thiorphan 0.3μM. [^{3}H]dynorphin 1-8 (stippled column), [^{3}H][Leu]enkephalin (hatched columns), [^{3}H]N-terminal fraction (solid column).

Values represent mean ± standard error of mean | n = 3

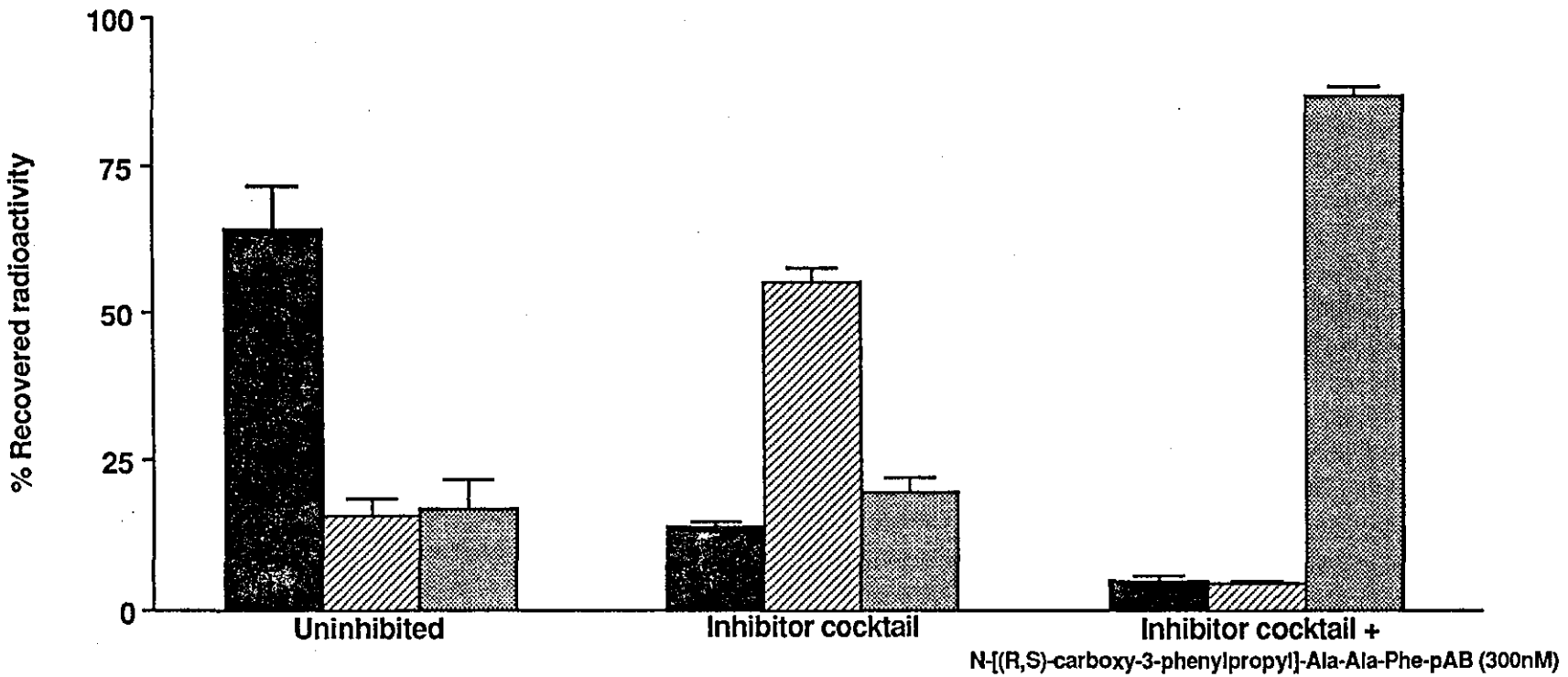


Figure 4.5 : The effect of various incubation conditions on the metabolism of [^{3}H]dynorphin 1-8 by rat ventricle. Inhibitor cocktail consists of bestatin 10μM, captopril 10μM and thiorphan 0.3μM. [^{3}H]dynorphin 1-8 (stippled column), [^{3}H][Leu]enkephalin (hatched columns), [^{3}H]N-terminal fraction (solid column).

Values represent mean ± standard error of mean n = 3

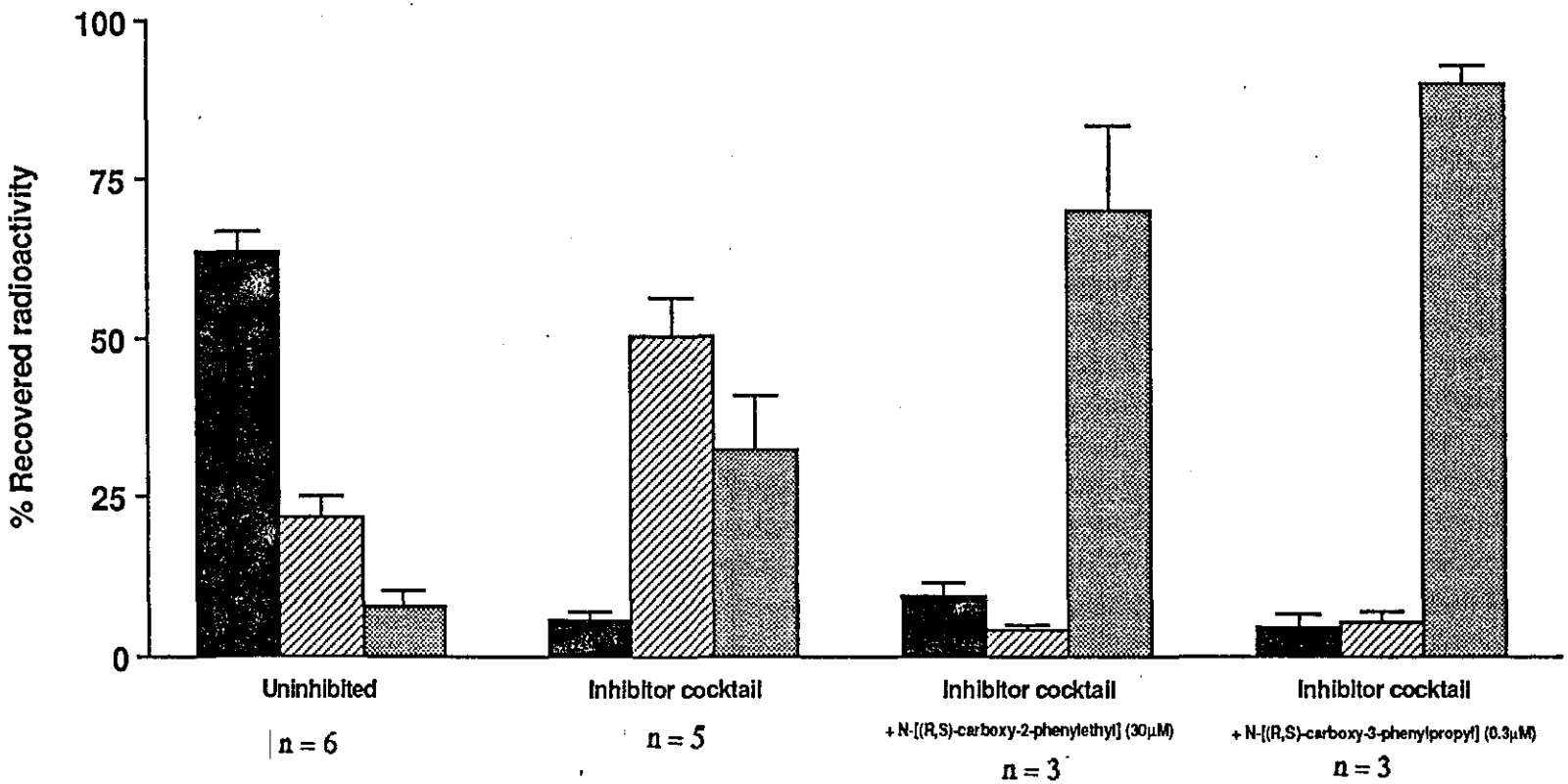


Figure 4.6 : The effect of various incubation conditions on the metabolism of [^{3}H]dynorphin 1-8 by rat spinal cord. Inhibitor cocktail consists of bestatin 10μM, captopril 10μM and thiorphan 0.3μM. [^{3}H]dynorphin 1-8 (stippled column), [^{3}H][Leu]enkephalin (hatched columns), [^{3}H]N-terminal fraction (solid column).

Values represent mean ± standard error of mean

DISCUSSION

Inclusion of the usual cocktail of enzyme inhibitors (bestatin, captopril and thiorphan) in the incubation medium stabilizes [Leu]enkephalin liberated from dynorphin 1-8 in addition to protecting the parent peptide as shown in Chapter 3. These incubation conditions thus allow for the ready assessment of the enzymic activity responsible for the hydrolysis of the Leu^5-Arg^6 bond.

Using competingsubstrates the optimum chain length for recognition by the enzymatic active site was seen to be between 8-13 amino acids. Indeed, dynorphin 1-13 displayed the most potent inhibitory action whilst dynorphin 1-17 was completely inactive as were [Met] and [Leu]enkephalin.

The production of [Leu]enkephalin from dynorphin 1-8 was not affected by the serine protease inhibitor phenylmethylsulphonylfluoride (PMSF) (1mM). It is therefore unlikely that the enzyme presently under investigation is a serine protease. Such an enzyme has been purified by Nyberg and colleagues from human cerebrospinal fluid [114]. This is of interest as it is able to hydrolyse the Arg^6-Arg^7 bond in dynorphin A and dynorphin B and the Arg^6-Lys^7 bond of alpha-neo-endorphin.

However the enzyme studied in this thesis could be similar to a thiol protease purified from rat brain and designated endo-oligopeptidase A [18, 19]. This enzyme is able to hydrolyse a variety of opioid peptides. It reportedly liberates [Met]enkephalin from BAM-12P via cleavage of the Met^5-Arg^6 bond [15]. Similarly it is able to release [Leu]enkephalin fron dynorphin 1-8, dynorphin B, alpha-neoendorphin and beta-neoendorphin through cleavage between amino acid residues 5 and 6. Consistent with the results obtained here, more extended forms of opioid peptides are resistant to hydrolysis, dynorphin 1-17, peptide E,

peptide F and BAM-12P showing no detectable metabolism. In addition the peptides [Met] and [Leu]enkephalin and the hexapeptide, [Leu]enkephalyl-Arg^6 are resistant to hydrolysis. This agrees with the present studies in which the inability of these peptides to inhibit [Leu]enkephalin production from dynorphin 1-8 is observed. The four fold increase in inhibitory effect between [Leu]enkephalyl-Arg^6 and [Leu]enkephalyl-Arg^6-Arg^7 also correlates well with the reported properties of endo-oligopeptidase A. The heptapeptides [Leu]enkephalyl-Arg^6-Arg^7, [Met]enkephalyl-Arg^6-Gly^7 and [Met]enkephalyl-Arg^6-Phe^7 are not converted to their respective pentapeptides by endo-oligopeptidase A but are cleaved at the 4-5 bond releasing Tyr^1-Gly^2-Gly^3-Phe^4. The octapeptide [Met]enkephalyl-Arg^6-Gly^7-Leu^8 is cleaved to give both [Met]enkephalin and Try^1-Gly^2-Gly^3-Phe^4 by purified endo-oligopeptidase A. Extension of [Met]enkephalyl-Arg^6-Gly^7-Leu^8 by a single lysine residue results in [Met]enkephalin being the sole metabolite. It is worth noting that in all cases where [Leu] and [Met]enkephalin are formed, the enkephalin sequence is followed by a pair of basic amino acids. However the primary amino acid sequence of the petides is not alone in determining the cleavage site as the enzyme cleaves a variety of substrates; bradykinin, neurotensin and dynorphin B similarly well [16].

The lack of inhibitory potency of the metal chelators EDTA and o-phenanthroline again suggests the enzyme is similar to endo-oligopeptidase A, as it to is unaffected by EDTA [15], indicating the lack of an essential metal ion. The thiol blocking agents N-ethylmalemide (NEM) and p-hydroxymercuribenzoate (p-HMB) caused a potent inhibition of [Leu]enkephalin production. Again these results are consistent with thiol protease activity, the purified form of endo-oligopeptiase A being strongly inhibited by p-CMB [15]. However endo-oligopeptidase A is activated by dithiothreitol whereas the enzymatic activity responsible for the production of

[Leu]enkephalin in the present system is inhibited by dithiothreitol suggesting differences between the two enzyme systems.

However a second enzyme has been reported that is also able to hydrolyse the Leu^5-Arg^6 bond of dynorphin 1-8. This enzyme was first purified from the soluble fraction of rat brain and designated EC 3.4.24.15, a zinc metalloendopeptidase. [118, 119]. In addition to a soluble form, 20% of the enzymatic activity is tightly associated with synaptosomal membranes [1]. Unlike endo-oligopeptdase A, EC 3.4.24.15 is strongly inhibited by metal chelating agents such as EDTA, EGTA and o-phenanthroline suggesting the presence of an essential metal ion. The lack of effect of EDTA in inhibiting the enzyme currently under investigation would appear to rule out the involvement of a metal ion. However this may not completely hold as EDTA is a relatively weak chelating agent, and therefore this enzyme may, like a variety of other enzymes, require dialysis against EDTA for several hours prior to any indication of inhibition of activity [162]. The variable response produced by o-phenanthroline is a better indication of the absence of an essential metal ion, especially considering o-phenanthroline is also capable of inhibiting enzymatic activity in Krebs buffer. A purified form of EC 3.4.24.15 displays a variable inhibition to thiol blocking agents, whereas the enzyme involved in the present metabolism is strongly inhibited by such compounds. However the site directed inhibitors of EC 3.4.24.15 namely N-[(RS)-carboxy-2-phenylethyl]Ala-Ala-Phe-pAB and N-[(RS)-carboxy-3-phenylpropyl]Ala-Ala-Phe-pAB [26, 120] markedly reduce the level of recoverable [^{3}H][Leu]enkephalin in all tissues tested, whilst increasing the amount of intact [^{3}H]dynorphin 1-8. Both inhibitors are effective against the soluble and membrane bound forms of the enzyme, preventing the hydrolysis of all the shorter opioid peptides to their respective pentapeptides and inhibiting the hydrolysis of other neuropeptides [1]. However the inhibitor N-[-(RS)-carboxy-2-phenylethyl]Ala-Ala-Phe-pAB

appears to have no inhibitory effect against the ability of endo-oligopeptidase A to liberate [Leu]enkephalin from dynorphin 1-8 [154].

In spite of the apparent differences between endo-oligopeptidase A and EC 3.4.24.15 there are a number of similarities. Both cleave the same bond within a variety of neuropeptides: neurotensin, bradykinin, dynorphin 1-8, alpha-neoendorphin, beta-neoendorphin, BAM 12-P and [Met]enkephalyl-Arg^6-Gly^7-Leu^8. Larger peptides such as BAM 22P are resistant to hydrolysis as are the pentapeptides [Leu] and [Met]enkephalin and both enzymes hydrolyse alpha-neoendorphin at a substantially slower rate than that of beta-neoendorphin [116].

Whether or not two separate enzymes are responsible for the hydrolysis of the Leu^5-Arg^6 bond in dynorphin 1-8 is a matter of dispute. A recent publication suggests that only one enzyme is responsible for the production of [Leu]enkephalin from dynorphin 1-8 [154]. Both EC 3.4.24.15 and endo-oligopeptidase A have similar molecular weights and are purified in the same manner. Following Sephadex G-100 gel filtration chromatography the two enzymes are contained within the same fraction. When a specific antibody, raised against endo-oligopeptidase A, is used to remove endo-oligopeptidase A activity within this mixed fraction, the remaining enzymic activity is unable to hydrolyse dynorphin 1-8 or metorphamide to [Leu]enkephalin. However, the remaining enzymic activity is able to hydrolyse the synthetic substrate of EC 3.4.24.15 namely Bz-Gly-Ala-Ala-Phe-pAB, a hydrolysis that was inhibited by N-[(RS)-carboxy-3-phenylpropyl]Ala-Ala-Phe-pAB, indicating the presence of EC 3.4.24.15. This coupled with the observation that the activity accorded to EC 3.4.24.15 displayed a variable inhibition to thiol blocking agents [1, 119] suggests that the ability to hydrolyse dynorphin 1-8 to [Leu]enkephalin was the result of contamination with endo-oligopeptidase A.

Futher anomalies are also evident in the present study. The lack of effect of EDTA questions the involvement of an essential metal ion. However this result is not conclusive as chelating agents vary in their efficacy to remove metal ions from proteins and often long periods of incubation are needed [162]. Likewise although the inhibitory properties of the thiol reagents NEM and pHMB are marked suggesting the involvement of a thiol protease such as endo-oligopeptidase A a definite assignment cannot be made considering endo-oligopeptidase A is activated by dithiothreitol [18] whereas in the present study dithiothreitol is inhibitory. In addition, the inhibitory action of the thiol reagents such as NEM of p-HMB can be ascribed to non-thiol interactions. Certainly NEM is not specific for thiols and will alkylate lysine and histidine residues whereas p-HMB may act non-specifically [85, 86]. It is, therefore, very difficult to ascertain which of the two enzymes is responsible for the hydrolysis of dynorphin 1-8 to [Leu]enkephalin. Indeed in the light of the work carried out by Toffoletto and colleagues, who demonstrated the presence of both enzymes in the same fraction of rat brain it is possible that the observations described above are the result of both metallo- and thiol-protease activity.

CHAPTER 5

Isolated Tissue Studies

INTRODUCTION

In the light of the results presented in the previous two chapters it appears that dynorphin 1-8 is widely and efficiently metabolised to [Leu]enkephalin via a direct hydrolysis of the Leu^5-Arg^6 bond by one or more enzymes. This hydrolysis can therefore be viewed as a conversion process rather than a metabolic inactivation since it generates a bioactive molecule. It is important therefore to investigate the effect of such metabolism on the agonist activity of dynorphin 1-8 in a functional assay. The myenteric plexus longitudinal muscle (MPLM) preparation of the guinea-pig provides an ideal tissue with which to examine the conversion of dynorphin 1-8 to [Leu]enkephalin. The MPLM of the guinea-pig contains only functional kappa and mu receptors and does metabolise dynorphin 1-8. This tissue will therefore allow the effect of the conversion of the kappa preferring dynorphin 1-8 to the delta/mu (in the case of the MPLM mu) preferring [Leu]enkephalin to be monitored.

The ability of dynorphin 1-8 to inhibit the electrically evoked contractions of the guinea-pig MPLM may be investigated using the octapeptide in the presence of various enzyme inhibitors. Construction of full Schild plots using various antagonists and agonists will ascertain which opioid receptor type is responsible for the inhibition of the twitch under all enzyme inhibitor conditions. It is hoped that the results obtained will provide preliminary information concerning what, if any, role is played by the conversion of dynorphin 1-8 to [Leu]enkephalin.

RESULTS

1.) *Naloxone as antagonist*

Dynorphin 1-8 caused a dose dependent inhibition of the electrically evoked contractions of the myenteric plexus longitudinal muscle (MPLM) of the guinea-pig ileum [Fig. 5.1]. The concentration of dynorphin 1-8 required to produce a 50% inhibition of contraction (IC_{50}) was 27.4 ± 3.7nM. Pre-incubation of the MPLM with varying concentrations of the opioid antagonist naloxone prior to addition of dynorphin 1-8 shifted the dynorphin 1-8 dose response curve to the right. From this data a dissociation constant (Ke) for the antagonist of 14.03 ± 2.4nM obtained from the Schild plot, with a slope of unity [Fig. 5.2] could be calculated.

The enzyme inhibitor cocktail of bestatin, 10μM; captopril, 10μM and thiorphan 0.3μM was added to the bath for 30min, to reduce peptidase activity, prior to repeating the dynorphin dose response curve. The IC_{50} of dynorphin 1-8 decreased to 1.8 ± 0.4nM under these conditions. When the effects of naloxone against the agonist activity of dynorphin 1-8 were studied in the presence of the peptidase inhibitory cocktail a concomitant decrease in the value of the naloxone Ke to 3.01 ± 0.79nM was observed. However the slope of the corresponding Schild plot decreased to 0.86 ± 0.08, a value significantly less than unity ($P<0.05$) [Fig. 5.3].

The non-labile mu opioid receptor agonist DAGOL was effective at inhibiting the electrically evoked contractions of the MPLM affording an IC_{50} of 15.2 ± 0.1nM. Repetition of the dose response to DAGOL in the presence of increasing concentrations of naloxone resulted in a shift of the dose-response curve to the right. Determination of the Schild plot from this data gave a Ke of

2.09 ± 0.54nM [Fig. 5.4] with a slope of unity. In contrast to dynorphin 1-8 a 30min preincubation with the enzyme inhibitory cocktail failed to alter either the naloxone Ke 3.07 ± 0.82nM or the IC_{50} 21.3 ± 7.4nM of DAGOL in this tissue. Indeed the value of the naloxone Ke obtained using the agonist DAGOL was not significantly different to that obtained using dynorphin 1-8 as agonist in the presence of the cocktail of enzyme inhibitors. Likewise, the naloxone Ke (1.63 ± 0.34nM) obtained using [Leu]enkephalin as agonist [Fig. 5.5], in the presence of the enzyme inhibitor cocktail, does not differ significantly from the Ke obtained with either DAGOL or dynorphin 1-8 in the presence of peptidase inhibition.

A 30min preincubation of the MPLM with the site directed inhibitor of EC 3.4.24.15, namely N-[-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (30μM) and the inhibitor cocktail of bestatin, 10μM; captopril, 10μM and thiorphan, 0.3μM prior to assaying dynorphin 1-8, did not significantly change the IC_{50} value of dynorphin 1-8 as compared with the IC_{50} value of the octapeptide obtained in the presence of the inhibitory cocktail alone. However under these inhibitory conditions a single dose of naloxone led to a shift to the right of the dose response curve to dynorphin 1-8. From this single dose response curve a single dose Ke [179] was calculated. Under these inhibitory conditions an increase in the naloxone Ke of dynorphin 1-8 from 3.01 ± 0.79nM to 14.31 ± 2.1nM in the absence and presence respectively of the inhibitor N-[-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (30μM) was observed. Replacing N-[-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB in the inhibitory cocktail with the more potent inhibitor N-[-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB (0.3μM) increased further the naloxone Ke against dynorphin 1-8 to 37.2 ± 8.3nM. The slope of the corresponding Schild plot was unity [Fig. 5.6]. Inclusion of the E.C. 3.4.24.15 inhibitor N-[-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (30μM) in the inhibitory

cocktail afforded a naloxone Ke calaculated against the mu agonist DAGOL of 3.0 ± 0.6nM a value not significantly different to the naloxone Ke calculated in the absence of N-[-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB.

Similarly the less labile opioid peptide, dynorphin 1-17, used in the absence of peptidase inhibitors, afforded a naloxone Ke of 33.6 ± 1.02nM with a Schild plot of slope unity [Fig. 5.7]. In addition using the stable kappa preferring U69 593 as agonist a naloxone Ke of 31.4 ± 9.69 nM, again with Schild plot slope of unity was obtained [Fig. 5.8]. The Ke values and the slope of the Schild plots for U69 593 and dynorphin 1-17 do not significantly differ from those values obtained using dynorphin 1-8 in the presence of the inhibitor cocktail and N-[-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB.

2.) *Nor-Binaltorphimine (nor-BNI) as antagonist*

Using the kappa receptor preferring nor-BNI to antagonise the agonist effects of dynorphin 1-8 in the MPLM a Schild plot could be constructed which afforded a Ke value of 0.17 ± 0.04nM and a slope of unity [Fig. 5.9]. In the presence of the inhibitor cocktail of, bestatin 10μM; captopril 10μM and thiorphan 0.3μM, the Ke of dynorphin 1-8 against nor-BNI significantly increased ($P<0.05$) to 0.62 ± 0.07nM, the slope of the Schild plot remained unity [Fig. 5.10]. When used as antagonist against the mu ligand DAGOL the Ke was 13.03 ± 1.91nM, the slope of the Schild plot remained close to unity [Fig. 5.11].

In the presence of both the inhibitor cocktail and N-[-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB (0.3μM), preincubated for 30min prior to the addition of agonist, a Ke for nor-BNI determined against the agonist effects of

dynorphin 1-8 of 0.098 ± 0.047nM with a slope of 0.99 ± 0.12 was found[Fig. 5.12]. Similarly when the agonist U69 593 was used, in the absence of peptidase inhibitors, a low Ke value of 0.067 ± 0.03nM and a slope of unity were obtained [Fig. 5.13].

3.) *M8008 (16methylcyprenorphine) as antagonist*

The Schild plot of M8008 using dynorphin 1-8 as agonist afforded a Ke of 76.9 ± 11.1nM, with a Schild plot slope of unity [Fig. 5.14]. The addition of the inhibitor cocktail of, bestatin 10μM; captopril 10μM and thiorphan 0.3μM to the tissue 30min prior to the addition of dynorphin 1-8 decreased the Ke against M8008 to 31.4 ± 8.8nM, the slope of the corresponding Schild plot was significantly less than unity [Fig. 5.15]. Using the mu receptor preferring agonist DAGOL the Ke obtained for M8008 was 3.5 ± 1.2nM from a Schild plot with a slope of unity [Fig. 5.16].

Addition of the potent inhibitor N-[-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB (0.3μM) to the standard inhibitor cocktail resulted in a large increase in the Ke of M8008 as measured against dynorphin 1-8 to 208.5 ± 70.3nM, again the slope of the Schild plot did not differ from unity [Fig. 5.17]. Similarly the agonist U69 593 afforded a high Ke value of 85.8 ± 14.4nM, with a Schild plot slope of unity [Fig. 5.18].

By analysis of variance (ANOVA followed by Dunnett's test) non of the dose response curves deviated from parallelism. All Schild plot slopes did not differ significantly from unity, except where stated (Mann Whitney U). Each concentration of antagonist produced a significant ($P<0.05$ ANOVA, Dunnett's test) increase in the IC_{50} value of the the agonist compared to the IC_{50} value obtained in the presence of the previous dose of antagonist.

4.) *Irreversible alkylation of the mu-receptors in MPLM by beta-funaltrexamine*

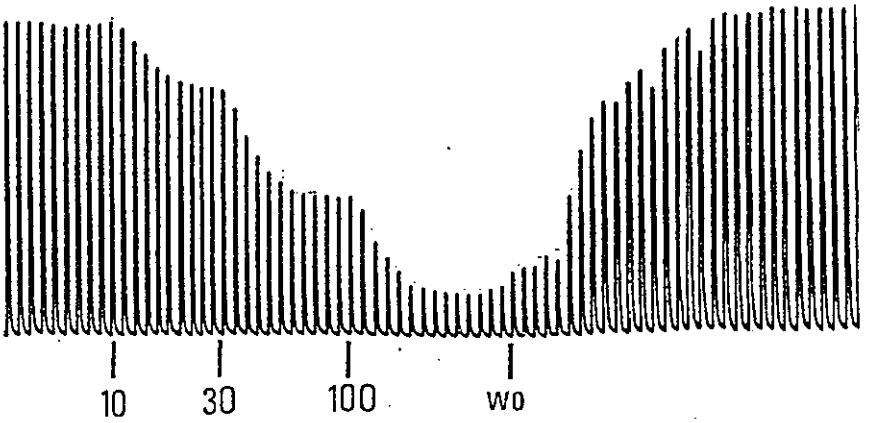
i.) *Standard Compounds*

The mu selective agonist DAGOL was used as a marker to test for successful alkylation. If the ratio of IC_{50} values determined from the dose response curves for DAGOL before and after treatment with 100nM beta-funaltrexamine (beta-FNA), incubated with the tissue for 1hour followed by a minimum of 1hour washout, was less than 20 the experiment was discarded.

As can be seen from Table 5.1 the mean dose ratio for DAGOL was 26.1 ± 7.49. In contrast the IC_{50} for the kappa selective agonist U50 488H was essentially unaffected by beta-FNA treatment affording a dose ratio of 2.1 ± 0.2. The dose response curve to the pentapeptide [Leu]enkephalin, in the presence of the same inhibitor cocktail, was greatly affected by beta-FNA treatment displaying a dose-ratio of 15.9.

ii.) *Dynorphin 1-8*

The potency of dynorphin 1-8 to inhibit the MPLM was unaffected by beta-FNA alkylation, affording a dose-ratio of 0.61 ± 0.08. However, upon the addition of the enzyme inhibitor cocktail of bestatin, 10μM; captopril, 10μM and thiorphan, 0.3μM, the IC_{50} after alkylation shifted to the right affording a dose-ratio of 1.57 ± 0.27, a value not significantly different to that of uninhibited dynorphin 1-8. Inclusion of the site directed inhibitor N-[-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB (0.3μM) in the inhibitor cocktail significantly reduced ($P<0.05$) the dose-ratio of dynorphin 1-8 to 0.80 ± 0.05 again this value is not significantly different to that obtained using dynorphin 1-8 in the absence of any peptidase inhibition.



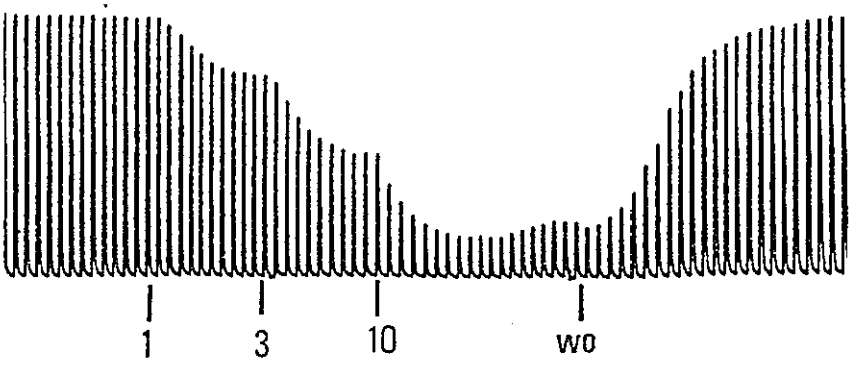


Figure 5.1 : Inhibition of the electrically induced contractions of the myenteric plexus longitudinal muscle of the guinea-pig by dynorphin 1-8 (upper trace) and dynorphin 1-8 in the presence of bestatin, 10μM; captopril, 10μM and thiorphan, 0.3μM (lower trace). Numbers are concentration of dynorphin 1-8 (nM).
wo = wash out

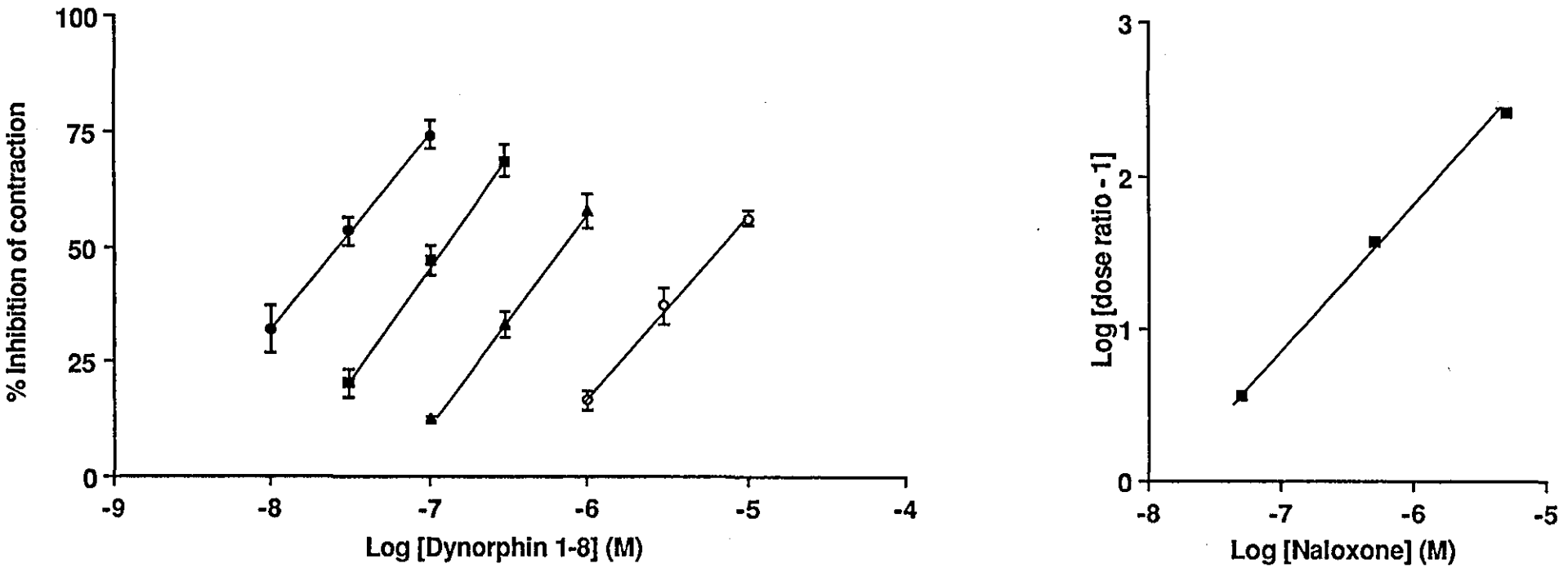


Figure 5.2 : Inhibition of electrically induced contractions of the myenteric plexus longitudinal muscle of the guinea-pig by dynorphin 1-8 in the abscence (●) and in the presence of 50nM (■), 500nM (▲) and 5000nM (○) naloxone. Sample of a corresponding Schild plot (■) shown on the right.

[n=19]

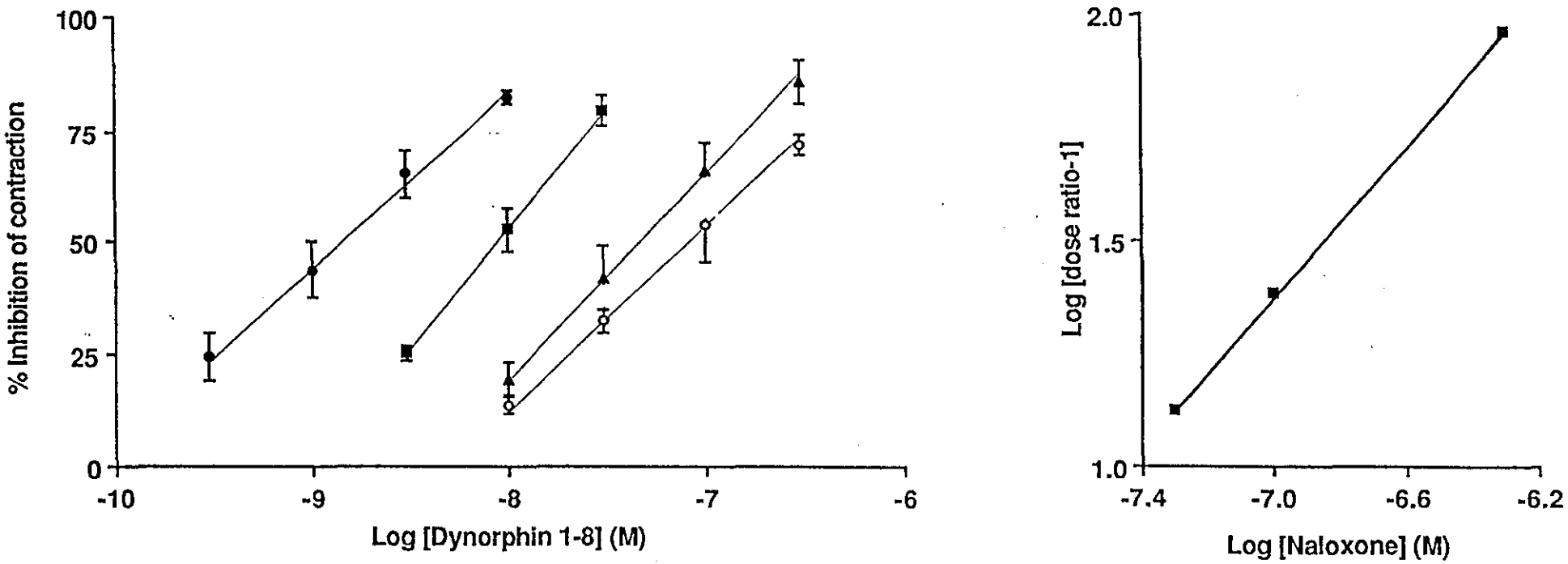
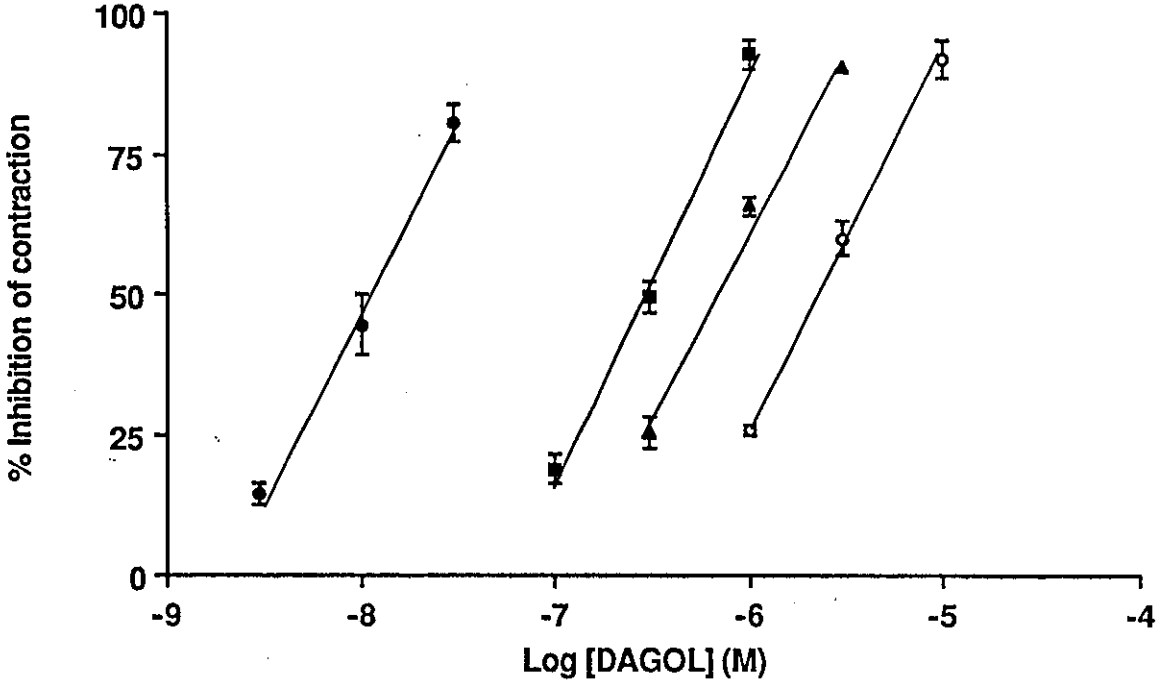


Figure 5.3 : Inhibition of electrically induced contractions of the myenteric plexus longitudinal muscle of the guinea-pig, in the presence of bestatin, 10μM; captopril, 10μM and thiorphan, 0.3μM by dynorphin 1-8 in the abscence (●) and in the presence of 50nM (■), 100nM (▲) and 500nM (○) naloxone. Sample of a corresponding Schild plot (■) shown on the right.

[n=9]



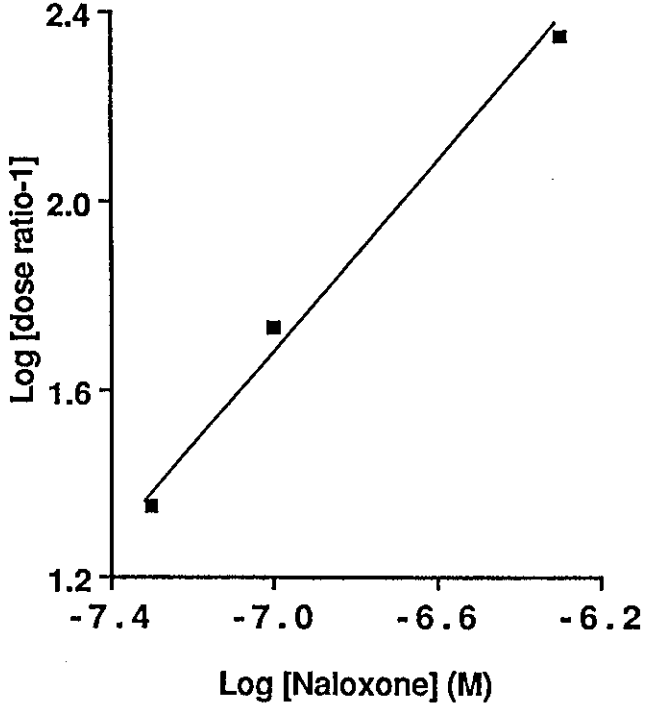


Figure 5.4 : Inhibition of electrically induced contractions of the myenteric plexus longitudinal muscle of the guinea-pig by DAGOL in the abscence (●) and in the presence of 50nM (■), 100nM (▲) and 500nM (○) naloxone. Sample of a corresponding Schild plot (■) shown on the right.

[n=3]

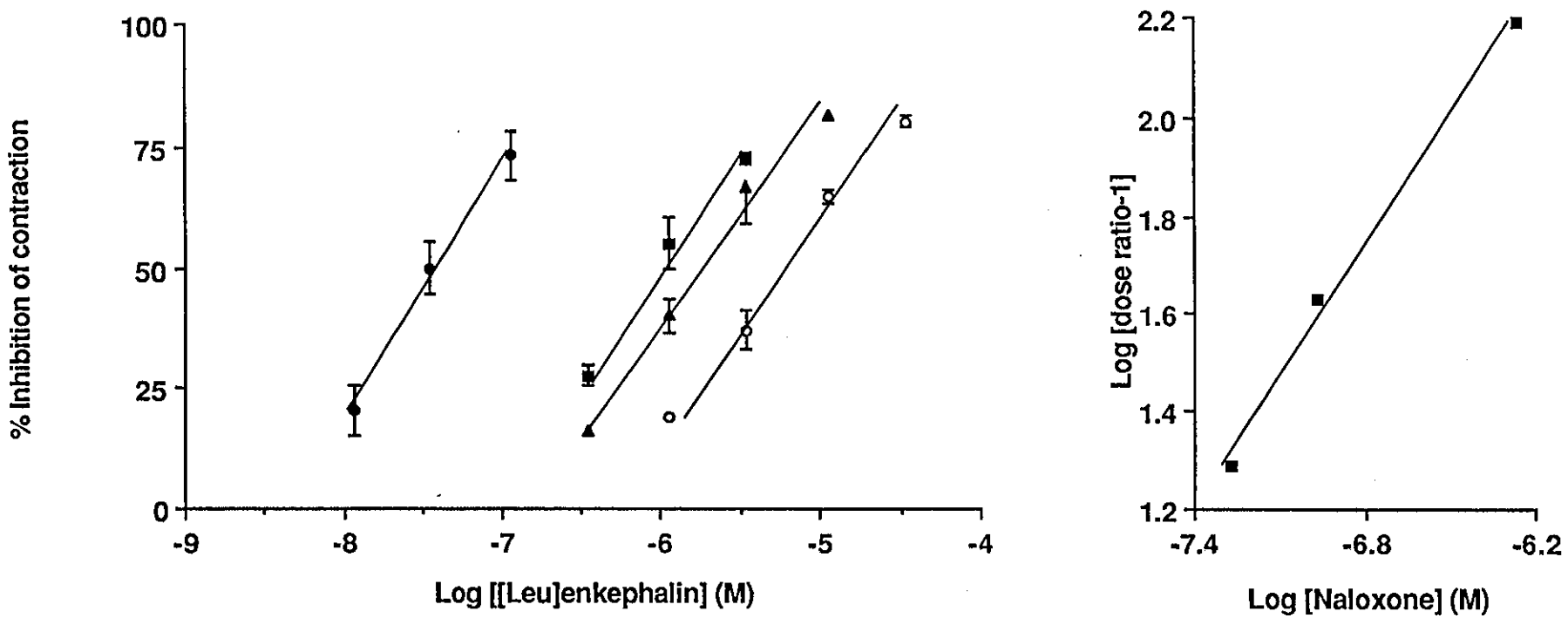


Figure 5.5 : Inhibition of electrically induced contractions of the myenteric plexus longitudinal muscle of the guinea pig, in the presence of bestatin, 10μM; captopril, 10μM and thiorphan, 0.3μM by [Leu]enkephalin in the absence (●) and in the presence of 50nM (■), 100nM (▲) and 500nM (○) naloxone. Sample of a corresponding Schild plot (■) shown on the right.

[n=8]

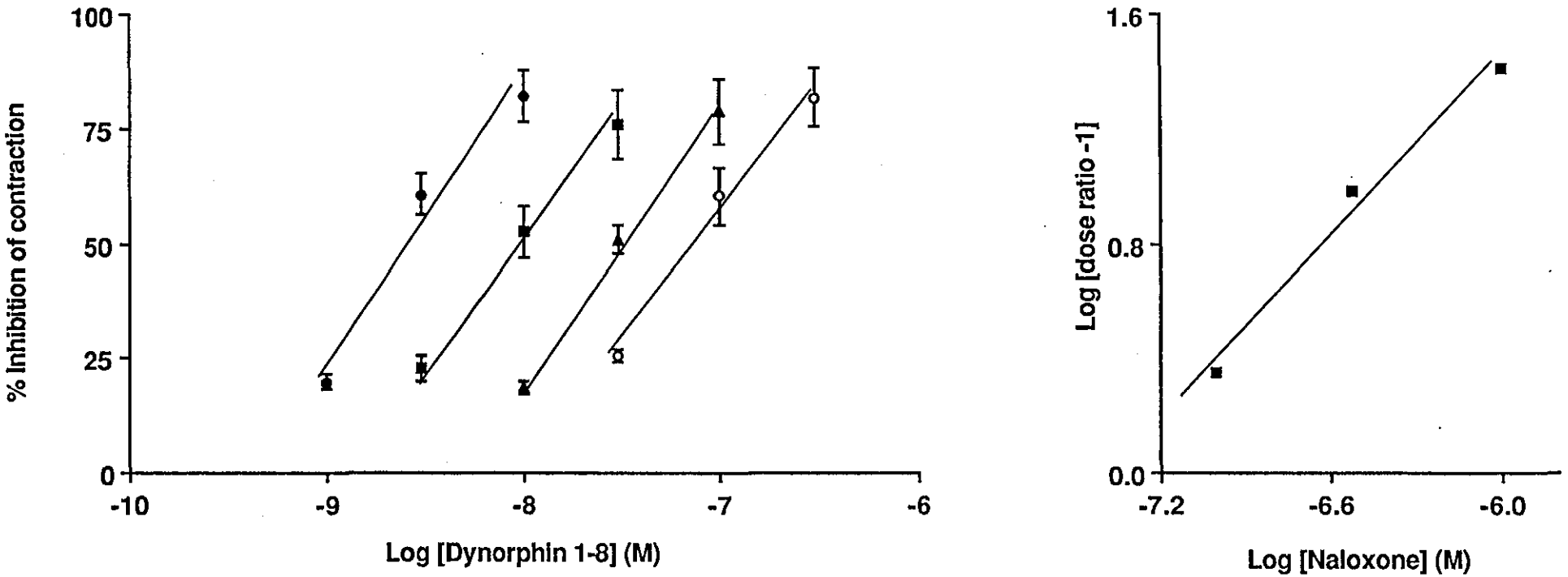


Figure 5.6 : Inhibition of electrically induced contractions of the myenteric plexus longitudinal muscle of the guinea-pig, in the presence of bestatin, 10μM; captopril, 10μM; thiorphan, 0.3μM and N-[(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB, 0.3μM, by dynorphin 1-8 in the abscence (●) and in the presence of 100nM (■), 300nM (▲) and 1000nM (○) naloxone. Sample of a corresponding Schild plot (■) shown on the right.

[n=5]

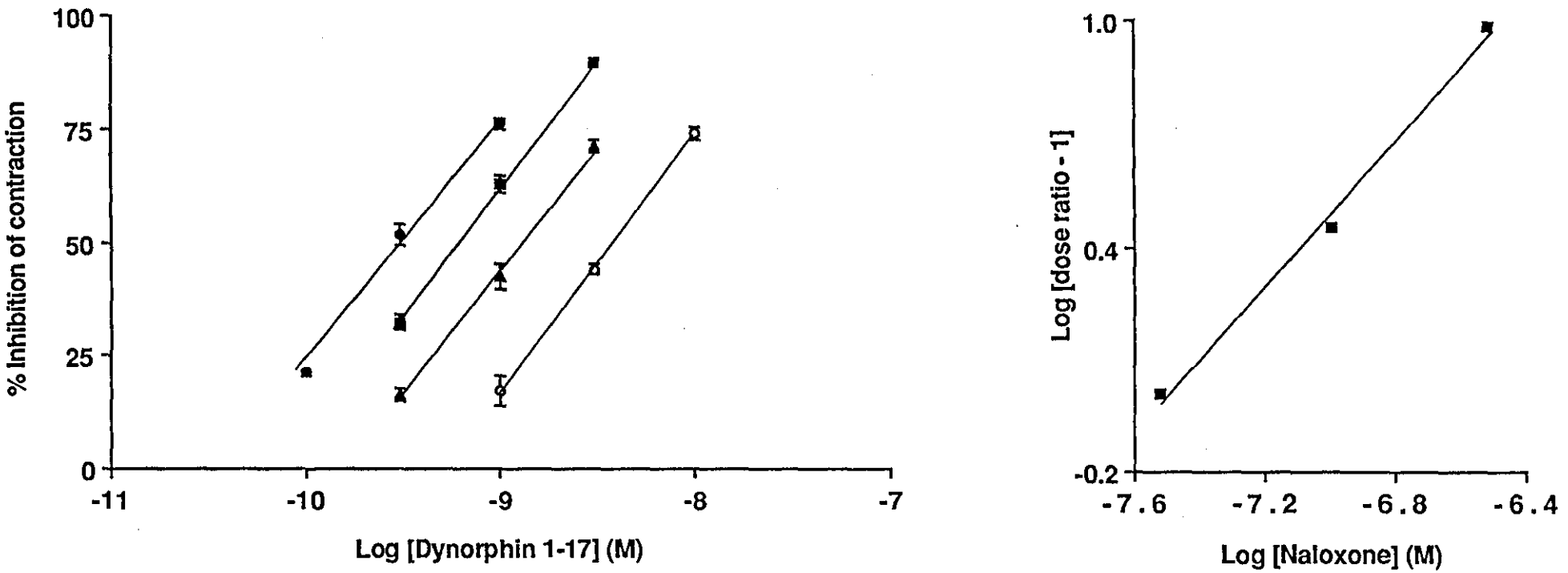


Figure 5.7 : Inhibition of electrically induced contractions of the myenteric plexus longitudinal muscle of the guinea-pig by dynorphin 1-17 in the abscence (●) and in the presence of 30nM (■), 100nM (▲) and 300nM (○) naloxone. Sample of a corresponding Schild plot (■) shown on the right.

[n=4]

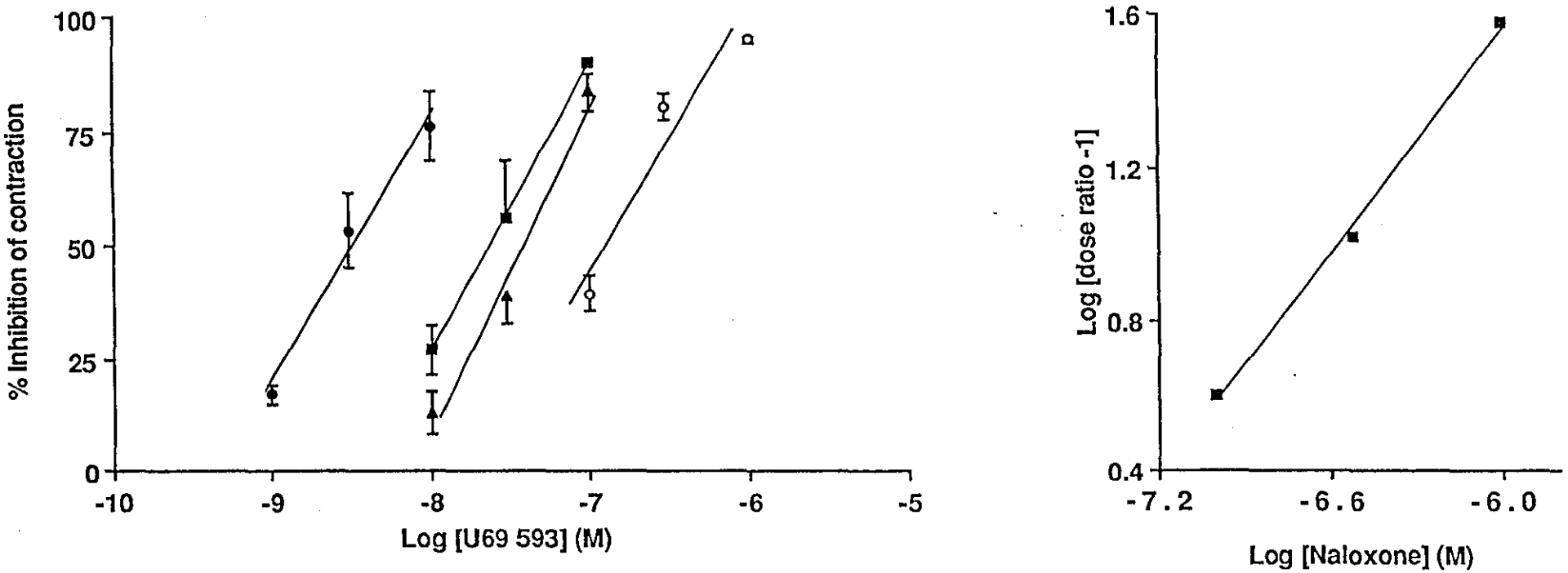


Figure 5.8 : Inhibition of electrically induced contractions of the myenteric plexus longitudinal muscle of the guinea-pig by U69 593 in the abscence (●) and in the presence of 100nM (■), 300nM (▲) and 1000nM (○) naloxone. Sample of a corresponding Schild plot (■) shown on the right.
[n=4]

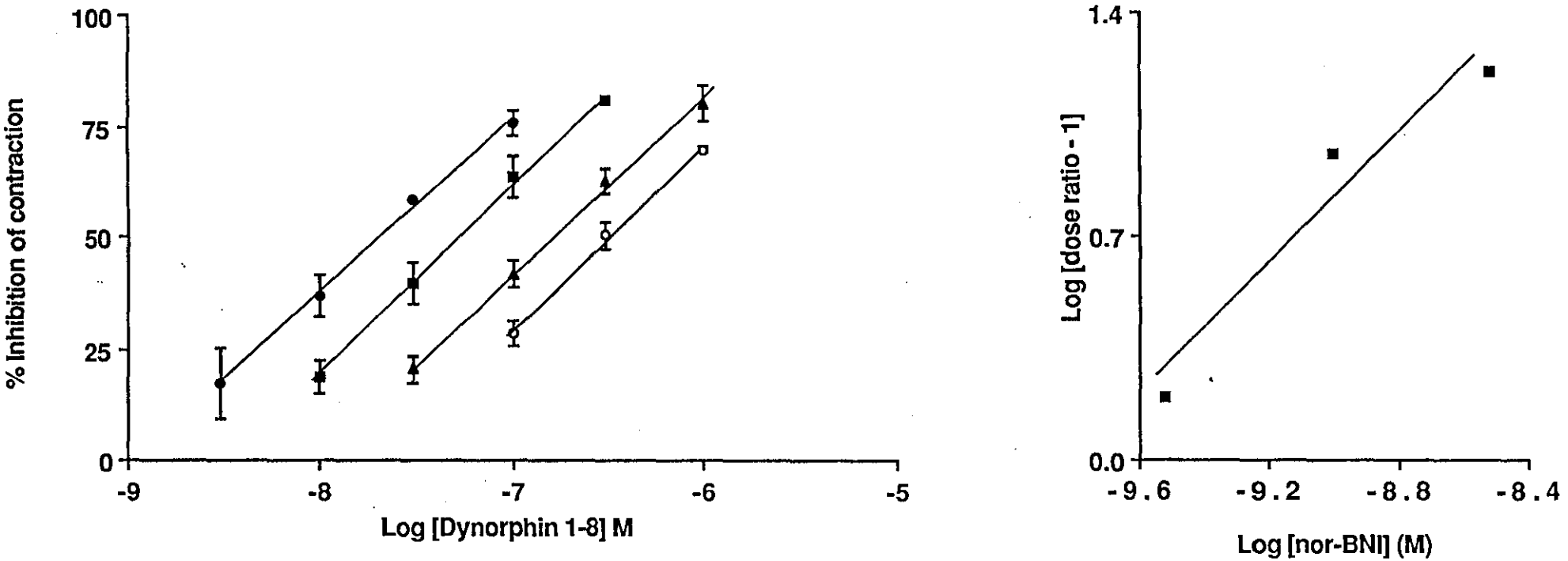


Figure 5.9 : Inhibition of electrically induced contractions of the myenteric plexus longitudinal muscle of the guinea-pig by dynorphin 1-8 in the abscence (●) and in the presence of 0.3nM (■), 1nM (▲) and 3nM (○) nor-binaltorphamine. Sample of a corresponding Schild plot (■) shown on the right.

[n=4]

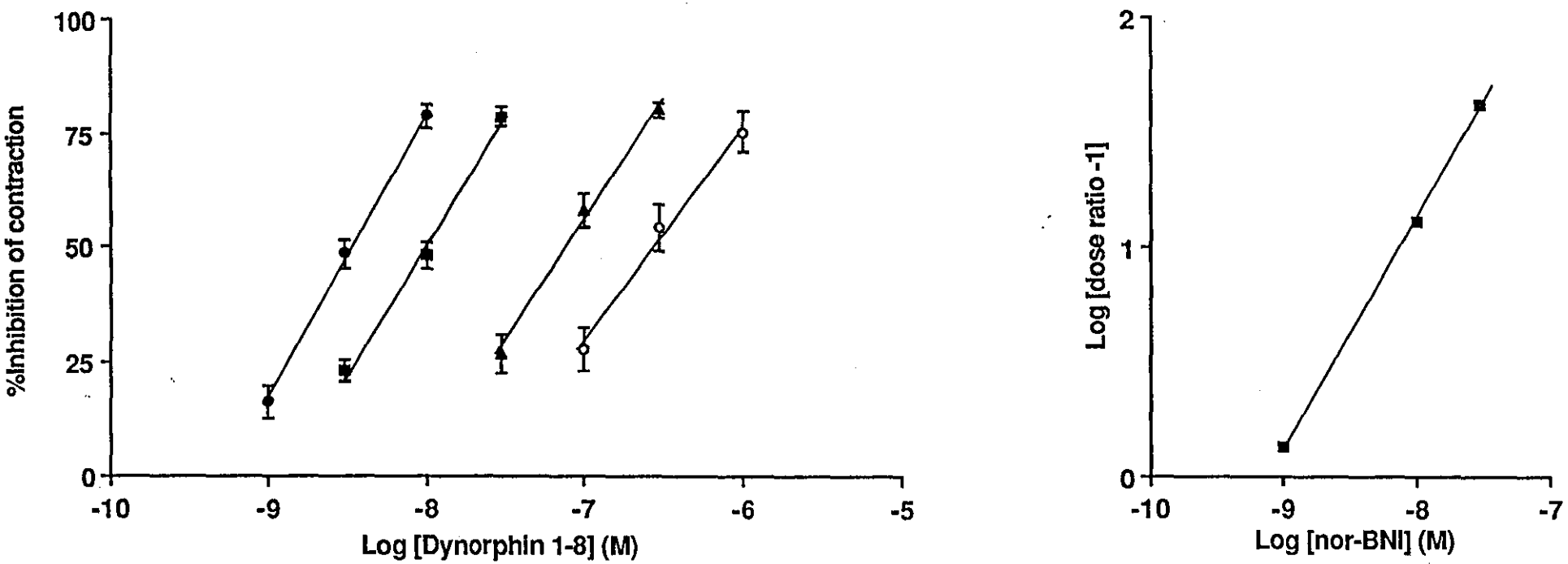


Figure 5.10 : Inhibition of electrically induced contractions of the myenteric plexus longitudinal muscle of the guinea-pig, in the presence of bestatin, 10μM; captopril, 10μM and thiorphan, 0.3μM by dynorphin 1-8 in the abscence (●) and in the presence of 1nM (■), 10nM (▲) and 30nM (○) nor-binaltorphamine. Sample of a corresponding Schild plot (■) shown on the right.

[n=7]

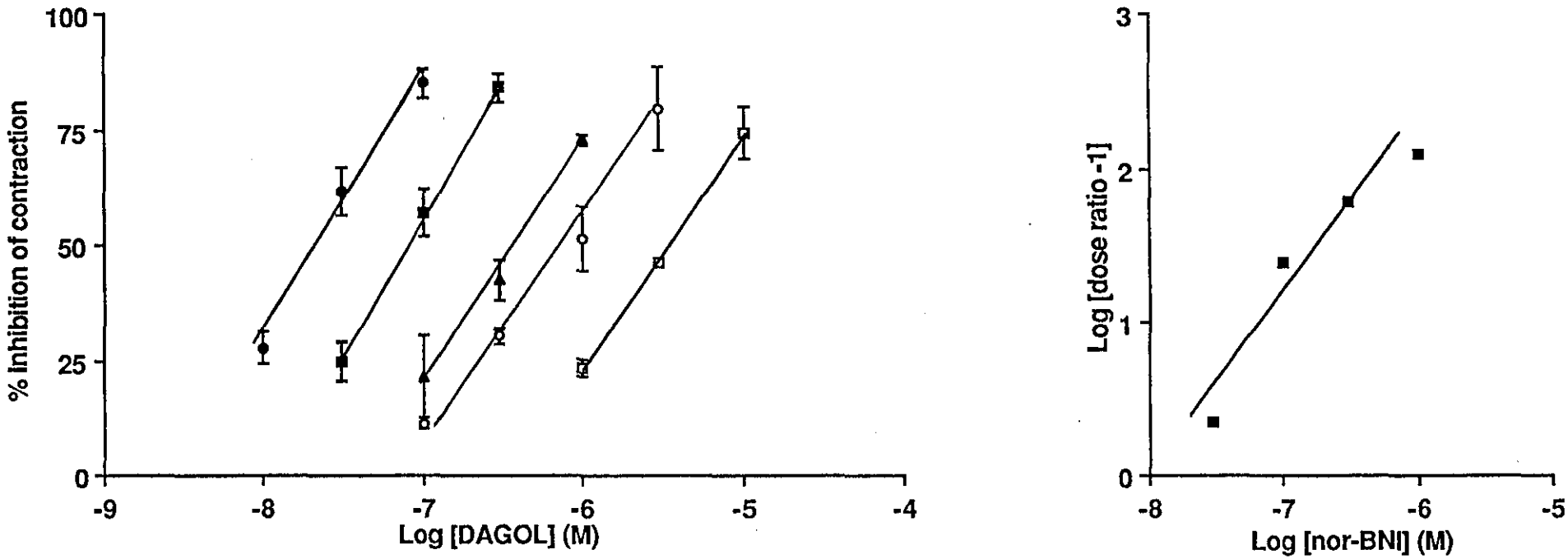
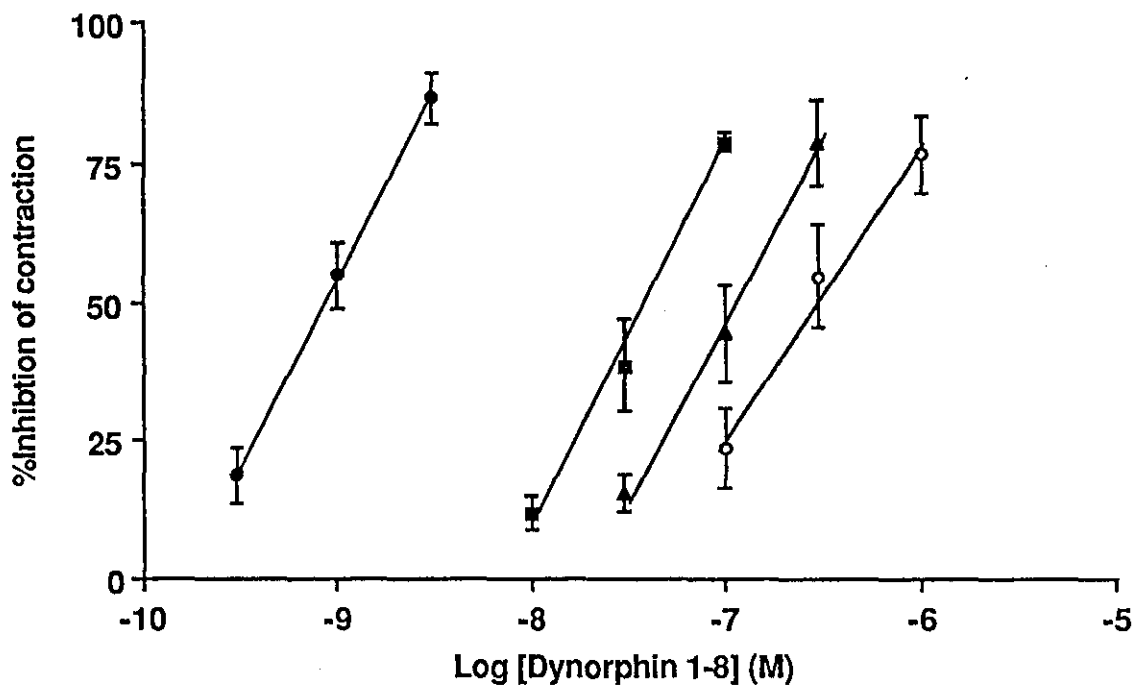


Figure 5.11 : Inhibition of electrically induced contractions of the myenteric plexus longitudinal muscle of the guinea-pig by DAGOL in the abscence (●) and in the presence of 30nM (■), 100nM (▲), 300nM (○) and 1000nM (□)nor-binaltorphamine. Sample of a corresponding Schild plot (■) shown on the right.

[n=5]



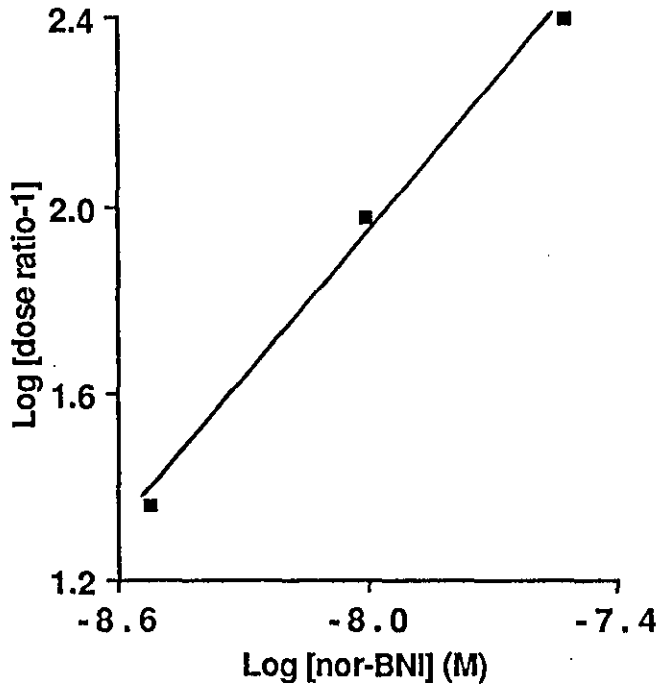


Figure 5.12 : Inhibition of electrically induced contractions of the myenteric plexus longitudinal muscle of the guinea-pig, in the presence of bestatin, 10μM; captopril, 10μM; thiorphan, 0.3μM and N-[(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB, 0.3μM, by dynorphin 1-8 in the abscence (●) and in the presence of 100nM (■), 300nM (▲) and 1000nM (○) nor-binaltorphamine. Sample of a corresponding Schild plot (■) shown on the right.
[n=4]

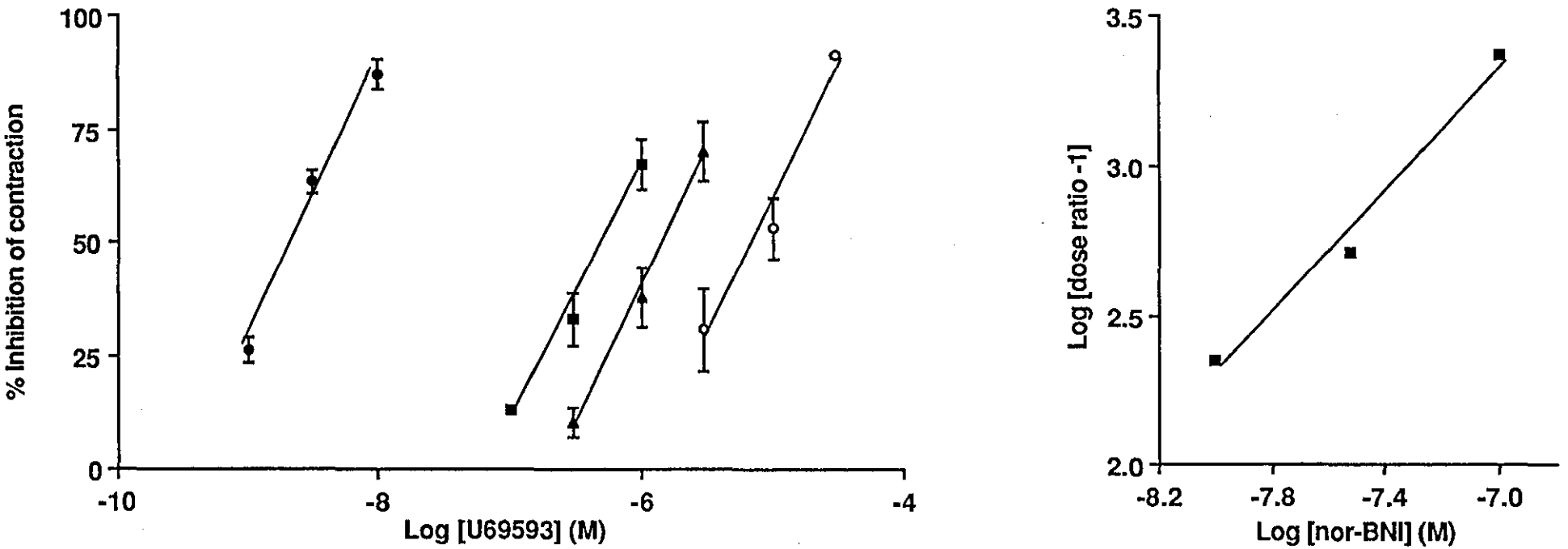


Figure 5.13 : Inhibition of electrically induced contractions of the myenteric plexus longitudinal muscle of the guinea-pig by U69 593 in the abscence (●) and in the presence of 10nM (■), 30nM (▲) and 100nM (○) nor-binaltorphamine. Sample of a corresponding Schild plot (■) shown on the right.

[n=4]

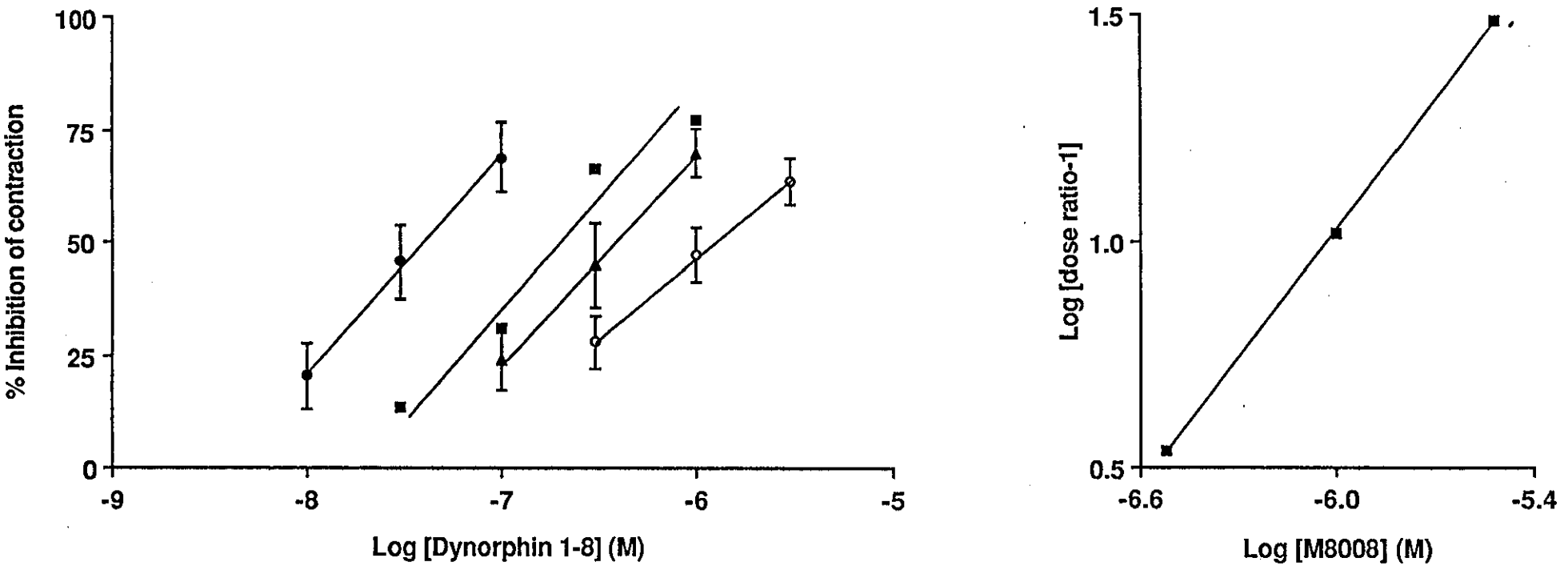


Figure 5.14 : Inhibition of electrically induced contractions of the myenteric plexus longitudinal muscle of the guinea-pig by dynorphin 1-8 in the abscence (●) and in the presence of 300nM (■), 1000nM (▲) and 3000nM (○) M8008. Sample of a corresponding Schild plot (■) shown on the right.

[n=4]

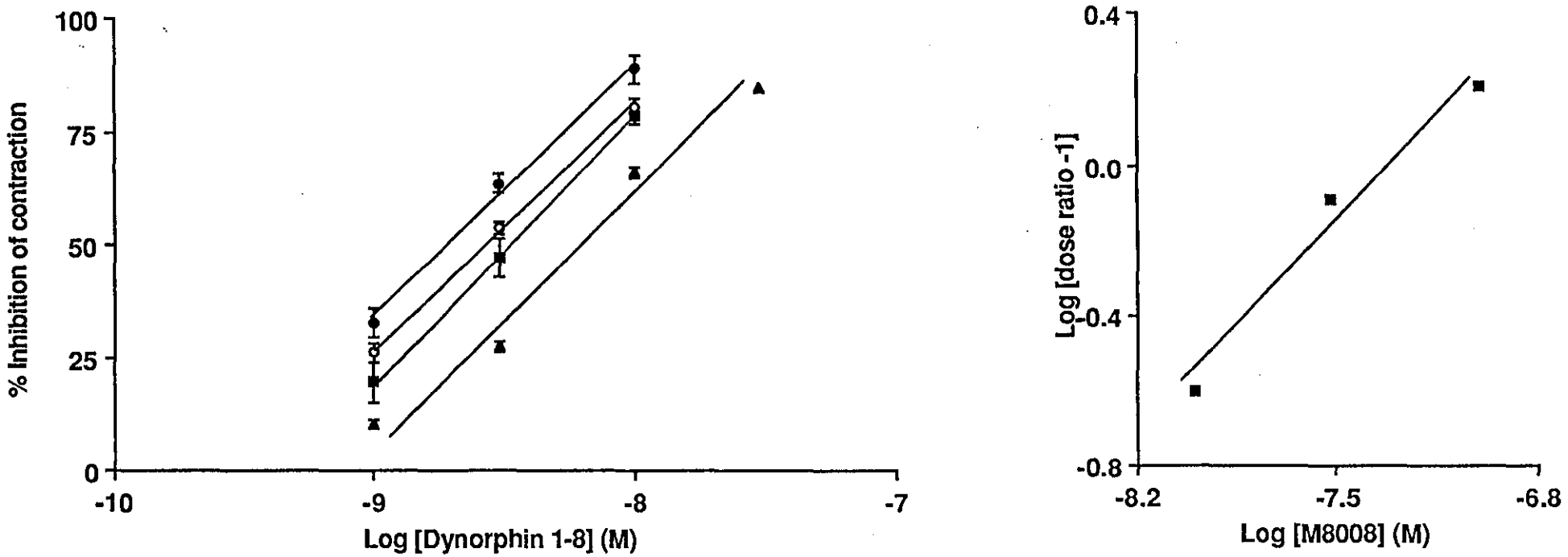
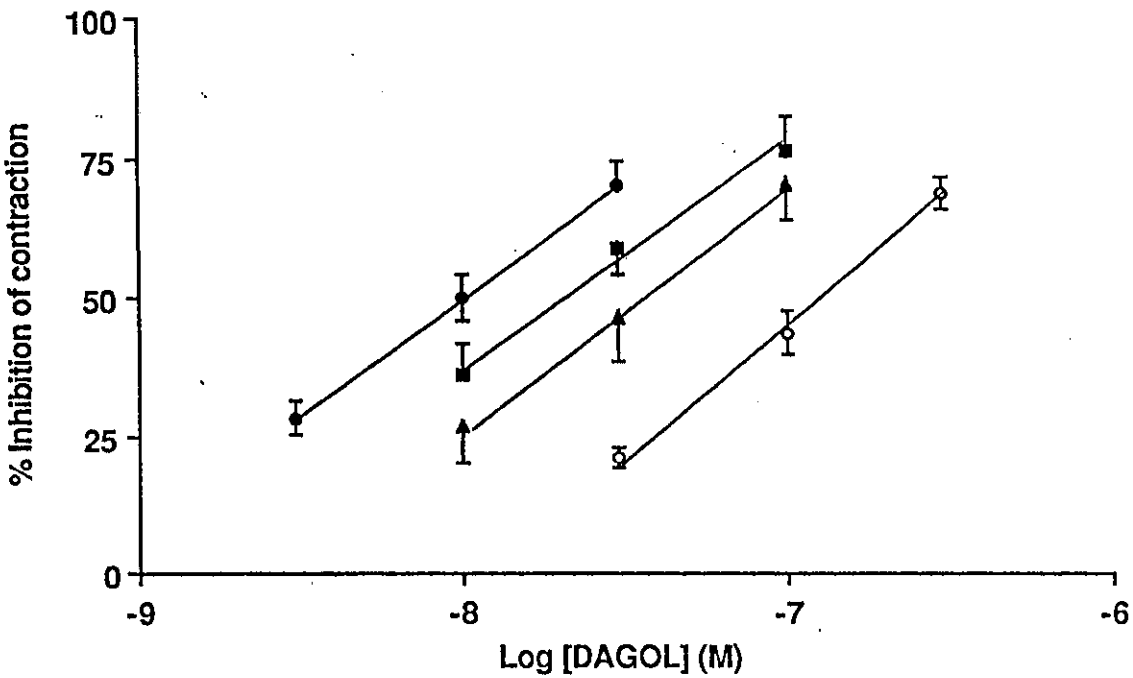


Figure 5.15 : Inhibition of electrically induced contractions of the myenteric plexus longitudinal muscle of the guinea-pig, in the presence of bestatin, 10μM; captopril, 10μM and thiorphan, 0.3μM by dynorphin 1-8 in the abscence (●) and in the presence of 10nM (■), 30nM (▲) and 100nM (○) M8008. Sample of a corresponding Schild plot (■) shown on the right.

[n=4]



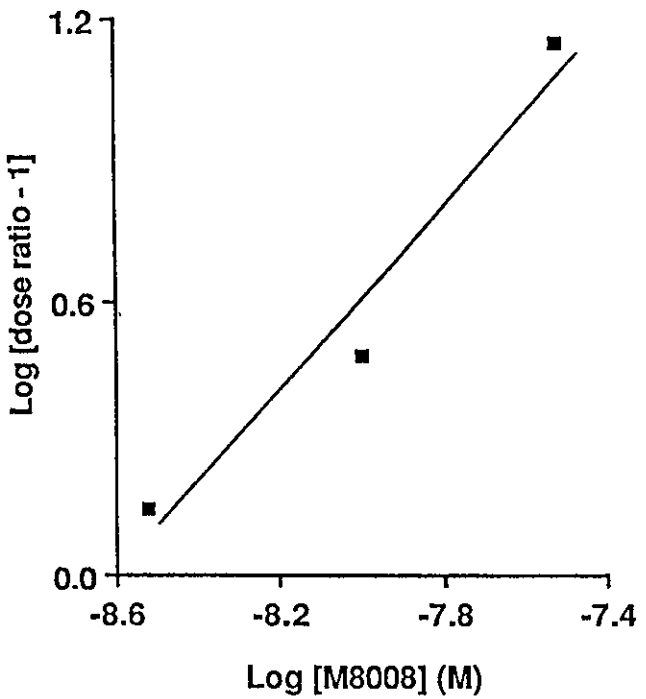


Figure 5.16 : Inhibition of electrically induced contractions of the myenteric plexus longitudinal muscle of the guinea-pig by DAGOL in the abscence (●) and in the presence of 3nM (■), 10nM (▲), 30nM (○) M8008. Sample of a corresponding Schild plot (■) shown on the right.

[n=4]

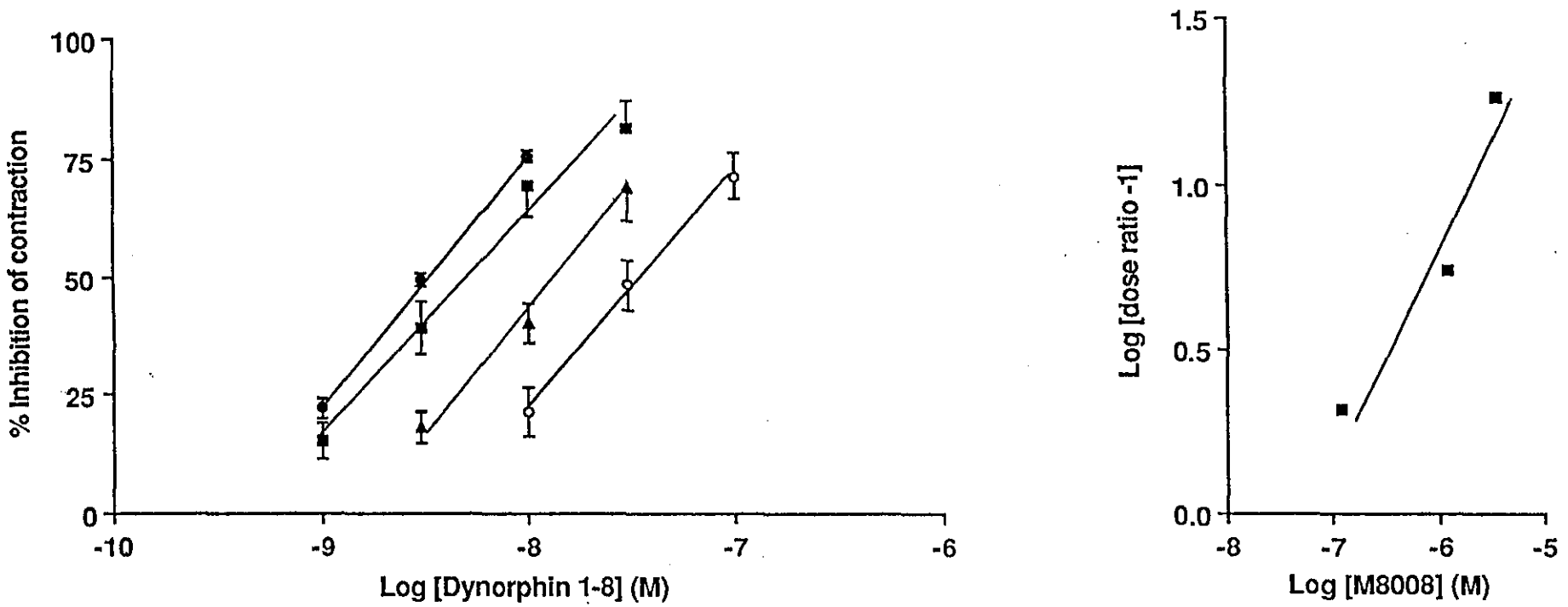
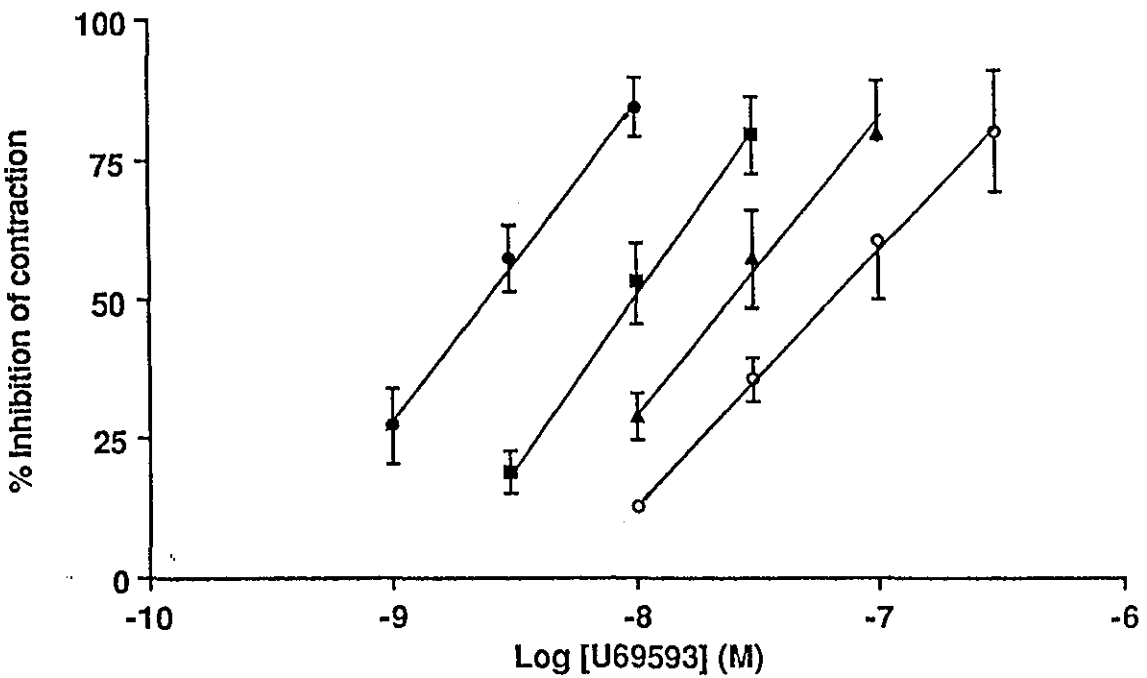


Figure 5.17 : Inhibition of electrically induced contractions of the myenteric plexus longitudinal muscle of the guinea-pig, in the presence of bestatin, 10μM; captopril, 10μM; thiorphan, 0.3μM and N-[(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB, 0.3μM, by dynorphin 1-8 in the abscence (●) and in the presence of 300nM (■), 1000nM (▲) and 3000nM (○) M8008. Sample of a corresponding Schild plot (■) shown on the right.

[n=4]



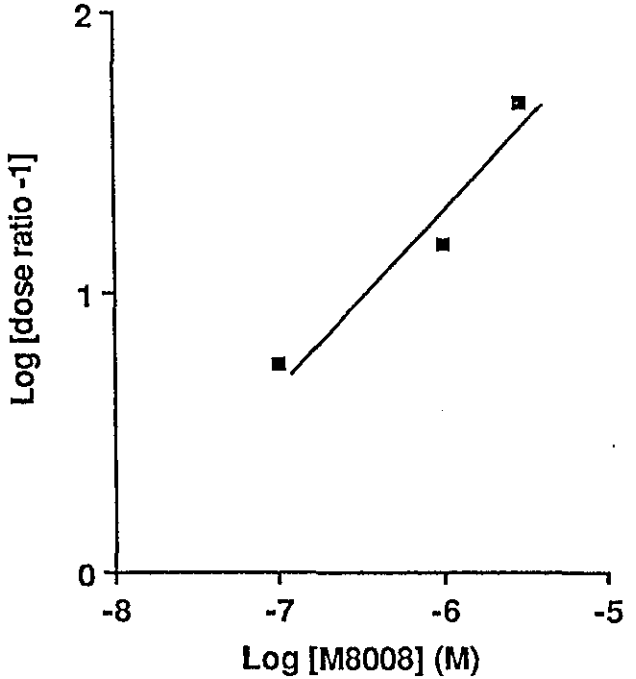


Figure 5.18 : Inhibition of electrically induced contractions of the myenteric plexus longitudinal muscle of the guinea-pig by U69 593 in the abscence (●) and in the presence of 300nM (■), 1000nM (▲) and 3000nM (○) M8008. Sample of a corresponding Schild plot (■) shown on the right.

[n=4]

DISCUSSION

The increase in potency (measured as a decrease in the IC_{50} value) of dynorphin 1-8 in the MPLM upon the addition of peptidase inhibitors indicates a stabilization of the dynorphin molecule. This phenomenon has been widely reported, occurring in a variety of tissues including the mouse and rabbit vas deferens, in addition to the MPLM of the guinea-pig [28, 84, 130, 133]. However the effect of peptidase inhibition on the naloxone Ke against dynorphin 1-8, calculated using the Schild plot technique, had not been previously investigated. Such studies with naloxone as antagonist are essential to define the receptor population at which the agonist is exerting its effect. Earlier studies revealed naloxone whilst not ideal, to be an antagonist capable of clearly distinguishing mu from kappa receptors in the MPLM of the guinea-pig ileum [82, 96]. Calculation of naloxone Ke values against agonists at the mu and kappa receptor population in the MPLM fall within the ranges of <5 and >14nM respectively [82, 96]. In addition using the Schild plot method to calculate antagonist Ke values also indicates whether the agonist is able to displace the antagonist from more than one receptor type. Schild plot slopes of less than unity indicate an interaction with more than one receptor type.

Single dose Ke values for dynorphin 1-8, obtained in both the absence and presence of peptidase inhibitors had been reported by James and co-workers [84]. These workers observed a decrease in the naloxone Ke against dynorphin 1-8, in guinea-pig MPLM, from 15 ± 1.5nM to $8.3 \pm .07$nM upon the addition of 10μM bestatin; 10μM captopril; 0.3μM thiorphan and 2mM leucyl-leucine. This decrease in the naloxone Ke, after peptidase inhibition broadly agrees with the results obtained in this thesis. However in the discussion of their findings they conclude that the inclusion of peptidase inhibitors did not significantly change the naloxone Ke, its value remaining at an intermediate

level between that expected for an interaction at mu or kappa receptors and is in line with the unselective nature of dynorphin 1-8 [28]. However because only the single dose method was used to calculate the naloxone Ke in the previous work it was not possible to tell whether one or more receptor types were involved. The decrease obtained in the present studies was however, greater in magnitude, the naloxone Ke decreasing from 14nM, in the absence of inhibitors, to 3nM in the presence of peptidase inhibitors, suggesting a change in receptor preference profile from kappa to mu .

Considering that in the results presented in this chapter the Ke value of dynorphin 1-8, in the presence of peptidase inhibitors, against naloxone does not differ from that obtained using DAGOL it would appear that dynorphin 1-8, in the presence of the inhibitor cocktail, is able to interact with the mu receptor population within the MPLM of the guinea-pig. As demonstrated in Chapter 3 the MPLM is able to metabolise [^{3}H]dynorphin 1-8 to [^{3}H][Leu]enkephalin. It is therefore possible that the Ke value obtained in the presence of inhibitors is a result of dynorphin 1-8 being metabolised to [Leu]enkephalin which subsequently activates the mu receptor population. This theory is further supported by the naloxone Ke value obtained using inhibited [Leu]enkephalin as agonist, affording a value that does not differ from those obtained using either DAGOL or inhibited dynorphin 1-8. The interferance of delta receptor occupation can be ruled out as the MPLM of the guinea-pig ileum contains only functional mu and kappa receptors [21, 96].

Addition of the site directed inhibitor of EC 3.4.24.15, namely N-[-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB [26] to the enzyme inhibitor cocktail resulted in an increase in the naloxone Ke of the dynorphin 1-8 to a value not dissimilar to that obtained using dynorphin 1-8 in the absence of any peptidase inhibitors and within the range associated with interaction with a kappa receptor

population. Unfortunately lack of availability of the inhibitor only allowed for the use of the single dose Ke method. Substituting the more potent N-[-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB for the more potent N-[-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB [120] in the inhibitor cocktail increased further the naloxone Ke against dynorphin 1-8 to a value not significantly different to the Ke values obtained against the kappa preferring ligands U69 593 [89] and dynorphin 1-17 [84]. Results reported in Chapter 4 have shown that inclusion of N-[-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB in the inhibitor cocktail almost completely protects the dynorphin 1-8 molecule from degradative attack. It would appear therefore that intact dynorphin 1-8 is selective for the kappa opioid receptor under these conditions.

Use of the kappa preferring antagonist nor-BNI [127, 150] again highlights the selectivity of dynorphin 1-8, when correctly stabilized, for the kappa receptor. Previously reported Ke values for nor-BNI range from 0.1nM to 0.005nM [12] measured against U50 488H or ethylketocyclazocine as kappa selective ligands. The more selective kappa agonist U69 593, as used in the present study afforded Ke values within this range, as did dynorphin 1-8 in the presence of the usual inhibitor cocktail plus N-[-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB. The uninhibited octapeptide displayed a higher Ke value outside this range. Birch and co-workers using DAGOL as agonist obtained Ke values ranging from 50.1nM to 0.8nM. The results obtained here for DAGOL were within this range although with a much smaller variation. Dynorphin 1-8 in the presence of the inhibitor cocktail afforded a Ke value just outside the lower end of the range previously reported, but again with a far smaller standard error. All Schild plots had slopes of unity indicating the high selectivity of nor-BNI for the kappa receptor.

The large standard errors obtained by Birch and colleagues could be the result of insufficient antagonist equilibration time. This would result in large errors in the estimate of Ke values. In the present study using U69 593 as agonist, a concentration of 0.3nM nor-BNI required a minimum of 30min preincubation in order to obtain equilibrium. The lower concentration of 0.1nM nor-BNI, incubated for 30min, used by Birch would therefore require a longer incubation period. In order to be confident that complete equlibrium conditions were obtained a 45min incubation period was used throughout each Schild plot, even when higher concentrations of nor-BNI were used.

Previous studies have shown M8008 to be a pure antagonist at opioid receptors that displays a preference for mu and delta over kappa receptors. However, considering the MPLM of the guinea-pig contains only mu and kappa receptors [22] any delta receptor affinity displayed by M8008 is irrelevant in this tissue. M8008 is able therefore to distinguish between mu and kappa receptors displaying a kappa/mu selectivity ratio of 33.6 in the mouse vas deferens [144]. A Ke for M8008 against uninhibited dynorphin 1-8 of 76.9 ± 11.1nM, indicative of an action at a kappa receptor population was determined. This kappa Ke value for M8008 is substantially greater than those previously published. Using the kappa agonist ethylketocyclazocine (EKC) which afforded a Ke of 33.1nM in MPML [144] Smith obtained the highest reported kappa Ke value of 62.4 ± 18nM in the rabbit vas deferens using ethylketocyclazocine as agonist. The kappa selective agonist U69 593 also afforded a Ke value greater than any previously reported. Whilst the mu preferring agonist DAGOL gave a low value Ke somewhat more in line with data published by Smith who obtained a Ke of 0.65 ± 0.05nM using normorphine as agonist.

The addition of the enzyme inhibitor cocktail decreased the Ke of dynorphin 1-8 to 31.4nM, a value that correlates well with that previously published using

EKC as agonist. However the slope of the corresponding Schild plot was less than unity, indicating an action at more than one receptor type. Addition of the inhibitor N-[-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB increased the Schild plot slope to unity and increased the Ke to 208 ± 70.3nM a value far in excess of previously published data in a wide variety of tissues [144]. These results confirm dynorphin 1-8 as a kappa preferring ligand which, when correctly stabilized, displays an increased potency and an apparent increase in selectivity towards the kappa receptor. In addition it would appear that in the present studies the antagonist M8008 may have a higher kappa/mu selectivity ratio than previously reported.

The ability of dynorphin 1-8 in the presence of the inhibitor cocktail to interact with the mu receptors, within the MPLM, through its metabolism to the pentapeptide [Leu]enkephalin could be further revealed using the mu selective alkylating agent beta-FNA. Previous studies have shown beta-FNA to be 100 times more selective for mu over kappa receptors [60]. Indeed beta-FNA is an agonist at kappa receptors and an antagonist at mu receptors Treatment with beta-FNA failed to elicit a shift in the dose-response curve to the kappa selective U50 488H. The dose response curves to uninhibited dynorphin 1-8 and dynorphin 1-8 in the presence of the inhibitor cocktail and N-[-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB were also unaffected by beta-FNA treatment. These results are indicative of an interaction with a kappa receptor population following the selective alkylation of the mu receptors within the MPLM which had been achieved as indicated by the large shift in the IC_{50} of DAGOL. Similarly [Leu]enkephalin, in the presence of the peptidase inhibitors suffered a large decrease in potency after beta-FNA treatment. Dose response curves constructed for dynorphin 1-8 in the presence of the inhibitor cocktail were displaced to the right, however this displacement failed to produce shifts in dose responses following beta-FNA that were significantly different to those

obtained for U50 488H, uninhibited dynorphin 1-8 and inhibited dynorphin 1-8 in the presence of N-[-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB.

These results indicate that a proportion of the response caused by inhibited dynorphin 1-8 is afforded through conversion to [Leu]enkephalin which then interacts with the mu receptor population. However sufficient dynorphin 1-8 remains intact to allow for the activation of kappa receptors, when the mu receptor population is severely reduced following beta-FNA alkylation.

CHAPTER 6

Ontogenic Development of the Enzyme Responsible for the Hydrolysis of the Leu^5-Arg^6 bond within [^{3}H]Dynorphin 1-8

INTRODUCTION

It is well known that both opioid receptor density [106] and opioid peptide levels [106] increase from birth to adulthood. The ratios of the various opioid prohormone products also vary with age [177]. Little work however has been carried out on the ontogenic development of the enzymes responsible for the metabolism of neuropeptides [177]. General studies investigating the changes in aminopeptidase and enkephalinase (the enzyme responsible for cleaving the Gly^3-Phe^4 bond within the pentapeptides) have been carried out [122]. However no study has traced the development of either endo-oligopeptidase A or EC 3.4.24.15. If these enzymes play an important role in the metabolism of dynorphin 1-8 and the consequent conversion of the octapeptide to [Leu]enkephalin has an important physiological function, it is possible to speculate that the development of the enzyme responsible for this conversion should be related to the ontogeny of the opioid peptides and their receptors within the c.n.s.

This chapter investigates the ontogenic development of the production of dynorphin 1-8 to [Leu]enkephalin and relates this development to the ontogeny of both opioid receptors and opioid peptide levels within the maturing rat c.n.s.

RESULTS

In order to examine the ontogenic development of the enzyme responsible for the release of [^{3}H][Leu]enkephalin fron [^{3}H]dynorphin 1-8 the following experiments were all performed in the presence of the peptidase inhibitor cocktail (bestatin 10μM; captopril 10μM and thiorphan 0.3μM) to allow the measurement of [^{3}H][Leu]enkephalin production as discussed in previous chapters.

Slices of spinal cord from 1 day old rats were able to release [^{3}H][Leu]enkephalin from [^{3}H]dynorphin 1-8. Following a 10min incubation period at 37°C 32.3 ± 1.7% of recovered radioactivity co-eluted with the [Leu]enkephalin marker peptide [Fig 6.1]. The largest portion of radioactivity, 67.7 ± 1.7%, co-eluted with the dynorphin 1-8 marker peptide. Little aminopeptidase activity remained in the presence of the inhibitors as the N-terminal fraction accounted for only 2.4 ± 2.4% of the recovered radioactivity.

The level of [^{3}H]dynorphin 1-8 recovered following a 10min incubation decreased sharply from day 1 until day 7, when it reached a value of 28.5 ± 6.5%. The metabolism had then reached a plateau that remained until day 14. However from day 14 the level of [^{3}H]dynorphin 1-8 continued to decline reaching 11.9 ± 5.3% by day 21. This increased metabolism of [^{3}H]dynorphin 1-8 corresponded to a peak in measurable aminopeptidase activity. An increase in the amount of N-terminal fraction accounting for 36.3 ± 11.9% of recovered radioactivity, even in the presence of the aminopeptidase inhibitor bestatin (10μM) was found. However, this peak of aminopeptidase activity decreased by day 28 to a stable level of 12.6 ± 2.2%. The level of [^{3}H][Leu]enkephalin rose steadily as the level of [^{3}H]dynorphin 1-8 decreased, reaching a peak at day 10 accounting for 59.6 ± 8.8% of

recovered radioactivity. This level of [^{3}H][Leu]enkephalin was maintained until day 28 when at 56.3 ± 2.5% the level began to increase reaching 65.7 ± 3.0% at day 56.

This pattern of metabolism displayed by the spinal cord was closely paralled by the cortex. Again as early as day 1 after birth cortex tissue was able to liberate [^{3}H][Leu]enkephalin from [^{3}H]dynorphin 1-8. After a 10min incubation at 37°C [^{3}H][Leu]enkephalin and [^{3}H]dynorphin 1-8 accounted for 30.4 ± 4.5% and 74.0 ± 1.1% of the recovered radioactivity respectively [Fig 6.2]. The presence of the inhibitor cocktail of, bestatin 10μM; captopril 10μM and thiorphan 0.3μM, completely prevented the degradation of the N-terminal at day 1 in cortex tissue. However, aminopeptidase activity overcame this inhibition and was evident at day 21 when the N-terminal fraction accounted for 34.6 ± 16.8% of eluted radioactivity even after the supposed inhibition of aminopeptidases. As in spinal cord tissue, this peak in aminopeptidase activity corresponded to a trough in the level of recoverable [^{3}H]dynorphin 1-8 which dropped to 15.7 ± 1.7%. This level of recoverable [^{3}H]dynorphin 1-8 then increased to reach a stable value of 32.4 ± 3.1% at day 28, this level was unchanged on day 56 at 29.2 ± 4.7%. The amount of recoverable [^{3}H][Leu]enkephalin steadily increased with age reaching a peak at day 7 accounting for 57.7 ± 1.2% and 48.1 ± 7.93 on day 14. The level of [^{3}H][Leu]enkephalin produced began to increase from day 14 reaching 60.5 ± 4.3% on day 56.

Striatal tissue, from 1 day old rats was also able to produce [^{3}H][Leu]enkephalin (31.7%) from exogenous [^{3}H]dynorphin 1-8 [Fig 6.3]. The level of [^{3}H]dynorphin 1-8 remaining decreased slowly from an initial value of 65.4% at day 1 to 13.2 ± 4.1% at day 21, thereafter rising to 27.1 ± 4.0% by day 56. The lowest level of [^{3}H]dynorphin 1-8 occurring at

day 21 corresponded to the highest level of recoverable N-terminal fraction at 38.7 ± 9.9%. This peak in aminopeptidase activity also paralleled the trough in the level of [^{3}H][Leu]enkephalin (46.3 ± 4.4%), which reached its peak value at day 10 accounting for 62.4 ± 5.8% of the recovered radioactivity. The amount of recoverable [^{3}H][Leu]enkephalin rose from day 21 to day 28, thereafter maintaining a steady value between 55 to 69 percent.

The pattern of metabolism produced by the cerebellum was somewhat different to that displayed by the spinal cord, cortex and striatum. Again the level of recoverable [^{3}H]dynorphin 1-8 decreased steadily from day 1 (74.0 ± 1.1%) to day 21 (14.4 ± 10.1%). displaying a plateau phase between day 7 and day 14, when the level remained at approximately 30% [Fig 6.4]. After day 21 the amount of [^{3}H]dynorphin 1-8 began to increase back to the 30% level. In this case the trough in the level of recoverable [^{3}H]dynorphin 1-8 was not accompanied by an increase in aminopeptidase activity, which reached a maximum on day 14 represented by the high level of N-terminal fraction recovered (33.6 ± 10.1%). The peak in aminopeptidase activity, at day 14, was paralleled by a sharp decrease in recoverable [^{3}H][Leu]enkephalin which contributed only 37.6 ± 2.8% to the total recovered radioactivity. The highest level of [^{3}H][Leu]enkephalin was recorded on day 21 (63.1 ± 1.9%) corresponding to the lowest level of [^{3}H]dynorphin 1-8, this level of [^{3}H][Leu]enkephalin was maintained until day 56.

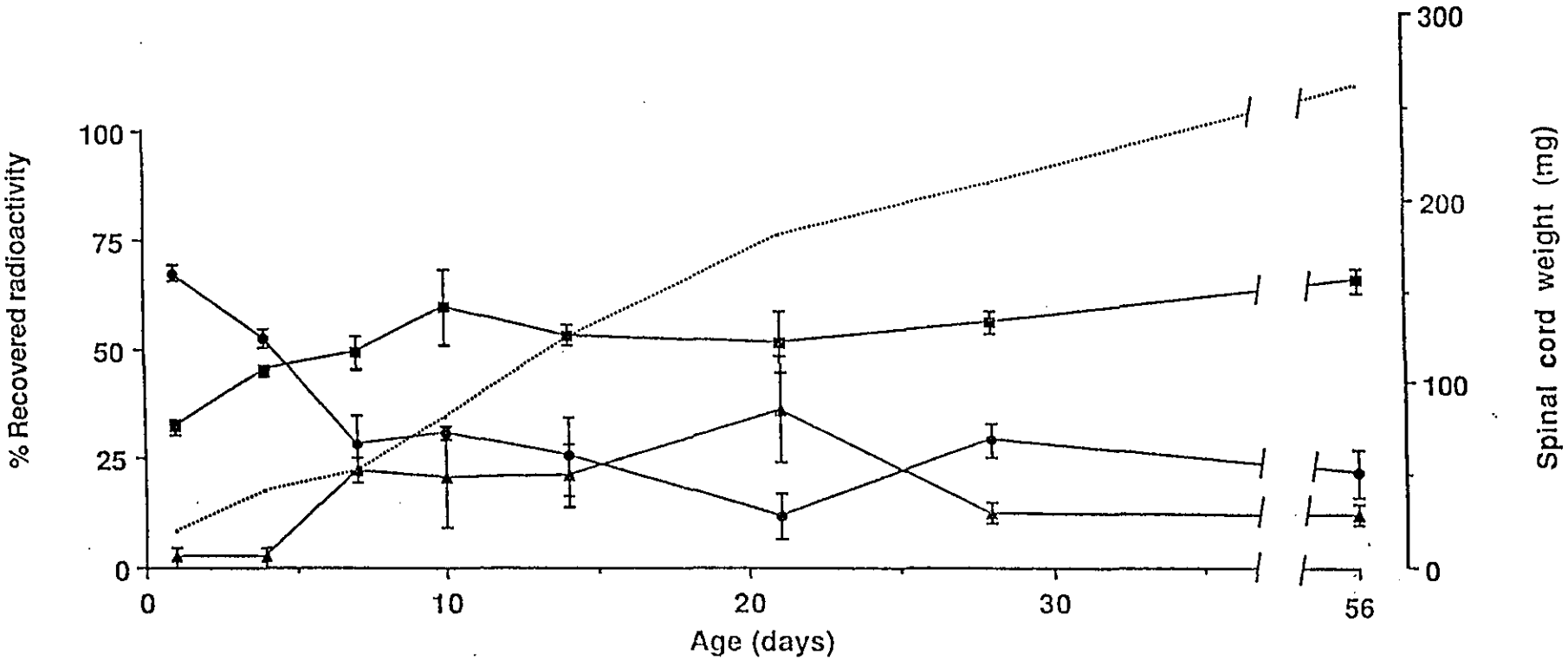


Figure 6.1 : Ontogenic development (solid lines) of the metabolism of [^{3}H]dynorphin 1-8 (●) to [^{3}H][leu]enkephalin (■) and [^{3}H]N-terminal fraction (▲) following a 10min incubation with slices of rat spinal cord in the presence of the peptidase inhibitors bestatin, 10µM; captopril, 10µM and thiorphan, 0.3µM. Corresponding increase in spinal cord weight is recorded by the dotted line.

[n=4]

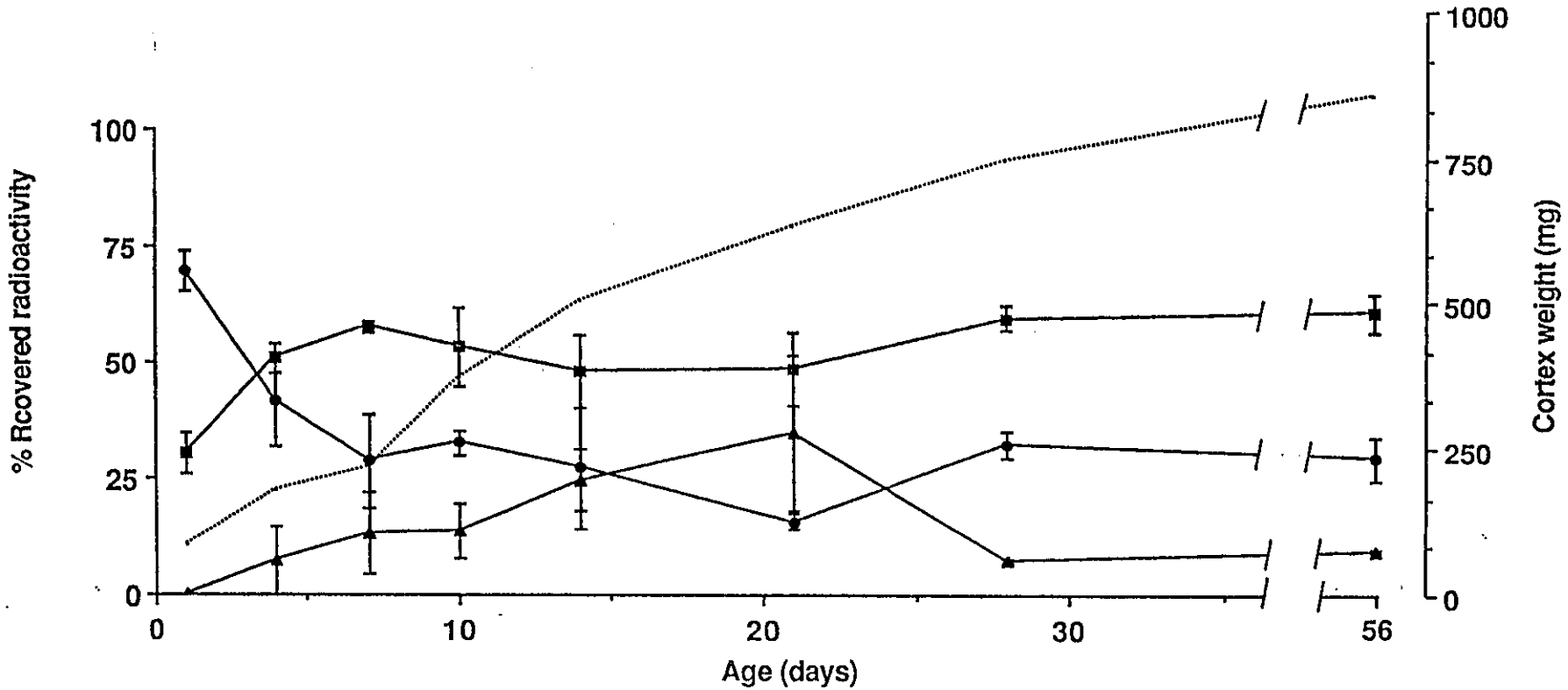


Figure 6.2 : Ontogenic development (solid lines) of the metabolism of [^{3}H]dynorphin 1-8 (●) to [^{3}H][leu]enkephalin (■) and [^{3}H]N-terminal fraction (▲) following a 10min incubation with slices of rat cortex in the presence of the peptidase inhibitors bestatin, 10µM; captopril, 10µM and thiorphan, 0.3µM. Corresponding increase in cortex weight is recorded by the dotted line. [

[n=4]

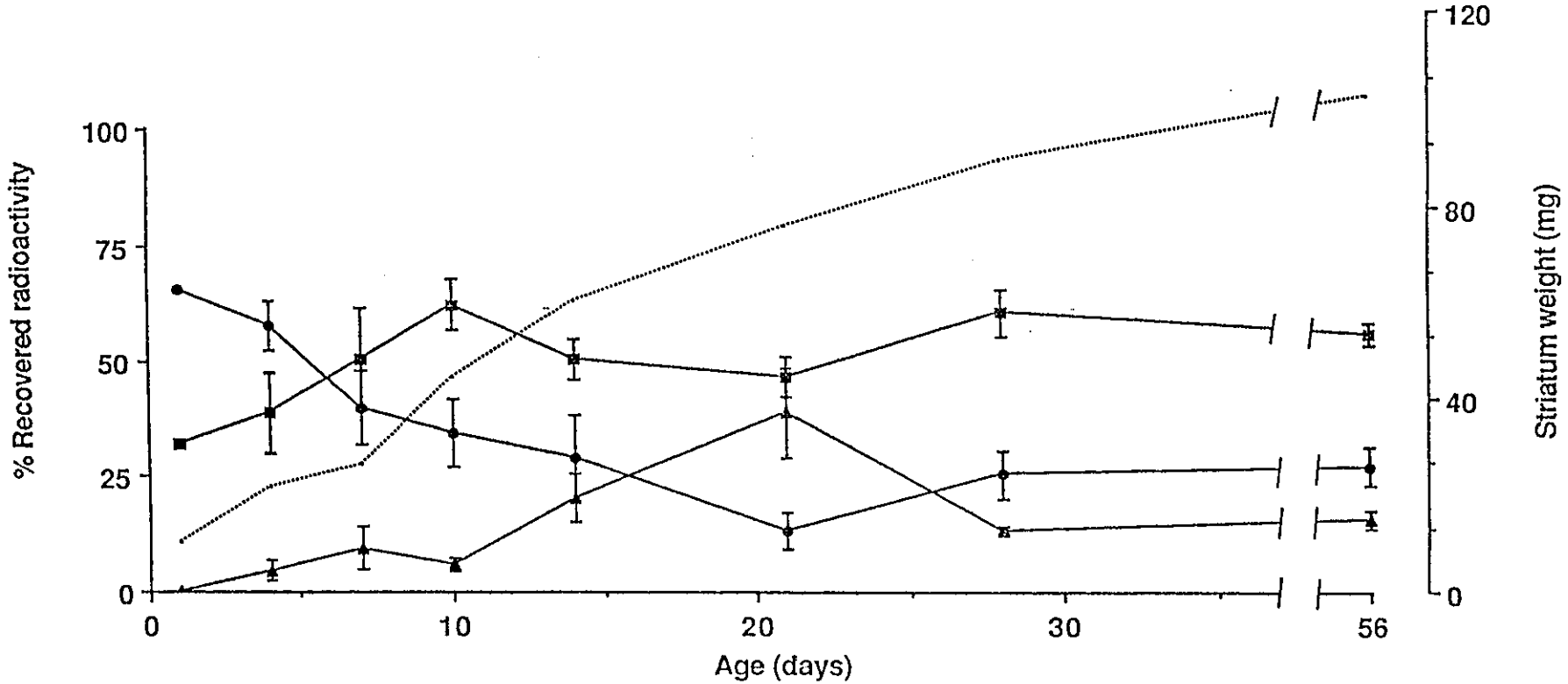


Figure 6.3 : Ontogenic development (solid lines) of the metabolism of [^{3}H]dynorphin 1-8 (●) to [^{3}H][leu]enkephalin (■) and [^{3}H]N-terminal fraction (▲) following a 10min incubation with slices of rat striatum in the presence of the peptidase inhibitors bestatin, 10μM; captopril, 10μM and thiorphan, 0.3μM. Corresponding increase in striatum weight is recorded by the dotted line.

[n=4]

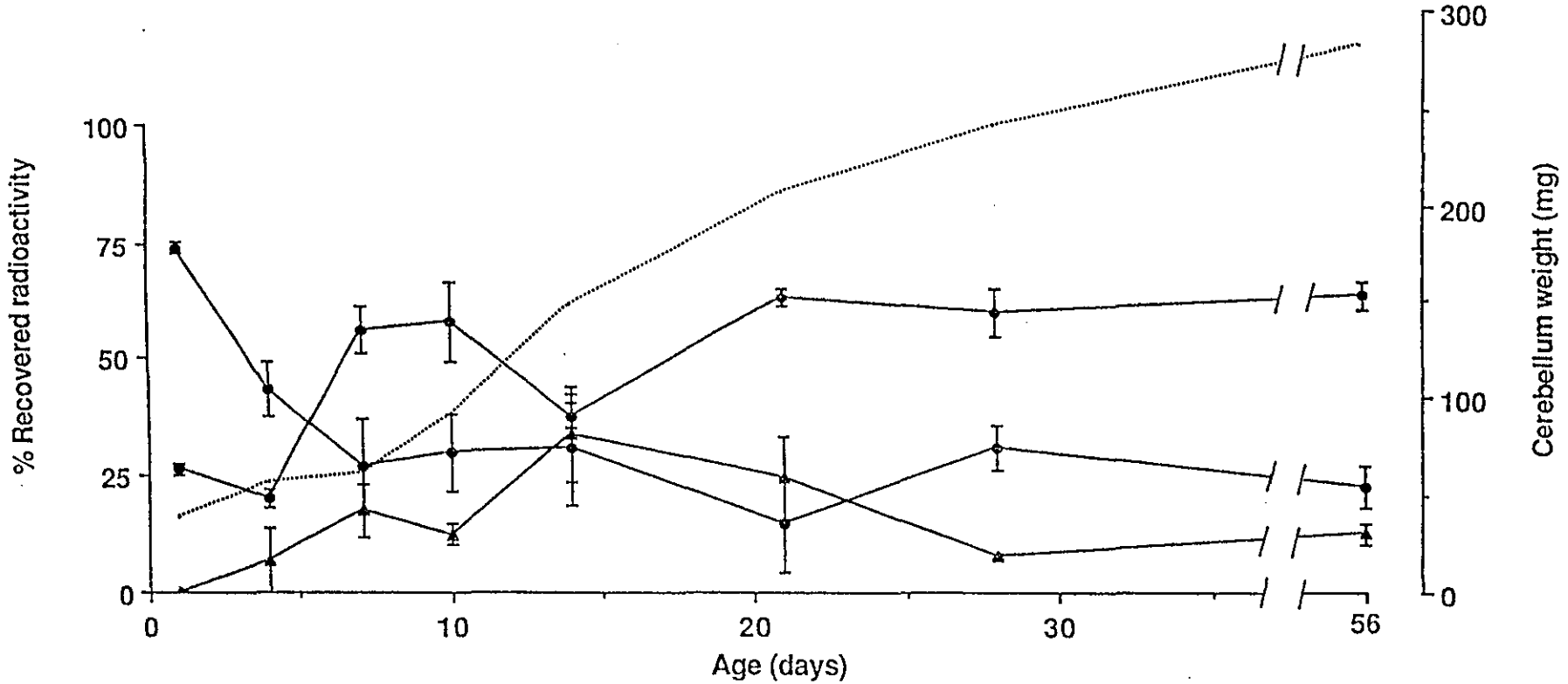


Figure 6.4 : Ontogenic development (solid lines) of the metabolism of [^{3}H]dynorphin 1-8 (●) to [^{3}H][leu]enkephalin (■) and [^{3}H]N-terminal fraction (▲) following a 10min incubation with slices of rat cerebellum in the presence of the peptidase inhibitors bestatin, 10μM; captopril, 10μM and thiorphan, 0.3μM. Corresponding increase in cerebellum weight is recorded by the dotted line.

[n=4]

DISCUSSION

It would appear from the results described above that aminopeptidase activity at postnatal day 1 in all four areas investigated is a bestatin sensitive aminopeptidase as inclusion of the aminopeptidase inhibitor bestatin in the incubation medium completely inhibits the cleavage of the N-terminus of dynorphin 1-8. A previous study reported aminopeptidase activity at birth, with only a two fold increase in activity in the adult rat [106]. However the ontogenic development of the total aminopeptidase activity, as measured by Patey and co-workers could differ from the ontogenic development of any bestatin resistant aminopeptidase measured in this study. Alternatively the amount of bestatin present in the incubation medium may be sufficient to inhibit the lower levels of aminopeptidase present at birth but is not sufficient to totally block the cleavage of the N-terminal tyrosine of dynorphin 1-8 as the levels of aminopeptidase increase with age.

The level of recoverable dynorphin 1-8 displays a steady decrease from birth to adulthood, and all c.n.s. regions studied are able to liberate [Leu]enkephalin as early as post-natal day 1. Since [Leu]enkephalin may be the endogenous ligand at the delta receptor it is important to compare these findings with the ontogeny of delta receptors. However these findings contrast with the ontogeny of the delta opioid receptor. Using the delta selective ligand [D-Pen^2,D-Pen^5]enkephalin, delta receptors cannot be detected before post-natal day 10, with peak binding seen at day 25 [106]. However [Leu]enkephalin also has a high affinity for mu receptors and mu binding sites are present as early as post-natal day 1 [146], although the numbers decline for several days after birth, prior to increasing to adult levels within two weeks. Spain and co-workers [146] also report an increase in delta receptor binding after post-natal day 10 which corresponds to a peak in [Leu]enkephalin production seen in all four

c.n.s. regions. The rat c.n.s. is, therefore, able to produce [Leu]enkephalin before the development of substantial delta-binding sites for [Leu]enkephalin. However the early peak in the enzyme activity that liberates the pentapeptide from dynorphin 1-8 corresponds to the start of an increase in both delta and mu receptor binding capacities.

Kappa receptor binding capacity is low at birth and only increases twofold in the adult rat [146]. However using [^{3}H]ethylketocyclazocine, with binding to mu and delta suppressed as the kappa ligand, identifies two binding sites, one of very low affinity. This could explain the discrepancies found between laboratories where different studies have revealed peak kappa binding to occur as early as day 16 [8] or as late as day 35 [146]. The development of kappa binding does not therefore appear to be related to the development of dynorphin 1-8 metabolism. However, as the ratio of [Leu]enkephalin to dynorphin 1-8 increases with age so does the ratio of delta/kappa receptors, as the increase in the level of delta receptor binding is more pronounced than that of kappa receptor binding [146]. Kappa receptors also display a differential development pattern in the fore and hind (cerebellum and brainstem) brain areas. Initial densities are the same in both brain areas, however, hindbrain levels rise substantially until day 14 when they begin to decline as forebrain levels increase [146]. This rostral to caudal development is seen in other opioid receptor development [29, 156].

The levels of all opioid peptides within the c.n.s. increase from birth to adult animals [139, 177]. Zamir and co-workers reported the main increase in levels of proenkephalin and prodynorphin derived peptides in rat hippocampus occurs during the period from post-natal day 7 to day 14 when dynorphin A, dynorphin 1-8, dynorphin B and alpha-neoendorphin reach adult levels. Again

this period corresponds with the peak in enzymatic activity liberating [Leu]enkephalin from dynorphin 1-8 .

The ratio of dynorphin 1-17 to dynorphin 1-8 in the neurointermediate lobe of the pituitary in neonates is 3:1 whereas in the adult the levels of the two peptides are equal. It would appear therefore, that the enzyme responsible for cleaving the single arginine residue in dynorphin 1-17 to liberate dynorphin 1-8 is not fully developed at birth [139]. In addition, a decrease in the concentration of immunoreactive dynorphin 1-8 recovered from the neurointermediate pituitary of the rat is seen at post-natal day 7 [139], with no corresponding decrease in the concentration of dynorphin 1-17. This decrease in dynorphin 1-8 could be explained by an increase in enzyme activity seen to occur at day 7 in brain and spinal tissue resulting in an increased metabolism of dynorphin 1-8 to [Leu]enkephalin. Seizinger [139] also reported an increase in [Leu]enkephalin content in the anterior pituitary from birth to adulthood but unfortunately did not measure the level of dynorphin 1-8 in this area.

The development of enkephalin levels in the c.n.s. proceeds in a caudal to rostral direction, in a similar manner to the observed development in opioid receptor binding. Peak enkephalin levels are seen within the first week of development in the cerebellum, the second week in the brainstem and the third week in the forebrain [156]. This caudal to rostral development is not seen in the present study of the liberation of [Leu]enkephalin from dynorphin 1-8. However, other studies have described more consistent general increases in enkephalin levels across all brain regions [13].

It would appear therefore that in general the ontogeny of the enzyme responsible for the liberation of [Leu]enkephalin from dynorphin 1-8 and the ontogeny of the opioid peptides and their receptors do display a degree of similarity. This is

a possible indication of the importance of the conversion process within the c.n.s.

CHAPTER 7

General Discussion

GENERAL DISCUSSION

As with many other neuropeptides dynorphin 1-8 is rapidly inactivated by c.n.s. tissue [10, 57, 77, 91, 173]. This inactivation, especially the cleavage of the N-terminal tyrosine residue, creates problems when short dynorphin peptides are used to define the kappa receptor in ligand binding studies as the metabolic inactivation leads to an underestimation of the potency of the dynorphin peptide [28, 84, 130, 133]. Inclusion of enzyme inhibitors, such as bestatin, captopril and thiorphan in the incubation medium results in the partial stabilization of the dynorphin 1-8 molecule against peptidase attack [48]. However, the enzyme inhibitors afford additional problems as they themselves interfere with binding studies to reduce the levels of bound peptide [108]. Lower incubation temperatures is an alternative strategy used to decrease enzymatic activity, however, lower temperatures not only slow the rate of metabolism but greatly lengthen the time required for ligands to reach equilibrium.

The problem of metabolic inactivation is not confined to isolated receptor binding studies. Isolated tissues such as the mouse vas-deferens [108] and the MPLM of the guinea-pig also rapidly inactivate dynorphin 1-8. This once again leads to an under estimation of the potency of dynorphin 1-8, as illustrated by the decrease in IC_{50} of the octapeptide in the MPLM upon the addition of peptidase inhibitors.

In addition to the inactivation of dynorphin 1-8 via the removal of the N-terminal tyrosine residue, results presented in this thesis show that there is a direct cleavage of the Leu^5-Arg^6 bond within the octapeptide affording the pentapeptide [Leu]enkephalin. This process of producing [Leu]enkephalin is not a simple sequential attack on the C-terminal of dynorphin 1-8 because:

1.) The metabolites dynorphin 1-6 and dynorphin 1-7 form less than 2% of the recovered radioactivity and addition of the enzyme inhibitor cocktail does not increase the level of either peptide.

2.) The level of recoverable [Leu]enkephalin is increased upon the addition of the cocktail of enzyme inhibitors, indicating that the enzyme responsible for the production of the [Leu]enkephalin is unaffected by such peptidase inhibitors and that any [Leu]enkephalin formed is itself protected from metabolism by the inhibitor cocktail.

3.) Site directed inhibitors of the enzyme EC 3.4.24.15 substantially reduce the level of [Leu]enkephalin produced from dynorphin 1-8 without affecting any other metabolite.

If one imposes the criteria that only a direct cleavage of the Leu^5-Arg^6 bond within dynorphin 1-8 can be viewed as a conversion process then the kappa preferring dynorphin 1-8 is converted to the delta/mu preferring [Leu]enkephalin. This problem is further exacerbated in the presence of enzyme inhibitors which, in addition to protecting the dynorphin 1-8 stabilize any [Leu]enkephalin formed, thereby amplifying the interference of the delta/mu preferring pentapeptide. This will be of great importance when one is using dynorphin 1-8, in the presence of enzyme inhibitors, to define a kappa receptor population in a tissue that contains a heterogenous population of opioid receptors.

This conversion process appears to take place in vivo. Zamir and colleagues observed a dynorphinergic pathway for the production of [Leu]enkephalin in the substantia nigra of the rat [176]. It is worth noteing that levels of [Met] and [Leu]enkephalin are altered independently in nigral areas of Parkinson diseased

brains, a process suggestive of a dynorphinergic pathway of [Leu]enkephalin production in man [152]. In Parkinson diseased brains [Leu] and [Met]enkephalin levels decrease in both the internal and external portions of the globus pallidus and the putamen. However in the substantia nigra only [Met]enkephalin levels are decreased with no corresponding change in [Leu]enkephalin or dynorphin 1-17. These observations suggest that in the putamen and pallidus dynorphin neurones are not the major source of [Leu]enkephalin and only proenkephalin A neurones are affected by Parkinsons Disease. Alternatively in these areas proenkephalin A could be located within dopaminergic neurones and the observed decrease in proenkephalin A products is secondary to the atrophy of dopamine containing neurones associated with Parkinsons Disease [152].

The observation of the wide distribution of the enzyme(s) capable of cleaving the Leu^5-Arg^6 bond and their availability at very early stages in development suggests an important physiological role for this conversion. This role would, however vary throughout the c.n.s.. Thus, although there is sufficient evidence to view the production of [Leu]enkephalin from dynorphin 1-8 as a conversion process eg. in areas containing a mixed population of opioid receptors, this would not be an appropriate view in areas containing a homogenous kappa receptor population. In such areas the production of [Leu]enkephalin would best be regarded, within the current boundaries of evidence, as a metabolic inactivation since [Leu]enkephalin has a very low, if any, affinity at kappa receptors [96, 177]. It would be of special interest if this process was under some form of variable control [78]. For example there is a clear association between stress and the release of opioid peptides [2].

However, before it will be possible to address fully the question of physiological relevance of the production of [Leu]enkephalin from dynorphin 1-

8, the controversy surrounding the nature and number of enzymes involved in the conversion process must be answered. More work remains to be done in this area. The precise location of the enzyme is required, especially with regard to the synaptic junction. It is important to know whether the enzyme has a specific location for example, in the synaptic cleft or within the terminals themselves. It is interesting to note that in the rat pituitary [Met]enkephalin levels are maintained under opioid inhibition [46]. Administration of the opioid antagonist naloxone causes a significant increase in the level of [Met]enkephalin found in both the neurointermediate and anterior lobes of the pituitary. However similar treatment with the mu preferring agonist [D-Ala^2,MePhe^4,Met(o)^{5}ol]enkephalin failed to affect [Met]enkephalin levels. The increase in [Met]enkephalin levels could result from a increase in synthesis or decrease in release, as in vitro naloxone has been shown to decrease the release of [Met]enkephalin from rat striatum [134]. This may be tha first indication of an opioid autoregulation system It is interesting to speculate the role of metabolism of the kappa preferring dynorphin 1-8 to the mu/delta preferring [Leu]enkephalin in such a system.

As discussed in Chapter 4 controversy remains regarding the exact nature of the enzyme involved in the hydrolysis of the Leu^5-Arg^6 bond within dynorphin 1-8. The two candidate enzymes one a thiol protease namely endo-oligopeptidase A [18] the other a metalloendopeptidase classified E.C. 3.4.24.15 [119] both appear to be able to produce [Leu]enkephalin from dynorphin 1-8. However evidence has been published suggesting that the metalloendopeptidase E.C. 3.4.24.15 is devoid of the ability to hydrolyse the Leu^5-Arg^6 bond of dynorphin 1-8 [154]. Results described in this thesis are also inconclusive with regard to the type of enzyme involved. The metabolism displays characteristics both of a metalloendopeptidase and a thiolprotease. it is therefore possible that a

mixture of the two enzymes is responsible for the metabolism described in this thesis.

Perhaps the most important observation contained within this thesis is that extreme care must be taken when using labile peptides to define or characterise any given receptor type, especially when enzyme inhibitors are used in an attempt to stabilize the peptide. This observation cannot be confined to the opioid system. In addition the results presented in Chapter 5 demonstrate that, when correctly and sufficently stabilized, dynorphin 1-8 is a potent and very selective peptide for kappa over mu opioid recptors, its selectivity for the kappa opioid receptor being comparable to that displayed by dynorphin 1-17 and the synthetic ligand U69 593.

FUTURE WORK

Future work must consider the physiological role for this enzymatic conversion, for example does the metabolism form the basis of an autoinhibition process as discussed above. The question of the exact nature of the enzyme responsible for the metabolism remains to be resolved. This resolution is necessary to determine whether the process is a specific or a non-specific hydrolysis.

REFERENCES

REFERENCES

1. Acker, G.R., Molineaux, C. and Orlowski, M. (1987) J. Neurochem. 48. 284-292

2. Akil, H., Richardson, D.E., Hughes, J. and Barchas, J.D. (1978) Science 201. 463-465

3. Almenhoff, A., Wilks, S. and Orlowski, M. (1981) Biochem. Biophys. Res. Commun. 95. 141-144

4. Almenhoff, J., Wilk, S. and Orlowski, M. (1981) Biochem. Biophys. Res. Commun. 102. 206-214

5. Arunlakshana, O. and Schild, H.O. (1956) Br. J. Pharmacol. 14. 48-58

6. Atweh, S.F. and Kuhar, M.J. (1977) Brain Res. 124. 53-67

7. Atweh, S.F., Murrin, L.C., Kuhar, M.J. (1978) Neuropharmacol. 17. 65-71

8. Barr, M.T., Bhatnagar, R.K. and Gebhart, G.F. (1983) Neuropharmacol 22. 453-461

9. Bayon, A., Shoemaker, W.J., Bloom, F.E., Mauss, A. and Guilleman, R. (1979) Brain Res. 179. 93-101

10. Benuck, M. and Marks, N. (1979) Biochem. Biophys. Res. Commun. 88. 215-221

11. Benuck, M. and Marks, N. (1980) Biochem. Biophys. Res. Commun. 95. 822-828

12. Birch, P.J., Hayes, A.G., Sheehan, M.J. and Tyers, M.B. (1987) Eur. J. Pharmac. 144. 405-408

13. Booth, A.G., Hubbard, M.L. and Kenny, A.J. (1979) Biochem. J. 179. 397-405

14. Botticelli, L.J., Cox, B.M. and Goldstein, A. (1981) Proc. Natl. Acad. Sci.. 78. 7783-7786

15. Camargo, A.C.M., Caldo, H. and Emson, P.C. (1983) Biochem. Biophys. Res. Commun. 116. 1151-1159

16. Camargo, A.C.M., Oliveira, E.B., Toffoletto, O., Metters, K.M. and Rossier, J. (1987) J. Neurochem. 48. 1258-1263

17. Camargo, A.C.M., Ribeiro, M.J.U.F. and Schwartz, W.N. (1985) Biochem. Biophys. Res. Commun. 130. 932-938

18. Camargo, A.C.M., Shapanka, R. and Green, L.J. (1973) Biochemistry 12. 1838-1844

19. Carvalho, K. M. and Camargo, A.C.M. (1981) Biochemistry 20. 7082-7088

20. Chaillet, P., Marcais-Collado, H., Costentin, H.,Yi, C.C., De la Baume, S. and Schwartz, J-C. (1983) Eur. J. Pharmac. 86. 329-336

21. Chavkin, C. and Goldstein, A. (1981) Nature 291. 591-593

22. Chavkin, C. and Goldstein, A. (1981) Proc. Natl. Acad. Sci.. 78. 6543-6547

23. Chavkin, C., James, I.F. and Goldstein, A. (1982) Science 215. 413

24. Cherot, P., Devin, J., Fournie-Zaluski, C. and Roques, B.P. (1986) Mol. Pharmacol. 30. 338-344

25. Cherot, P., Fournie-Zaluski, M.C. and Laval, J. (1986) Biochemistry 25. 8184-8191

26. Chu, T.G. and Orlowski, M. (1984) Biochemistry 23. 3598-3603

27. Cohen, M.L., Geary, L.E. and Wiley, K.S. (1983) J. Pharmacol. Exptl. Thera. 244. 379-385

28. Corbett, A.D., Paterson, S.J., McKnight, A.T., Magnan, J. and Kosterlitz, H.W. (1982) Nature 299. 79-81

29. Coyle, J.T. and Pert, C.B. (1976) Neuropharmacology 15. 555-560

30. Craviso, G. L. and Musacchio, J.M. (1978) Life Sci. 23. 2019-2030

31. Cuello, A.C. and Paxinos, G. (1978) Nature 271. 178-180

32. Danielson, E.M., Vyas, J.P. and Kenny, A.J. (1980) Biochem. J. 191. 645-648

33. De la Baume, S., Patey, G. and Schwartz, J.C. (1980) Neuroscience 6. 315-321

34. Devi, L. and Goldstein, A. (1986) J. Neurochem. 47. 154-157

35. Dickenson, A.H., Sullivan, A.F., Fournie-Zaluski, M.C. and Roques, B.P. (1987) Brain Res. 408. 185-191

36. Dupont, A., Cusan, L., Garon, M., Alvarado-Urbina, G. and Labrie, F. (1977) Life Sci. 21. 907-914

37. Faden, A.I., Jacobs, T.P. and Holaday, J.W. (1981) Science 211. 493-502

38. Faden, A.I., Molineaux, C.J., Rosenberger, J.G., Jacobs, T.P. and Cox, B.M. (1985) Ann. Neurol. 17. 386-390

39. Fallon, J.H. and Leslie, F.M. (1986) J. Comp. Neurol. 249. 293-336

40. Fang, F.G., Fields, H.L. and Lee, N.M. (1986) J. Pharmacol. Exptl. Thera. 238. 1039-1044

41. Fields, H.L. and Basbaum, A.I. (1978) Ann. Review Physiol. 40. 217-248

42. Fields, H.L., Emson, P.C., Leigh, B.K., Gilbert, R.F.T. and Iversen, L.L. (1980) Nature 284. 351-353

43. Fournie-Zaluski, M.C., Chaillet, P., Bouboutou, R., Coulaud, A., Cherot, P., Waksman, G., Costentin, J. and Roques, B. (1984) Eur. J. Pharmac. 102. 525-528

44. Gafford, J., Skidgel, R.A., Erdos, E. and Hersh, L.B. (1983) Biochemistry 22. 3265-3271

45. Gee, N.S., Bowes, M.A., Buck, P. and Kenny, A.J. (1985) Biochem. J. 228. 119-126

46. Gerge, S.R. and Kertes, Z.M. (1987) Eur. J. Biochem. 140. 95-98

47. Gilbert, P.E. and Martin, W.R. (1976) J. Pharmacol. Exptl. Thera. 198. 66-82

48. Gillan, M.C.G., Robson, L.E., McKnight, A.T. and Kosterlitz, H.W. (1985) J. Neurochem. 45. 1034-1042

49. Gillan, M.G.C., Kosterlitz, H.W. and Magnan, J. (1981) Br. J. Pharmacol. 72. 13-15

50. Glazer, E.J. and Basbaum, A.I. (1981) J. Comp. Neurol. 196. 377-389

51. Glowinski, J. and Iverson, L.L. 1966 J. Neurochem. 13. 655-669

52. Goldstein, A., Lowney, L.I. and Pal, B.K. (1971) Proc. Natl. Acad. Sci. 70.

53. Gorenstein, C. and Snyder, S.H. (1979) Life Sci. 25. 2065-2070

54. Gray, B.G. and Dostrovsky, J.O. (1983) J. Neurophysiol. 49. 932-947

55. Gubler, U., Seeburg, P., Hoffman, B.J., Gage, L.P. and Udenfriend, S. (1982) Nature 295. 206-208

56. Guthrie, J. and Basbaum, A.I. (1984) Neuropeptides 4. 437-445

57. Hambrook, J.M., Morgan, B.A., Rance, M.J. and Smith, C.F. (1976) Nature 262. 782-783

58. Han, J.S. and Xie, C.W. (1982) Life Sci. 31. 781-1784

59. Han, J.S., Xie, G.X. and Goldstein, A. (1984) Life Sci. 34. 1573-1579

60. Hayes, A.G., Sheehan, M.J. and Tyers, M.B. (1985) Br. J. Pharmacol. 86. 899-904

61. Haynes, L.W., Smyth, D.G. and Zakarian, S. (1982) Brain Res. 232. 115-128

62. Hazato, T., Inagaki-Shimamura, M., Katayama, T. and Yamamoto, T. (1982) Biochem. Biophys. Res. Commun. 105. 470-475

63. Herman, B.H. and Goldstein, A. (1985) J. Pharmacol. Exptl. Thera. 232. 27-32

64. Herman, Z.S., Stachura, Z., Laskawiec, G., Kowalski, J. and Obuchowicz, J. (1985) J. Pharm. Pharmacol. 37. 133-140

65. Hersh, L.B. (1981) Biochemistry 20 2345-2350

66. Hersh, L.B. (1982) Mol. Cell. Biochem 47. 35-43

67. Hersh, L.B. (1984) J. Neurochem. 43. 487-493

68. Hersh, L.B. (1985) J. Neurochem. 44. 1427-1435

69. Hersh, L.B., Aboukhair, N. and Watson, S. (1987) Peptides 8. 523-532

70. Hokfelt, T., Ljungdhal, A., Terenius, L., Elde, R. and Nilsson, G. (1977) Proc. Natl. Acad. Sci. 74. 3081-3085

71. Hokfelt, T., Tsuruo, Y., Ulfhake, B., Cullheim, S., Aruidsson, U., Foster, G.A., Schultzberg, M., Schalling, M., Arborelius, L., Freedman, J., Post, C. and Visser, T. (1989) in TRH : Biomedical Significance [Ed. Metcalf, G. and Jackson, I.M.D] 553. 76-105

72. Holaday, J.W. and Loh, H.H. (1979) Adv. Biochem. Psycopharmacol. 20. 227-258

73. Hollt, V. (1986) Ann. Rev. Pharmacol. Toxicol. 26. 59-77

74. Hollt, V. (1982) Eur. J. Pharmac. 85. 355-356

75. Hollt, V., Sanchez-Blazquez, P. and Garzon, J. (1985) Phil. Trans. R. Soc. Lond. Series B. 308. 299-310

76. Hook, V.Y., Eiden, L.E. and Brownstein, M.J. (1982) Nature 295 341-342

77. Hughes, J. (1975) Brain Res. 88. 295-308

78. Hughes, J. (1983) Brit. Med. Bull. 39. 17-24

79. Hughes, J., Smith, T., Morgan, B. and Fothergill, L. (1975) Life Sci. 16. 1753-1758

80. Hughes, J., Smith, T.W., Kosterlitz, H.W., Fothergill, L.A., Morgan, B.A. and Morris, H.R. (1975) Nature 258. 577-579

81. Hui, K.S., Gioannini, T., Hui, M. Simon, E.J. and Lajtha, A. (1985) Neurochem. Res. 10. 1047-1058

82. Hutchinson, M., Kosterlitz, H.W., Leslie, F.M., Waterfield, A.A. and Terenius, L. (1975) Br. J. Pharmacol. 55. 541-46

83. Iadarola, M.J., Panula, P., Majane, E.A. and Yang, H. Y-T. (1985) Brain Res. 330. 127-134

84. James, I.F., Fischli, W. and Goldstein, A. (1984) J. Pharmacol. Exptl. Thera. 288. 88-93

85. Jocelyn, P.C. (1987) in Methods in Enzymology: Sulphur and Sulphur Amino-Acids [Ed. Jackoby, W.B. and Griffith, O.W.] 143. 44-67 (Academic Press)

86. Jocelyn, P.C. (1987) in Methods in Enzymology: Sulphur and Sulphur Amino-Acids [Ed. Jackoby, W.B. and Griffith, O.W.] 143. 246-256 (Academic Press)

87. Kakidani, H., Furutani, y., Takahashi, H., Noda, M., Morimoto, Y., Hirose, T., Asai, M., Inayama, S., Nakanishi, S. and Numa, S. (1982) Nature 298. 245-249

88. Kosterlitz, H.W. and Paterson, S. (1985) Phil. Trans. R. Soc. Lond. Series B 308. 291-297

89. Lahti, R.A., Mickelson, M.M., McCall, J.M. and Von Voigtlander, P.F. (1985) Eur. J. Pharmacol. 109. 281-284

90. Lemoine, H. and Kaumann, A.J. (1983) Naunyn-Schmiedeberg's Arch. Pharmacol. 322. 111-120

91. Leslie, F.M. and Goldstein, A. (1982) Neuropeptides 2. 185-196

92. Llorens, C. and Schwartz, J-C. (1981) Eur. J. Pharmacol. 69. 113-116

93. Long, J.B., Kinney, R.C., Malcolm, D.S., Graeber, G.M. and Holaday, J.W. (1987) Brain Res. 436. 374-381

94. Long, J.B., Martinez-Arizala, A., Echevarria, E.E., Tidwell, R.E. and Holaday, J.W. (1988) Eur. J. Pharmac. 153. 45-54

95. Lord, J.A.H., Waterfield, A.A., Hughes, J. and Kosterlitz, H.W. (1976) in Opiates and Endogenous Opioid Peptides [Ed. Kosterlitz, H.W.] 275-280

96. Lord, J.A.H., Waterfield, A.A., Hughes, J. and Kosterlitz, H.W. (1977) Nature 267. 495-499

97. Majane, E.A., Idarola, M.J. and Yang, H-Y. (1983) Brain Res. 280. 95-103

98. Malfroy, D., Swerts, J.P., Guyon, A., Roques, B.P. and Schwartz, J.C. (1978) Nature 276. 523-526

99. Marks, N., Grynbaum, A. and Neidle, A. (1977) Biochem. Biophys. Res. Commun. 74. 1552-1559

100. Martin, W.R. 1967 Pharmacological Reviews 19. 463-521

101. Martin, W.R. and Gordetzky, C.W. (1965) J. Pharmacol. Exptl. Thera. 150. 437-442

102. Martin, W.R., Eades, C.G., Thompson, J.A., Huppler, R.E. and Gilbert, P.E. (1976) J. Pharmacol. Exptl. Thera. 197. 517-532

103. Martin, W.R., Eades, C.G., Thompson, W.O., Thompson, J.F. and Flanary, H.G. (1974) J. Pharmacol. Exptl. Thera. 189. 759-771

104. Martin, W.R., Faser, H.F., Gorodetzky, C.W. and Rosenburg, D.E. (1965) J. Pharmacol. Exptl. Thera. 150. 426-436

105. Matsas, R., Fulcher, I.S., Kenny, A.J. and Turner, A.J. (1983) Proc. Natl. Acad. Sci. 80. 3111-3115

106. McDowell, J. & Kitchen, I. 1987. Brain Res. 434 397-421 39

107. McKnight, A.T., Corbett, A.D. and Kosterlitz, H.W. (1983) Eur. J. Pharmac. 86. 393-402

108. Millar, L., Rance, M.J., Shaw, J.S. and Traynor, J.R. (1985) Eur. J. Pharmac. 116. 159-163

109. Nagy, A., Graf, L. and Lajtha, A. (1983) Life Sci. 33. 835-840

110. Nagy, J.I., Vincent, S.R., Staines, W.A., Fiberger, H.C. Reisine, T.D. and Yamamura, H.I. (1980) Brain Res. 186. 435-444

111. Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A.C.Y., Cohen, S.N. and Numa, S. (1979) Nature 278. 423-427

112. Ninkovic, M., Hunt, S.P. and Kelly, J.S. (1981) Brain Res. 230. 111-119

113. Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Hirose, T., Inayama, S., Nakanishi, S. and Numa, S. (1982) Nature 295. 202-206

114. Nyberg, F., Nordstrom, K. and Terenius, L. (1985) Biochem. Biophys. Res. Commun. 131. 1069-1074

115. Oka, T., Negishi, K., Suda, M., Matsumiya, T., Inazu, T. and Ueki, M. (1980) Eur. J. Pharmac. 73. 235-236

116. Orlowski, M. and Chu, T.G. (1985) Endocrinology 116. 1418-1425

117. Orlowski, M. and Wilk, S. (1981) Biochem. Biophys. Res. Commun. 101. 814-822

118. Orlowski, M. and Wilk, S. (1981) Biochemistry 20. 4942-4950

119. Orlowski, M., Michaud, C. and Chu, T.G. (1983) Eur. J. Biochem. 135. 81-88

120. Orlowski, M., Michaud, C. and Molineaux, C.J. (1988) Biochemistry 27. 597-602

121. Pasternak, G.W., Goodman, R. and Snyder, S.H. (1975) Life Sci. 16. 1765-1769

122. Patey, G., Dela Baume, S., Gros, C. and Schwartz, J-C. (1980) Life Sci. 27. 245-252

123. Paterson, S.J., Robson, L.E.and Kosterlitz, H.W. (1983) Brit. Med. Bull. 39. 31-36

124. Pert, C.A., Pasternak, G.W. and Snyder, S.H. 1973 Science 182. 1359-1361

125. Pert, C.B. and Snyder, S.H. 1973 Proc. Natl. Acad. Sci. 70. 2243-2247

126. Pert, C.B. and Snyder, S.H. 1973 Science 179. 1011-1014

127. Portoghese, P.S., Lipkowski, A.W. and Takemori, A.E. (1987) Life Sci. 40. 1287-1297

128. Przewlocki, R., Gramsch, C., Pasi, A. and Herz, A. (1983) Brain Res. 280. 95-103

129. Przewlocki, R., Shearman, G.T. and Herz, A. (1983) Neuropeptides 3. 233-240

130. Rezvani, A., Hollt, V. and Leong-Way, E. (1983) Life Sci. 33. 271-274

131. Robson, L.E., Foote, R.W., Maurer, R. and Kosterlitz, H.W. (1984) Neurosci. 12. 621-627

131a. Roques, B.P., Fournie-Zaluski, M.C., Soroca, E., Leconte, J.M., Malfroy, B., Llorens, C. and Schwartz, J.C. (1980) Nature 288. 286-288

132. Llorens, C. and Schwartz, J.C. (1980) Nature 288. 286-286

133. Sanchez-Blazquez, P., Garzon, J. and Lee, N.M (1984) Eur. J. Pharmacol. 98. 389-396

134. Sawynok, J., Labella, F.S. and Pinsky, C. (1980) Brain Res. 181 483-486

135. Schmauss, C. and Yaksh, T.L. (1984) J. Parmacol. Exptl. Thera. 228. 1-12

136. Schmauss, C., Shimohigashi, Y., Jensen, T.S., Rodbard, D. and Yaksh, T.L. (1985) Brain Res. 337. 209-215

137. Schwartz, J.C., Malfroy, D. and De la Baume, S. (1981) Life Sci. 29. 1715-1740

138. Seizinger, B.R., Grimm, C., Hollt, V. and Herz, A. (1984) J. Neurochem. 42. 447-457

139. Seizinger, B.R., Liebisch, D.C., Grimm, C. and Herz, A. (1984) Neuroendocrinology 39. 414-422

140. Seizinger, B.R., Maysinger, D., Hollt, V., Grimm, C. and Herz, A. (1982) Life Sci. 31. 1757-1760

141. Shook, J.E., Kazmierski, W., Lemcke, P.K., Hruby, V.J. and Burks, T.F. (1988) J. Pharmacol. Exptl. Ther. 246. 1018-1025

142. Simon, E.J., Hiller, J.M. and Edelman, I. 1973 Proc. Natl. Acad. Sci 70. 1947-1949

143. Slater, P. and Patel, S. (1983) Eur. J. Pharmacol. 92. 159-160

144. Smith, C.F. (1987) Life Sci. 40. 267-274

145. Smith, C.F.C. and Rance, M.J. (1983) Life Sci. 72. 327-330

146. Spain, J.W., Roth, B.L. and Coscia, C.J. (1985) J. Neurosci. 5. 584-588

147. Stevens, C.W. and Yaksh, T.L. (1986) J. Pharmacol. Exptl. Thera. 238. 833-838

148. Suda, H., Aoyagi, T., Takeushi, T. and Omezawa, H. (1976) Arch. Biochem. Biophys. 117. 196-200

149. Suh, H.H. and Tseng, L-F. (1988) J. Pharmacol. Exptl. Ther. 245. 587-593

150. Takemori, A.E., Ho, B.Y., Nasseth, J.S. and Portoghese, P.S. (1988) J. Pharmacol. Exptl. Ther. 246 255-258

151. Tam, S.W. (1983) Proc. Natl. Acad. Sci. 80. 6703-6707

152. Taquet, H., Javoy-Aquid, F., Giraud, P., Legrand, J.C., Agid, Y. and Cesselin, F. (1985) Brain Res. 341. 390-392

153. Terenius, L. 1973 Acta Pharmacol. et Toxicol. 32. 317-320

154. Toffoletto, O., Metters, K.M., Camargo, C.M. and Rossier, J. (1988) Biochem. J. 252. 35-38

155. Traynor, J.R. and Wood, M.S. (1987) Neuropeptides 10. 313-320

156. Tsang, D. and Ng, S.C. (1980) Brain Res. 188. 199-206

157. Tsang, D., Ng, S.C., Ho, K.P., Ho, W.K.K. and Dev, B.R. (1982) Dev. Brain Res. 5. 257-261

158. Tseng, L.F., Cheng, S.S. and Fujimoto, J.M. (1983) J. Pharmacol. Exptl. Thera. 244. 51-54

159. Tseng, L.F., Higgino, M.J., Hong, J.S., Hudson, P.M. and Fujimoto, J.M. (1985) Brain Res. 343. 60-69

160. Udenfriend, S. and Kilpatrick, D.L. (1984) in The Peptides [Ed. E. Gross] vol. 6. 25-68 (Academic Press)

161. Van Buren, K.J.H., Van Amsterdam, J.G.C., Mulder, J.R.A. and Soudijn, W. (1985) Neuropeptides 6. 381-389

162. Wagner, F. (1988) in Methods in Enzymology: Metallobiochemistry [Ed. Riordan, J.F. and Vallee, L.B.] 158. 21-32 (Academic Press)

163. Waksman, G., Bouboutou, R., Devin, J., Bourgoin, S., Cesselini, F., Hamon, M., Fournie-Zaluski, M-C. and Roques, B.P. (1985) Eur. J. Pharmacol. 117. 233-243

164. Walker, J.M., Moises, H.C., Coy, D.H., Baldrighi, G. and Akil, H. (1982) Science 218. 1136-1138

165. Watson, S.J., Akil, H., Richardson, C.W. and Barchas, J.D. (1978) 275. 226-228

166. Weber, E., Roth, K.A. and Barchas, J.D. (1981) Biochem. Biophys. Res. Commun. 103. 951-958

167. Weber, E., Roth, K.A. and Barchas, J.D. (1982) Proc. Natl. Acad. Sci. 79. 3062-3066

168. Weihe, E., Millan, M.J., Leibold, A., Nohr, D. and Herz, A. (1988) Neurosci. Letts. 85. 187-192

169. Wong, D.T. and Hong, J.S. 1973 Life Sci. 13. 1543-1556

170. Wood, P.L. (1982) Neuropharmacol. 21. 487-497

171. Yaksh, T.L. (1983) J. Parmacol. Exptl. Thera. 226. 303-316

172. Yaksh, T.L. and Noueihed, R. (1985) Ann. Review Pharmacol. Toxicol. 25. 433-462

173. Young, E.A., Walker, J.M., Houghten, R. and Akil, H. (1987) Peptides 8. 701-707

174. Zajac, J.M., Ling, N., Rossier, J. and Roques, B.P. (1983) Eur. J. Pharmacol. 90. 147-148

175. Zamir, N., Palkovits, M., Weber, E. and Brownstein, M.J. (1984) Brain Res. 300. 121-127

176. Zamir, N., Palkovits, M., Weber, E., Mezey, E. and Brownstein, M.J. (1984) Nature 307. 643-645

177. Zamir, N., Quirion, R. and Segal, M. (1985) Neuroscience 15. 1025-1034

178. Zamir, N., Weber, E., Palkovits, M. and Brownstein, M. (1984) Proc. Natl. Acad. Sci. 81. 6886-6889

179. Kosterlitz, H.W. and Watt, A.J. (1968) Br. J. Pharmacol. 33. 266-270

APPENDIX

This appendix contains the tabulated data from which the graphs in Chapters 3-6 were constructed.
The first number of each table refers to the chapter in which the respective graph can be found.
For abbreviations see front of thesis.

Incubation Time (min)	% Recovered Radioactivity		
	N-Terminal	[Leu]Enkephalin	Dynorphin 1-8
0	0.8 ± 0.4	0.3 ± 0.2	98.3 ± 0.9
0.5	12.0 ± 3.6	6.0 ± 1.4	79.7 ± 5.2
1	18.8 ± 4.1	9.9 ± 2.5	68.8 ± 5.1
2	28.3 ± 3.3	16.8 ± 3.4	50.3 ± 5.1
5	47.6 ± 4.6	22.5 ± 3.5	24.5 ± 5.2
10	63.5 ± 3.5	21.6 ± 3.3	7.9 ± 2.3
20	81.4 ± 4.1	11.0 ± 3.3	2.4 ± 1.3

Table 3.1 Time course of metabolism of [^{3}H]dynorphin 1-8 by slices of rat spinal cord

n = 6

Incubation Time (min)	% Recovered Radioactivity		
	N-Terminal	[Leu]Enkephalin	Dynorphin 1-8
0	0.0 ± 0.0	5.83 ± 3.9	91.7 ± 4.2
0.5	1.2 ± 1.0	6.7 ± 2.3	90.7 ± 3.8
1	1.2 ± 0.6	14.2 ± 5.0	84.0 ± 6.9
2	2.4 ± 1.1	20.7 ± 5.9	70.3 ± 9.8
5	4.8 ± 1.6	34.6 ± 6.5	50.7 ± 9.5
10	5.9 ± 2.2	60.5 ± 2.2	16.2 ± 4.2

Table 3.2 Time course of metabolism of [^{3}H]dynorphin 1-8 by rat spinal cord in the presence of 10μM bestatin; 10μM captopril; 0.3μM thiorphan and 2mM leucyl-leucine.

n = 5

Tissue	% Recovered Radioactivity		
	N-terminal	[Leu]Enkephalin	Dynorphin 1-8
Cortex	5.3 ± 1.0	67.0 ± 3.6	17.8 ± 5.0
Striatum	4.6 ± 0.8	52.4 ± 3.0	33.5 ± 2.2
Hypothalamus	4.6 ± 0.6	45.7 ± 6.7	42.2 ± 6.2
Hippocampus	5.0 ± 1.0	57.4 ± 4.0	30.3 ± 5.3
Medulla	6.0 ± 2.6	49.0 ± 1.1	37.7 ± 3.2
Cerebellum	15.5 ± 2.9	57.8 ± 3.4	17.3 ± 1.3
Spinal Cord	5.9 ± 1.0	50.25 ± 6.1	32.2 ± 8.4

Table 3.3 Metabolism of [^{3}H]dynorphin 1-8 by various areas of the rat c.n.s. in the presence of the inhibitor cocktail (bestatin 10μM; captopril 10μM and thiorphan 0.3μM) following a 10min incubation period.

n = 4

Metabolite	% Recovered Radioactivity
N-terminal	49.5 ± 3.2
[Leu]Enkephalin	35.2 ± 11.7
Dynorphin 1-8	6.3 ± 2.1

Table 3.4 Metabolism of [^{3}H]dynorphin 1-8 by guinea-pig cerebellum following a 10min incubationperiod.

n = 9

Metabolite	% Recovered Radioactivity
N-terminal	22.2 ± 2.0
[Leu]Enkephalin	55.9 ± 2.4
Dynorphin 1-8 + Inhibitor Cocktail	8.5 ± 1.3

Table 3.5 Metabolism of [^{3}H]dynorphin 1-8 by guinea-pig cerebellum in the presence of the inhibitor cocktail (bestatin 10μM, captopril 10μM and thiorphan 0.3μM) following a 10 min incubation period.

n = 9

Incubation Time (min)	% Recovered Radioactivity		
	N-terminal	[Leu]Enkephalin	Dynorphin 1-8
0	3.1 ± 0.2	0.4 ± 0.4	96.3 ± 0.5
2.5	40.1 ± 2.3	9.7 ± 0.4	44.9 ± 4.6
5	55.3 ± 5.0	12.7 ± 0.6	25.1 ± 8.4
10	80.5 ± 4.2	8.2 ± 1.2	7.0 ± 3.0
20	96.3 ± 1.5	3.3 ± 0.3	1.3 ± 0.4

Table 3.6 Time course of metabolism of [^{3}H]dynorphin 1-8 by slices of guinea-pig myenteric plexus longitudinal muscle.

n = 3

Incubation Time (min)	% Recovered Radioactivity		
	N-Terminal	[Leu]Enkephalin	Dynorphin 1-8
0	1.2 ± 0.3	0.5 ± 0.5	98.3 ± 0.4
2.5	4.7 ± 0.6	12.9 ± 3.8	79.6 ± 4.0
5	7.4 ± 0.8	24.4 ± 4.2	57.7 ± 5.7
10	14.9 ± 1.6	34.2 ± 1.2	38.2 ± 8.0
20	31.4 ± 4.9	41.7 ± 1.0	14.0 ± 3.8

Table 3.7 Time course of the metabolism of [^{3}H]dynorphin 1-8 by slices of guinea-pig myenteric plexus longitudinal muscle in the presence of: bestatin, 10μM, captopril, 10μM and thiorphan, 0.3 μM.

n = 3

Competing Drug	% Inhibition of [^{3}H][Leu]Enkephalin Production	% Increase in Recoverable [^{3}H]Dynorphin
[Leu]Enkephalin	5.56 ± 4.56	1.30 ±1.30
[Met]Enkephalin	2.17 ± 2.17	0.53 ± 0.53
Dynorphin 1-6	5.92 ± 4.09	15.45 ± 6.92
Dynorphin 1-7	22.8 ± 9.84 *	35.10 ± 17.56 *
Dynorphin 1-8	52.91 ± 5.54 *	84.48 ± 6.15 *
[Met]Enkephalyl-Arg^6-Gly^7-Leu^8	52.1 ± 2.50 *	96.64 ± 3.81 *
Dynorphin 1-9	51.0 ± 5.62 *	82.7 ± 6.12 *
Dynorphin 1-13	88.8 ± 3.52 *^	158.6 ± 14.3 *^
Dynorphin 1-17	8.6 ± 4.3	1.6 ± 1.6
ß-Endorphin	N.E.	N.E.
DAGOL	N.E.	N.E.
DADLE	N.E.	N.E.
Morphine	N.E.	N.E.

Table 4.1 The effect of various peptides and drugs on the ability of rat spinal cord slices to produce [^{3}H][Leu]enkephalin from [^{3}H]dynorphin 1-8.
N.E. = No Effect
n = 4

* P < 0.05 cp to control
*^ P < 0.01 cp to control

Students t-test

Inhibitor	Concentration (mM)	% Inhibition of [Leu]Enkephalin Production	
		TRIS	Krebs/Hepes
PMSF	1	6.85 ± 4.4[a]	N.T.
DTT	2	42.22 ± 17.2*	46.1[b]
NEM	1	45.83 ± 13.7*	56.45 ± 11.8*
pCMB	0.2	92.2 ± 2.17 *^	93.2 ± 3.83*^
o-Phen	1	28.16 ± 8.6*	60.49 ± 30.3*
EDTA	1	0.0 ± 0.0	0.0 ± 0.0

Table 4.2 Inhibition of [^{3}H][Leu]enkephalin production from [^{3}H]dynorphin 1-8 following a 10 min incubation period, by rat spinal cord tissue preincubated with the inhibitor cocktail (bestatin 10μM; captopril 10μM and thiorphan 0.3μM) and a variety of chemical inhibitors, in either TRIS buffer or Krebs buffered with Hepes (for abbreviations see text).

n = 3 except where indicated.

a, n = 6

b, n = 2

N.T. = Not Tested

* $P < 0.05$ cp to control

*^ $P < 0.01$ cp to control

Students t-test

Agonist	IC_{50} (nM)	Naloxone Ke	Slope	n
U50488H	1.5 ± 0.14	19.62 ± 1.4	S.D.	6
U69593	2.8 ± 0.47	31.4 ± 9.69	0.98 ± 0.08	4
Dynorphin 1-17	0.32 ± 0.02	33.55 ± 1.02	1.07 ± 0.03	4
DAGOL	16.6 ± 3.75	2.09 ± 0.54	0.92 ± 0.04	3
[Leu]Enkephalin + Inhibitor cocktail	36.8 ± 7.0	1.63 ± 0.34	0.94 ± 0.03	8
Dynorphin 1-8	27.37 ± 3.7	14.03 ± 2.4	0.92 ± 0.03	19
Dynorphin 1-8 + Inhibitor cocktail	1.8 ± 0.34	3.01 ± 0.79	0.86 ± 0.08*	9
Dynorphin 1-8 + Inhibitor cocktail + N-(R,S)-ethyl	1.89 ± 0.72	14.31 ± 2.1	S.D.	3
Dynorphin 1-8 + Inhibitor cocktail+ N-(R,S)-propyl	2.77 ± 0.22	37.2 ± 8.3	0.99 ± 0.09	5

Table 5.1 Values of IC_{50}, Ke against naloxone and Schild plot slopes for a variety of ligands in the guinea-pig myenteric plexus longitudinal muscle preparation. * value is significantly different from unity $P<0.05$ Mann Whitney U
n = number of observations
S.D. = single dose method used to calculate Ke

Agonist	IC_{50} (nM)	Ke (nM)	Slope	n
U69 593	2.22 ± 0.21	0.06 ± 0.01	1.07 ± 0.03	4
DAGOL	23.14 ± 3.27	13.03 ± 1.91	1.04 ± 0.08	5
Dynorphin 1-8	19.66 ± 4.2	0.17 ± 0.04	0.98 ± 0.08	4
Dynorphin 1-8 + Inhibitor Cocktail	3.66 ± 0.40	0.62 ± 0.07	1.07 ± 0.03	7
Dynorphin 1-8 + Inhibitor Cocktail + N-(R,S)-phenyl	0.88 ± 0.16	0.098 ± 0.047	0.99 ± 0.12	4

Table 5.2 IC_{50}, Ke against nor-BNI and Schild plot slope values for various agonists on the guinea-pig myenteric plexus longitudinal muscle preparation.

n = number of experimental observations

Agonist	IC_{50} (nM)	Ke (nM)	Slope	n
U69 593	2.56 ± 0.62	85.8 ± 14.4	0.91 ± 0.05	4
DAGOL	10.18 ± 2.5	3.5 ± 1.2	0.91 ± 0.05	4
Dynorphin 1-8	42.9 ± 11.0	76.9 ± 11.1	0.91 ± 0.03	4
Dynorphin 1-8 + Inhibitor Cocktail	1.7 ± 0.3	31.4 ± 8.8	0.79 ± 0.01*	4
Dynorphin 1-8 + Inhibitor Cocktail + N-(R,S)-propyl	2.8 ± 0.6	208.5 ± 70.3	1.05 ± 0.05	4

Table 5.3 IC_{50}, Ke against M8008 and Schild plot slope values for various agonists on the guinea-pig myenteric plexus longitudial muscle preparation.

n = number of experimental observations

* = significantly different from unity $P<0.05$ (Mann-Whitney U test)

Agonist	IC_{50} (nM)		Dose Ratio	n
	Before ß-FNA	After ß-FNA	(after/before)	
DAGOL	12.3 ± 1.7	263.4 ± 55.8	26.1 ± 7.5	12
U50 488H	1.73 ± 0.56	1.96 ± 0.43	1.08 ± 0.08	4
[Leu]Enkephalin + Inhibitor Cocktail	33.0	551.5	15.9	2
Dynorphin 1-8	18.56 ± 5.6	11.6 ± 2.5	0.61 ± 0.08	4
Dynorphin 1-8 + Inhibitor Cocktail	1.08 ± 0.22	2.53 ± 0.23	2.57 ± 0.35	6
Dynorphin 1-8 + Inhibitor Cocktail+ N-(R,S)-phenyl	2.08 ± 0.13	1.75 ± 0.08	0.8 ± 0.05	3

Table 5.4 The effect of ß-BFNA (100nM for 1hr) on the IC_{50} values of a variety of opioid agonists.on the guinea-pig myenteric plexus longitudinal muscle

n = number of experimental observations