# BEHAVIORAL AND SOMATIC RESPONSES TO PHARMACOLOGICAL AND BIOCHEMICAL MANIPULATION OF THE ENDORPHIN-OPIATE RECEPTOR SYSTEM

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A Thesis

Presented to the

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In Partial Fulfillment

of the Requirements for the Degree

of Doctor of Philosophy

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by

ASHOK K. DUA

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To
Sushma
and
My Parents

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As this boat has arrived at the bank the memory of the whole journey and midstream turbulence, I am reminded of those people who helped me in sailing it on the river. My sincere thanks are due to:

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ABSTRACT

More than ten years have elapsed since the first serious postulation of and early evidence for the existence of endogenous opioids--now known as enkephalins and endorphins--in the mammalian brain. Evidence is still being sought intensively for a physiological role, or for a syndrome of deficiency or excess, associated with these substances. A search for such evidence was a prime objective of the work done for this thesis. In pursuing that objective it was demonstrated that enzymatic degradation of opioid peptides, a major limitation to our understanding of the action and effects of endogenous and of exogenously-administered endorphins and enkephalins, can be overcome by treatment in vivo with molecular site-selective inhibitors of peptides and proteinases. It has been confirmed here in vivo, with predictable behavioral concomitant, that enzymatic degradation of enkephalins may be blocked at specific peptide bond sites by selective and stereospecific inhibitors of peptidase action. Such concept had elsewhere been previously arrived at only from in vitro studies. Enkephalins are degraded at their amino end (Tyr-Gly bond) by an aminopeptidase and at the carboxyl end (Gly-Phe bond) by dipeptidylcarboxypeptidase. Amastatin, a potent and selective aminopeptidase inhibitor, produced significant analgesia when given to rats via intracerebroventricular (ICV) infusion; its stereoisomer, epiamastatin, was inactive. ICV amastatin significantly potentiated a nonanalgesic dose of ICV Met-enkephalin whereas epiamastatin did not. The amastatin-enhanced analgesic response was naloxone-reversible. These observations represent the first although indirect evidence for stereospecific inhibition in vivo of a peptide bond-selective enzyme.

ICV infusion of a highly-selective dipeptidylcarboxypeptidase inhibitor, Phe-Ala, produced significant analgesia by itself. Pretreatment with ICV Phe-Ala significantly enhanced the analgesic effect of ICV Met-enkephalin; the enhanced analgesia was reversed by naloxone. As aminopeptidase and dipeptidylcarboxypeptidase are the two major enkephalin-degrading enzymes, it was hypothesized that the simultaneous inhibiton of these enzymes -- produced by combining the two peptide bond-selective inhibitors -- should produce analgesia by itself and should significantly potentiate the analgesic response of enkephalin. Treatment of rats with amastatin/Phe-Ala produced a significantly higher analgesia compared with epiamastatin/Phe-Ala, saline vehicle control, or each drug given by itself. All analgesic responses were naloxone-reversible. Met-enkephalin, 100 µg, administered to amastatin/Phe-Ala-pretreated rats produced a profound analgesia which was significantly higher than the additive result of the effects of combination of either inhibitor with the enkephalin. The high levels of analgesia observed with the combined inhibitors have never heretofore been reported for opioid pentapeptides. This response, which illustrates a genuine pharmacological potentiation, was naloxone-reversible. The profound analgesia which was achieved by the novel <u>simultaneous</u> protection of the enkephalin molecule at two vulnerable sites strongly suggests that enkephalins are in fact highly analgesic substances whose action is terminated very rapidly by localized specific inactivating enzymes. Possession of such properties would permit these opioid pentapeptides to be regarded as true neurotransmitters, analogous to those found at classical synaptic sites.

Beta-endorphin is resistant to enzymatic degradation at its amino and carboxyl ends while its middle portion is available and sensitive to the action of endopeptidase. This enzyme, so far, is not well characterized. The serine proteinase inhibitors, aprotinin and phenylmethylsulfonyl fluoride (PMSF) produced naloxone-reversible analgesia, each by themselves; also, each inhibitor potentiated beta-endorphin-induced analgesia. PMSF, which is lipid-soluble, produced significant analgesia via systemic administration in rats (AD50 = 2.9 $\pm$ 1.5 (S.E.) mg $kg^{-1}$). Its toxicity as tested intraperitoneally in mice was low (LD50 = 215 $\pm$ 55 mg $kg^{-1}$), hence the use of PMSF as a parenteral analgesic has been suggested. ICV beta-endorphin, 15 µg, administered in rats pretreated with ICV aprotinin 18 ug, caused death due to respiratory arrest in two out of five rats so treated; death due to beta-endorphin is thus reported for the first time. ICV aprotinin 14.5 µg ameliorated morphine withdrawal and an associated epileptiform activity in rats. The withdrawal-associated epileptiform activity was unexpected for morphine dependency, but may correspond to the infantile seizures seen clinically in neonates born to opiate-narcotic dependent mothers.

The foregoing results of experiments done with selective peptidase inhibitors in vivo permit a new and clarifying nomenclature to be proposed for the peptidases important in the inactivation of enkephalins. Since the observations made here now suggest that both aminopeptidase and dipeptidylcarboxypeptidase are of physiological importance specifically in relation to enkephalins it would be appropriate to refer to both enzymes as enkephalinases, and not restrict the term only to the latter, as is currently done. This writer

therefore proposes that aminopeptidase should be designated aminoenkephalinase whereas dipeptidylcarboxypeptidase should be termed carboxy(l)enkephalinase. The proposed nomenclature has the virtue that each name would be descriptive both of the molecular site of enzyme action and of the important physiological role of the enzyme as has been discerned in this thesis.

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## Figures and Legends

## Tables and Legends

# I. INTRODUCTION AND REVIEW OF LITERATURE

Morphine, the prototype opiate narcotic substance, is a fascinating drug. Many believe it to be a gift from nature to human beings, to relieve their pain. A minority of people, however, consider morphine to be evil, due to its addiction liability. Many generations of scientists have engaged in exploring these two properties of morphine, and we do not know how many more generations may continue before the quest will come to a conclusion. Each time a new discovery or aspect of morphine action becomes known, the hope for the final conclusion arises, but with passing of time more questions than answers seem to be raised by these discoveries. The discovery of morphine receptor(s) and of the endogenous morphinelike substances (endorphins) has raised the hope, and it seems, thus far, that this time we hope are somewhat closer to the goal of understanding opiate narcotic action than with previous discoveries related to the pharmacology of morphine.

The explosion of knowledge in the field of endorphins is based on five steps of groundwork, these are: (i) Development of radioreceptor assay for opiate receptors in the brain. (ii) Postulation of endogenous ligands for these opiate receptors. (iii) Isolation of pentapeptides and polypeptides as endogenous ligands for these receptors. (iv) Recognition of the amino acid sequence of Met-enkephalin and beta-endorphin (B-END) within that of beta-Lipotropic hormone (B-LPH). (v) Discovery of a common precursor for ACTH, MSH, B-LPH and B-END.

The groundwork for the development of the opiate receptor assay was laid down quite early by a number of observations which suggested that opiates must act via specific opiate receptors. These observations were: (a) Opiates have been found to produce very characterstic effects even when they are injected in very small quantities (in Hollt and

Wuster, 1978). This suggested that opiates act through a small number of some specific sites. (b) Chemists - skilled in the art and science of organic synthetic medicinal chemistry - were able to identify and modify opioid molecules, thereby permitting detailed studies to be made on the critical structure-activity relationships of opioid narcotic drugs. The early studies in this field showed that only a very particular type of chemical structure is capable of exerting opiatelike effects (in Hollt and Wuster, 1978). Using various of such specific opioid structures, Beckett and Casy (1954) developed configuration-specific molecular modules for a postulated opioid receptor site (this writer holds that the organic/medicinal chemists have been denied of their rightful acclaim for the contributions they have made toward our current concepts of opioid receptors and their endogenous ligands). (c) Pharmacological experiments also with various isolated tissue organs (in Hollt and Wuster, 1978) and with whole animal preparations (ibid.) indicated the necessary requirement of a particular structure to produce a profound opiatelike effect with a very small dose of the structure-specific molecules. As the number of such observations increased, a pressing need was developing for the discovery of an opiate receptor (or, at least, for the identification of an opioid recognition site in the central nervous system). Even a specific assay system to study opioid structure-activity would have been welcomed by investigators in the opiate narcotic field during the mid-1950's. Availability of the opiate receptor antagonist naloxone became one of the major reasons for a relatively faster growth in the field of opiates. This growth was based on the presumption that naloxone is a pure opiate receptor antagonist. However the studies done

in this laboratory and elsewhere (reviewed by Sawynok et al., 1979) showed that naloxone does have its own effects. Now it is well accepted in the literature that it is necessary to show naloxone blockade in order to infer opioid effects. Such blockade, however, is no longer regarded as a sufficient criterion for the involvement of opiate receptors in any given pharmacological response.

The search for an opiate receptor was undertaken by different scientific groups using different approaches, depending on their expertise, background information and on techniques available at the time. Mule and Woods (1962) and later Ingoglia and Dole (1970) and Clouet and Williams (1973), adopted in vivo approaches to study the distribution of opiates after their microinjection into various areas of the brain. These studies were, however, inconclusive due to certain limitations of the techniques. On the other hand in vitro approaches were taken by Van Praag and Simon (1966), Kosterlitz et al. (1972), Scrafani et al. (1969), Hollt and Teschemacher (1972), and by Seeman et al. (1972). In these experiments attempts were made to demonstrate opiate receptors in nervous tissues. The reason for failure of these early experiments was, to a large extent, the nonavailability of very specific radioactive ligands which could have demonstrated a very specific binding. The first successful attempt to overcome the problem of nonspecific binding was made by Goldstein et al. (1971), who were able to show a specific binding of 2% in mouse brain. Two years elapsed while many laboratories sought to overcome the technical problems involved, and to refine the techniques. By 1973 four groups (Pert and Snyder, 1973; Simon et al., 1973; Wong and Hrong, 1973; Terenius, 1973) were able independently to demonstrate highly-specific opiate receptor

binding. The use of highly-specific radioactive ligands enabled these groups to use a very small quantity of radioactive ligand, which resulted in the desirable very low nonspecific binding.

The work of Goldstein et al. (1971) regarding the specific opiate receptor was not conclusive, but people working in this area of research saw it as the dim light of a sole and distant lighthouse; it was felt by a few farsighted workers that there had to be a physiological role for the putative opiate receptors, provided by a very parsimonious Mother Nature. In 1971, H.O.J. Collier postulated in public the existence of an endogenous neurohumoral factor which could interact with these receptors (Collier, 1972). He declared that he could not imagine a natural receptor existing primarily to recognize molecules of a substance that is foreign to the body (Collier, 1972). This elegant hypothesis gained experimental support from different laboratories, some of which are briefly mentioned here. Akil et al. (1972) found that analgesia could be produced by electrical stimulation of certain areas of the periaqueductal gray of the midbrain in the rat. This effect could be partially blocked by the opiate antagonist naloxone (Akil et al., 1976a, 1976b). Jacob et al. (1974) showed that mice injected with naloxone displayed hyperalgesia when they were subjected to a noxious stimulus. This laboratory (Gigliotti and Pinsky, 1974) showed independently, almost at the same time, that naloxone increased nociception in mice subjected to circumthreshold footshock stimulus. Lal et al. (1974) postulated that reversal of morphine withdrawal hyperthermia by a conditional stimulus may be due to release of activated endogenous opioid at sites intimately associated with thermoregulation. These landmark experiments and hypotheses set the stage for the isolation of endogenous ligands for opiate receptors.

To arrive conclusively at the sought-for endogenous agent, investigators had to decide upon a method to purify a chemical from the brain extract, the method depending on the nature of the chemical. Three types of chemicals were considered as candidates for the endogenous opioid ligand: (a) an alkaloid with an opiate structure (b) a peptide/polypeptide (c) a steroid. Another technical difficulty was to decide on a reliable bioasssay to test opiate activity of the outcome of these isolation experiments.

Various groups of workers looked into all three possible chemical species by different methods. The first attempt to isolate an endogenous opioid, was made by Goldstein's group in 1973 (Goldstein, 1973). In this attempt brain tissue was extracted with techniques appropriate to the isolation of a molecule with an opiatelike alkaloid structure. Unfortunately, the extract had no opiate activity in various bioassays. An attempt to consider steroids as endogenous opiates, was made by Pinsky and his co-workers in 1975 (Pinsky et al., 1975). This group used an in vivo (surgical) approach, removing all glandular source of steroid (gonadectomy, adrenalectomy) and testing these animals with a mildly noxious stimulus of 44.5$^{0}$C warmplate. Their "steroidectomized" animals were much less sensitive to morphine analgesia than were adrenalectomized or sham-operated controls. They suggested that endogenous steroids might act as modulators at opiate receptor sites, but did not pursue their hypothesis in detail. More recent work by other investigators also has suggested that steroids may modify opioid action (Mendelson and Mello, 1978).

The first successful attempt in the search for an endogenous opiate was reported by John Hughes, working out of the laboratory of H.

Kosterlitz in Aberdeen, at a meeting of Neurosciences Research Program in Boston in May, 1974 (Hughes, 1975a). Hughes described the extraction of a crude extract of rat brain in 80% acetone. The bioassay used to test this substance was guinea pig ileum longitudinal muscle with myenteric plexus preparation and the isolated mouse vas deferens; bioassay preparations that had been worked out meticulously by Kosterlitz, over the some ten years preceding the announcement by Hughes (Kosterlitz et al., 1972; Kosterlitz, 1975). It was the guinea-pig ileum that served as an active test object for the stereospecific structure-activity relationships of opioid agonist and antagonists at very small doses (Kosterlitz and Waterfield, 1975). Hughes (1975a) observed the inhibition of electrically-induced contraction in mouse vas deferens preparation and utilized it skillfully in directing the extraction procedure of opiatelike substance in brain extract. In the same year Terenius and Wahlstrom (1975), reported an opiatelike activity in an acidic aqueous extract of rat brain and guniea-pig ileum, in the dihydromorphine binding assay. Pasternak et al. (1975) used a different testing system for checking the opiatelike activity in acidic aqueous extract. They employed an opiate binding assay using 3H-naloxone as a ligand.

Beginning in 1975 the following characteristics of endogenous opiate ligands became known: (1) The extract was water-soluble, polyionic in character and resistant to heat and acid degradation. (2) The extract inhibited the binding of opiate receptors in the opiate receptor assay and this inhibition was a competetive one (Terenius and Wahlstrom, 1975). (3) Binding of the endogenous substance to the opiate receptor was increased in the presence of $Mn^{++}$ and decreased by $Na^{+}$

(Pasternak et al., 1975). (4) The distribution of endogenous material in the brain correlated well with that of opiate receptors (Pasternak et al., 1975; Hughes, 1975b). (5) The material was found in various species, for example: in the brains of pigs (Bradbury et al., 1976a), sheep (Chretien et al., 1976a), camel (Li and Chung, 1976), guinea-pigs (Hughes, 1976c) and calves (Pasternak et al., 1975), and in CSF of humans (Terenius and Wahlstrom, 1975). The latest additions to this growing list include the spinal cord of cat (Su et al., 1980), human pituitary (Li et al., 1976b), bovine adrenal medulla (Schultzberg et al., 1978), human prostate (Vallesti et al., 1980), salmon pituitary (Kawauchi et al., 1980), and neurons of invertebrates, e.g. earthworm (Aluments et al., 1979), and amoeba (Josefson and Johansson, 1979). (6) The potency of this endogenous substance in bioassay decreased with incubation time, suggesting degradation of the endogenous ligand (Terenius, 1975) and this was later found to be due to various peptidases. These findings made it urgent to purify and characterize its structure, which was suspected to be that of peptide(s).

Once the nature of the extract became clear, considerable efforts were directed to purify the peptide(s). The process of isolation was aided greatly by the avilable ultramodern instrumentation for such sophisticated techniques as ultrafiltration and high pressure ("high performance") liquid chromatography (HPLC). Elucidation of the peptide structure was speeded up by sequential degradation and mass spectrometry (Hughes et al., 1975).

A review of the present knowledge -- accumulated for this presentation -- has resulted in the summary of biochemical pathways for the formation and degradation, presented in Fig.1. The reader is

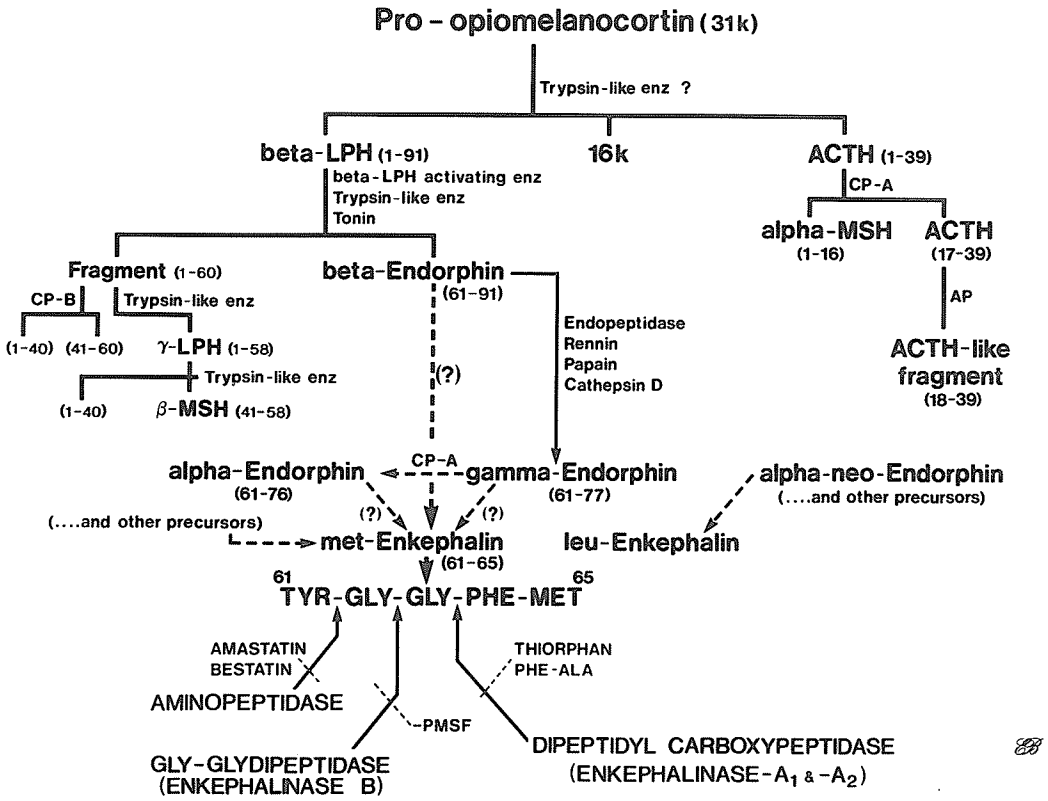
Pro - opiomelanocortin (31k)
Trypsin-like enz ?
beta-LPH (1-91)
16k
ACTH (1-39)
beta-LPH activating enz
Trypsin-like enz
Tonin
CP-A
alpha-MSH (1-16)
ACTH (17-39)
Fragment (1-60)
beta-Endorphin (61-91)
CP-B
Trypsin-like enz
AP
(1-40)
(41-60)
γ-LPH (1-58)
Endopeptidase
Rennin
Papain
Cathepsin D
Trypsin-like enz
(?)
ACTH-like fragment (18-39)
(1-40)
β-MSH (41-58)
CP-A
alpha-Endorphin (61-76)
gamma-Endorphin (61-77)
alpha-neo-Endorphin
(....and other precursors)
(?)
(?)
(....and other precursors)
met-Enkephalin
leu-Enkephalin
(61-65)
61
65
TYR-GLY-GLY-PHE-MET
AMASTATIN
BESTATIN
THIORPHAN
PHE-ALA
AMINOPEPTIDASE
PMSF
GLY-GLYDIPEPTIDASE (ENKEPHALINASE B)
DIPEPTIDYL CARBOXYPEPTIDASE (ENKEPHALINASE-A1 & -A2)

Fig. 1

Fig. 1. Pro-opiomelancortin metabolic pathways. AP = aminopeptidase; CP-A = carboxypeptidase-A; CP-B = carboxypeptidase-B. Amastatin, bestatin, Phe-Ala, PMSF and thiorphan are effective inhibitors at the indicated locations in metabolism of the 61-65 fragment (Met-enkephalin), as developed in the body of this thesis.

cautioned here that many steps in the pathways illustrated were elucidated in separate experiments by different investigators; this should be kept in mind before making inferences or extrapolations to further concepts from the scheme presented.

The term pro-opiocortin, to designate the protein that is a common precursor of opioid peptides and corticotropin, was first coined by Rubenstein et al., in 1978. Later on Chretien et al. (1979a) suggested that it should be called pro-opiomelanocortin, as this precursor contained also the sequence of the peptide melanophore-stimulating factor (MSF). Interestingly, the evidence to suggest a common precursor had been available long before this, and other lines of evidence were added on with time. These evidences were that: (1) most physiological and pathological conditions, or some stimuli, that alter ACTH release from the pituitary will alter B-LPH release in a parallel manner (Jones et al., 1977; Li and Chung, 1976b). (2) Ectopic ACTH-secreting tumors usually secrete peptides with B-MSH immunoreactivity (Roberts and Herbert, 1977; Palletier, 1973). (3) Immunochemical studies have shown that B-LPH, endorphins and ACTH occur in the same pituitary cells (Moriarty, 1973; Dubois et al., 1973; Bloom et al., 1977; Phifer, 1974) and within the same secretory granules (Moriarty, 1973). (4) Studies on human pituitary extracts suggested that antigenic determinants for ACTH and LPH occur in the same molecule (Lowry et al., 1974). (5) Identification and characterization of corticotropin peptide in the precursor molecule was done by isolating mRNA from cultures of At T 20/D-16V tumor cells and translating this in a mRNA-dependent reticulocyte cell-free system. A glycosylated product of molecular weight 31,000 ("big" ACTH) was isolated (Eipper et al., 1976). (6) When

the 30,000 molecular weight precursor was digested with trypsin, B-LPH and a peptide B-LPH(61-65) was obtained. These were identical to the fragments obtained by trypsin digestion of B-LPH or B-END, hence the 30K peptide is a precursor to endorphin and Met-enkephalin (however, the latter is disputable; Rubenstein et al., 1978). (7) Recently, the mRNA coding for pro-ACTH/endorphin has been purified from the bovine intermediate lobe (Kita et al., 1979). (8) A common precursor to corticotropin and endorphin was found in mouse tumor cells, by double-antibody immunoprecipitation technique with antisera to ACTH and endorphin (Mains et al., 1977, Mains and Eipper 1976). The enzyme which cleaves pro-opiocortin into B-LPH and ACTH has not as yet been purified. However, several studies have shown that tryptic treatment yields B-LPH and ACTH (Roberts and Herbert, 1977; Lewis et al., 1978; Rubenstein et al., 1978). (9) Weber et al. (1979) used the immunostaining technique to study the rat anterior pituitary and found that corticotropin, B-LPH, B-END and 16K fragments are all stored in the same cells of the anterior pituitary. The 16K fragment corresponds to the non-B-LPH-non-corticotropin part of corticotropin/B-endorphin precursor (Mains and Eipper, 1978; Eipper and Mains, 1978). (10) When At T-20 cells are labelled with radioactive amino acids, radioactive forms of the ACTH endorphin precursor are resolved by Na-dodecyl sulfate polyacrylamide gel electrophoresis, with apparent molecular weights of 29,000, 32,000 and 34,000 daltons (Roberts and Herbert 1977a).

Pulse-labelling studies with radioactive amino acids suggest that the first form of the ACTH endorphin precursor to be labelled in this cell is 29K ACTH and it appears to be a precursor of the 32K and 34K

form of ACTH endorphin. The difference in apparent molecular weights of these forms are very likely due to differing amounts of glycosylation attached after the peptide chain is formed (Hughes et al., 1980). All three forms of the precursor contain both the ACTH and endorphin antigenic determinants, suggesting a similarity in their peptide structure.

In 1979 Chretien et al. presented and reviewed much evidence in favour of the existence of a single common precursor for endorphin, ACTH and melanocortin. They suggested that it should be called pro-opio-melanocortin, an extension of the term proposed by Rubenstein et al. in 1978 (op cit.). Chretien's proposed nomenclature includes reference to all the important biological components of the postulated precursor. He proposed that B-END is liberated as the result of a maturation process of the larger precursor with B-LPH being an important transient intermediary. A site for cleavage of the large peptide into smaller ones is well provided for by the specifically vulnerable paired basic amino acids present in the sequence of B-LPH and proopiomelanocortin (Steiner et al., 1980).

Lewis et al. (1978) have shown that there are major differences between the precursors of the enkephalins in the striatum and those in pituitary. They found that there are two proteins which in guinea pig, rat and beef striatum give rise to opioid activity upon digestion with trypsin. Neither of the so-called proopiomelanocortin proteins found in the locations just abovementioned are precursors for enkephalin; moreover, pro-opiocortin is found at a different location than that of enkephalin in the pituitary. They found only met-and leu-enkephalin in the striatum, while endorphin(s) and B-LPH were not detected. This point is elaborated on later in a different subsection.

The precursor pro-opiocortin gives rise to the expected fragments B-LPH and ACTH on tryptic digestion (Rubenstein et al., 1978; Mains et al., 1977; Chretien et al., 1979a). There is some evidence that trypsinlike enzyme is present in the pituitary and may be responsible for this breakdown (Bradbury et al., 1976b). Direct evidence that trypsinlike enzyme is present in the pituitary has been provided by Kenessey et al. (1977, 1979). However, direct evidence that pro-opiocortin is degraded by pituitary extract has not yet been forthcoming.

Although preliminary indications showing that the pituitary gland contains lipolytic substances were given by Anselmino and Hoffman in 1931, the complete characterization of two of these factors was accomplished only some three and one-half decades later, by Li et al. (1965) and by Chretien and Li (1967). Working with bovine pituitary, Li and his associates characterized and named the weakly lipotropic molecules beta-and gamma-lipotropin. The former consists of 91 amino acids, of which residues 1-58 contain the sequence of gamma-lipotropin (Chretien and Li, 1967). Both molecules comprise within their sequence the full polypeptide segment B-MSH in positions 41-58 (Chretien and Li, 1967). Later, homologous lipotropins were isolated and characterized in bovine (Li and Chang, 1977; Lohmar and Li, 1967), porcine (Gilardeau and Chretien, 1970; Graf et al., 1970; Graf and Cseh, 1968; Graf et al., 1969) and in human (Chretien et al., 1976; Cseh, 1972; Cseh, Graf and Goth, 1968; Li and Chang, 1976; Scott and Lowry, 1974) pituitaries. From all these studies a common structural feature of lipotropins has become apparent. Their structure includes the species-specific B-MSH sequence. This was recognized by Chretien and Li in 1967, and those

workers proposed that B-MSH could be derived from B-LPH by enzymatic cleavage; with initial production of gamma-LPH as an intermediary peptide. The structural resemblance of the cleavage sites (according to basic amino acid sequence) to those seen in proinsulin (Chance et al., 1968; Steiner, 1968), and in other precursor prohormones supports such a view (Steiner et al., 1980).

The ACTH sequence as detected by radioimmunoassay is present in the pro-opiocortin molecule, and can be released from lung tumors by controlled tryptic digestion (Yalow, 1976). Bradbury et al. (1976a) isolated and sequenced a 38-residue peptide from the secretory granules of pig pituitary. The peptide was released with enzymatic treatment and is believed to be the N-terminal of an ACTH prohormone and B-MSH (an 18-amino acid sequence in pig and bovine pituitary). It must be noted that human B-MSH-like and B-LPH-like peptides are different from those in other species of animals.

H.R. Morris deserves considerable credit for finding the structure of enkephalins and for discerning the resemblance of amino acid sequence of met-enkephalins with that of B-LPH. It was Morris who, during a lecture on B-LPH, given by D. Smyth, noticed the resemblance of amino acid sequences of enkephalins in the structure of B-LPH along with that of B-END. This perception had a great impact in research on opioids, and a number of questions were raised as a result of Moriss' keen observation (Morris, 1977). The issues which became important at that time included such questions as: (i) Were the enkephalins isolated by Hughes a by-product of B-END, as it undergoes degradation during the isolation procedure? (ii) Was B-END a precursor of Met-enkephalin? (iii) Was B-LPH a prohormone for B-END? (iv) Was there a separate

precursor of Met-enkephalin and Leu-enkephalin, and was the latter yet to be isolated? Many of those early questions have, of course, been answered, as outlined in the foregoing sections here. Still more are undergoing current investigation.

Once it was noted that the B-LPH molecule contains in itself the structure of B-END, B-MSH and Met-enkephalin, efforts were made to identify those enzymes which could cleave the B-LPH precursor. Chretien et al. (1979a), postulated that the cleavage would likely result from the action of a trypsinlike enzyme acting at the site of two basic amino acid residues. This was by analogy to the formation of insulin from proinsulin and of parathyroid hormone from proparathyroid (Steiner et al., 1980). Bradbury et al. (1976) first inferred that the pituitary gland contains a trypsinlike enzyme capable of releasing the C-fragment of B-LPH by cleavage of the Arg60-Tyr61 bond. Cleavage of the paired lysine residue close to the N-terminal of the B-MSH sequence was slow, which led them to think that it was due to a different endopeptidase. Chretien et al. (1979) and Pezalla et al. (1978) have shown that in the pituitary gland, particularly in the intermediate lobe, B-END is derived from the de novo synthesis of its precursor B-LPH. Thus B-LPH is a proendorphin in that tissue. More definite proof that B-LPH is the biosynthetic precursor of B-END has been provided by more extensive studies with pulse-chase labelling technique (Hughes et al., 1980). Recently, Seidah et al. (1979) have described the enzyme, tonin, which is present in the pituitary and is responsible for the formation of B-END and B-MSH from B-LPH.

The first evidence for the formation of Met-enkephalin and gamma-END was presented by Smyth and Snell in 1977. They showed that

the extracellular degradation of the C-fragment of B-LPH in striatum is not initiated by the attack of an aminopeptidase, unlike the proteolysis catalysed in brain membrane preparations. Instead, the first step appeared to involve an attack by endopeptidase. Burbach et al. (1981) have recently shown that such endopeptidase action is responsible also for B-END cleavage in brain synaptosomes.

The degradation of endogenous endopeptidases was readily followed by gel filtration on Sephadex G-50. The disappearance of C-fragment was accompanied by the appearance of tyrosine and of two chromatography peaks (A and B) due to intermediate peptides containing the intact NH2-terminal region. Eluate of peak A yielded gamma-END on rechromatography and, as a minor component, alpha-END. It is not known whether alpha-END is formed directly from C-fragment of B-END by the action of an endopeptidase, or whether it arises by the loss of COOH terminal amino acid from gama-END by the action of a carboxypeptidase. Peak B on rechromatography eluted Met-enkephalin as the principal product along with a small amount of hexa- or heptapeptide.

Austen and Smyth (1977) found that the COOH-terminal sequence of C-fragment of B-LPH (END) is highly resistant to attack by carboxypeptidase. Hence, they concluded that both NH2-and COOH-terminals are in such a conformational position that those two ends are not available for enzymatic degradation, with only the middle part or central section of the chain exposed to degradation via enzymatic attack, presumably by the action of endopeptidases. Once the middle portion is cleaved, B-END undergoes stepwise degradation to form gamma-END, alpha-END and Met-enkephalin. In the central portion of the B-END chain the 77-78 bond is cleaved by rennin (not renin) and/or by

cathepsin D, releasing B-LPH 61-77 (gamma-END). The 78-79 bond is cleaved by chymotrypsin and armillaria protease while the 79-80 bond is split by trypsin. Austen and Smyth have suggested that gamma-END does not require an enzyme with specific activity to cleave the 77-78 bond. They argued that Met-enkephalin is slowly formed and, being vulnerable to exopeptidase, has only a transient existence. From this argument they concluded that the degradation of C-fragment of B-LPH in striatal slices leads to the formation of gamma-END, alpha-END and Met-enkephalin. They ascribed the proportions in which the various peptide fragments are formed in vivo as depending on the relative activities of the degrading enzymes (Austen and Smyth, 1977). Burbach et al. (1980, 1981) reported that B-END is first degraded by an endopeptidase to form gamma-END. This gamma-END, depending on the pH of the in vitro system, is rapidly converted to des-tyrosine-gamma-END by aminopeptidase action or to alpha-END or to further sequential degradation by carboxypeptidase-A action. Zakarian and Smyth (1982) reported that B-END is present in pituitary and different regions of brain with related peptides such as B-END 1-27, des-histidine derivative of B-END 1-26; each of these two are present also in an alpha, N-acetyl, form. These different forms of B-END are derived from a single precursor by differential proteolytic cleavage and acetylation. The proportions of the six B-END peptides are strikingly different in various regions of the brain.

There is growing contention that B-END is not the major precursor for Met-enkephalin. Following are listed some of the arguments that uphold such contention: (a) If B-END is Met-enkephalin precursor then the distribution of both of them should be the same. However, the

distributions of B-END and in brain differs from that of Met-enkephalin. B-END is distributed mainly in the basal hypothalamus, medial preoptic area, medial dorsal thalamus, periaqueductal gray matter and certain other nuclei, whereas Met-enkephalin is concentrated mainly in globus pallidus, nucleus caudatus and certain other areas. In these nuclei, B-endorphin is not present along with Met-enkephalin (Bloom et al., 1978; Dupont et al., 1980). In the periphery the pattern is even more striking. In the adrenal medulla -- which is the richest source of enkephalin in the periphery -- pro-opiocortin, B-LPH and B-END are almost nonexistent (Kimura et al., 1980; Viverous et al., 1976). (b) Immunofluorescence studies show that neurons may contain either B-END or Met-enkephalin, but never both of them (Rossier et al., 1977; Bloom et al., 1978, 1979; Watson et al., 1978; Larsson et al., 1979; Hughes et al.. 1977). (c) Hypophysectomy has no effect on enkephalin levels whereas it lowers the B-END levels (Ogawa et al., 1979). (d) Pulse-chase experiments with radiolabelled amino acids in vivo — a crucial test in the determination of precursor/product relationships — confirmed that formation kinetics for proopiocortin:ACTH:MSH:LPH:B-END are all interrelated, whereas there is no relationship between formation of B-END and Met-enkephalin (Pezalla et al., 1978; Seidah et al., 1978; Mains and Eipper, 1979; Loh et al., 1979). (e) In most of the known polypeptide precursor hormones the active molecule is present with a pair of basic amino acids at NH2- and COOH-terminals; this particular bond is first cleaved by a trypsinlike enzyme and later on by carboxypeptidase-B-like enzyme (Steiner et al., 1980; Hughes et al., 1980). However, in the case of B-END there is no pair of basic amino acids before nor after Met-enkephalin. Hence, if

B-END is the precursor for Met-enkephalin, its breakdown would require an unusual endopeptidase which can cleave a bond between hydrophobic and hydrophilic amino acids.

Alpha-END is possibly formed by the action of aminopeptidase, once the endopeptidase cleaves the central portion of the B-END molecule (Austen and Smyth, 1977). Another possibility could be that it is formed by the action of carboxypeptidase on gamma-END; however no convincing evidence is thus far available. Leu-enkephalin was isolated along with Met-enkephalin, it differs only from the latter only in that it contains leucine instead of methionine at the carboxy end (Hughes et al., 1975). It is interesting that the presence of the Met-enkephalin amino acid sequence in the B-LPH molecule has tended to obscure the importance of Leu-enkephalin, whose sequence does not occur in the B-LPH precursor. Nevertheless, Leu-enkephalin appears to be a purer agonist than is Met-enkephalin, at least in analgesic tests in vivo (Leybin et al., 1975; Ungar et al., 1976) and in vitro in sodium-shift test (Simontov and Snyder, 1976; Chang et al., 1976).

Japanese investigators have isolated the peptide, alpha-neoendorphin, from the hypothalamus and proposed it to be the precursor for Leu-enkephalin (Kangawa et al., 1979). There are no other reports so far, however, to confirm or to deny this hypothesis. The tryptic digestion of the alpha-neoendorphin leads to the formation of Leu-enkephalin (Kangawa et al., 1979). Goldstein et al. (1979) have described a novel opioid peptide in the pituitary, they reported it to be more potent than B-endorphin. Recently, Herman et al. (1980) have purified and characterized the peptide and named it dynorphin; it seems to contain the sequence of Leu-enkephalin. The other rich source of

Leu-enkephalin is in the periphery, especially in the adrenal medulla. A good number of other small- to intermediate-sized peptides have been isolated. Some of these peptides have either a single sequence of Leu-enkephalin while others contain multiple forms, usually in combination with that of Met-enkephalin (Schultzberg et al., 1978; Kimura et al., 1980; Viveros et al., 1976).

Soon after the isolation of the enkephalins it was realized that these peptides are subject to the action of peptidases. In recent years some specific enkephalinases have been isolated and characterized by a number of workers. This is leading to a somewhat clearer picture of the role of these enzymes, a scheme for which is presented in Fig.1.

During the isolation and characterization of enkephalins Hughes (1975) investigated several commercially-available enzymes showing that carboxypeptidase-A and, to a lesser extent, leucine-aminopeptidase, were effective in destroying the activity of the brain extract. To identify the mode of deactivation of peptides in human and rat brain homogenates, Hambrook et al. (1976) added different sequences of amino acids found in Met-enkephalin. They noted that deactivation took place through the cleavage of the Tyr-Gly amide bond, and that there was a release of tyrosine from its neighboring amino acid. Similar observations are obtained from different tissues: rat brain homogenate (Marks et al., 1977; Jacquet et al., 1976; Dupont et al., 1977; Knight and Klee, 1978; Pert et al., 1976; Vogel and Altstein, 1977), rat striatal membranes (Meek et al., 1977; Chang et al., 1976;), mouse brain extract (Marks et al., 1977), guinea pig ileum (Craviso and Musacchio, 1978) and from rat brain synaptosomal membrane preparations (Lane et al., 1977; Austen et al., 1979).

After intracerebroventricular (ICV) infusion of carrier-free 3H-(Leu)5-enkephalin (Meek et al., 1977) or of 3H-(Met)5-enkephalin (the latter given either by ICV infusion, or intravenously) in unanesthetized rats (Stein et al., 1980; Clement-Jones et al., 1980), more than 80-90% of the cerebral radioactivity as analyzed by HPLC was due to enkephalin metabolites. The half-life of this degradative, presumably enzymatic, process is about four min. The metabolites consist mainly of 3H-Tyr, with minor chromatographic peaks revealing 3H-Tyr-Gly-Gly or 3H-Tyr-Gly-Gly-Phe and two other unidentified peptides. When 3H-(Met)5-enkephalin is perfused into the cisterna magna via the lateral ventricles in rats under anesthesia, the catabolic half-life is prolonged to about ten min and the main metabolite is no longer 3H-Tyrosine, but becomes either 3H-Tyr-Gly-Gly or 3H-Tyr-gly (Craves et al., 1978). These results indicate that anesthetic agents could have some effect on the activity of enkephalin-degrading enzymes, for example by suppressing the aminopeptidase and slowing the activity of dipeptidylcarboxypeptidase.

Vogel and Altstein (1977) drew on work of Meek et al. (1977) and developed a simple assay for detecting the release of Tyr upon hydrolysis. It was at one time suggested that aminopeptidase activity associated with blood vessels possibly contributes most of the enkephalin-hydrolysing activity found in brain homogenates (Shaw and Cook, 1978). This may no longer be a valid observation, however, as a specific aminopeptidase enzyme has been isolated from brain tissues.

At the present time five groups of investigators have already purified to homogeneity a specific aminopeptidase from the soluble fraction of the brain from several species (Hayashi and Oshima, 1977;

Hayashi, 1978; Schnebli et al., 1979; Hersh and McKelvy, 1981; Traficante et al., 1980). Comparisons among their finding for the enzyme's physical and biochemical properties indicate several common features viz, a molecular weight of 100,000 daltons, except for the human enzyme; optimal pH around 7.0; presence of essential thiol and metal groups, and inhibition by puromycin. Due to various biochemical discrepancies it is not certain at this moment whether or not all of these five enzymes represent the identical substance.

There is good evidence to show that enkephalin-hydrolysing activity can be detected in extensively washed particulate fractions of rat or mouse brain (Craves et al., 1978; Malfroy et al., 1978; Knight and Klee, 1978; Sullivan et al., 1978). A certain line of intact and washed neuroblastoma cells contains an enkephalin-aminopeptidase activity, which is probably located at the cell surface since it gets inactivated when the intact cells are treated with trypsin (Hazum et al., 1979). Recently Hui et al., (1981) have isolated and purified a particulate-bound aminopeptidase from the rat brain. The purified enzyme splits enkephalin at a rate of 25 uMole per mg protein per min and is different in several biochemical properties from soluble aminopeptidase. This particulate-bound enzyme seems also to be the enkephalin-specific aminopeptidase.

Lane et al. (1977) proposed that the location of opiate receptors are not functionally related to the site of enkephalin-hydrolysing activity by aminopeptidase. Knight and Klee (1978) tested the possibility that degradation by aminopeptidase may be related to receptor occupancy. From their experiments they concluded that (a) rate of aminopeptidase action on Met-enkephalin is almost independent of

whether or not the peptide is bound to the receptor. (b) receptor binding of enkephalin acts as a concentrating mechanism for membrane bound aminopeptidase, which may be located in the vicinity of the enkephalin receptor on the membrane. Malfroy et al. (1979) presented evidence for the homogeneous distribution of solubilized aminopeptidase in mouse brain. Schwartz et al. (1980) showed that distribution of solubilized aminopeptidase is homogeneous compared with the heterogeneous distribution of opiate receptors and of the enzyme dipeptidylcarboxypeptidase. Gorenstein and Snyder(1980) reported that the localized distribution of enkephalinase-B and of aminopeptidase activity in brain contrasts with the marked variation in opiate receptor distribution.

Dipeptidase hydrolase or dipeptidases were found in the brain as far back as 1936 by Blum, then by Abderhalden and Caeser (1940), Kies and Schwimmer (1942) and by Price et al. (1947) (in Gabrielescu, 1975). Van den Moort and Uzman (1961) considered that a separate enzyme was responsible for the hydrolysis of Gly-Gly. This enzyme differs by its sensitivity to activation by $Co^{++}$ (Marks, 1968). Stern and Marks (1979) studied the distribution and role of glycylglycine hydrolase of rat brain, in cleavage of glycine-rich oligopeptides including Met-enkephalin. The enzymatic activity was elevated in the presence of $Co^{++}$. It was highest in striatum and cerebellum and was lowest in spinal cord. The activity was mainly in the cytosol and increased during the development. The cleavage of Met-enkephalin at the Gly-Gly bond was observed by Craves in 1978, while he was studying the <u>in situ</u> metabolism of tritiated enkephalins. That observation was confirmed by Gorenstein and Snyder in 1980. Gorenstein and Snyder (1980) attempted

to purify three enkephalinases. They isolated and partially purified enkephalinase-A1 and -A2, which are known to generate Tyr-Gly-Gly, and enkephalinase-B which is believed to generate Tyr-Gly. They reported that enkephalinase-B can be fully resolved from both enkephalinase-A1 and -A2 but overlaps in the isolation procedure with aminopeptidase and angiotensin converting enzyme. It is quite possible that this enkephalinase-B might be responsible for the final degradation of the Gly-Gly bond once enkephalin is inactivated either by aminopeptidase or by dipeptidylcarboxypeptidase. As accumulation of Gly-Gly dipeptide after enkephalin inactivation has been reported by Marks et al. (1977).

In his report on endogenous opioids, Hughes noted that enkephalins are degraded at amino and carboxy terminals. This observation has been confirmed by various workers (Craves et al., 1978; Erdos et al., 1978; Sullivan et al., 1978; Gorenstein and Snyder, 1980; Malfroy et al., 1978). Attempts have been made to isolate, purify and localize this enzyme. So far three groups of workers have succesfully isolated this enzyme (Gorenstein and Snyder, 1980; Swerts et al., 1980; Sullivan et al., 1980). Gorenstein and Snyder (1980) isolated two enzymes, which they named enkephalinase-A1 and -A2. This A1 and A2 designation is given to two peaks in Porapak column during the isolation procedures and they show some difference in sensitivity to enzyme inhibitors. However, it is quite possible that A1 and A2 are different states of the same enzyme, as was pointed out by the investigators. For some time it was believed that enkephalin dipeptidylcarboxypeptidase and membrane-bound angiotensin converting enzyme (ACE) are the same (Gorenstein and Snyder, 1980; Swerts et al., 1979; Benuck and Marks, 1979; Arreguiv et al., 1979). However, in the

last few years these suggestions have been withdrawn with the emerging clear distinction between the two enzymes (Schwartz et al., 1980; Sullivan et al., 1980; Gorenstein and Snyder, 1980). The dipeptidylcarboxypeptidase (enkephalinase) is more specific for enkephalin than for angiotensin-I and its specifity for other neuropeptides is very low. (Schwartz et al., 1980). Also, the distribution of the enzyme enkephalinase shows a large regional variation in mouse brain (Schwartz et al., 1980) and in rat brain (Sullivan et al., 1980), and is highly correlated with the distribution of opiate receptors. In lesioning studies, kainic acid was injected into mouse striatum, leading to a decrease in opiate receptor content as well as losses in enkephalinase activity (Schwartz et al., 1980; Gorenstein and Snyder, 1980). It has been reported that opiate receptors increase twofold between birth and adulthood in rat (Auguy-Valette et al., 1978; Clendenin et al., 1976; Schwartz et al., 1980) and, whereas there is also approximately a twofold increase in enkephalinase activity, the pattern of development of opiate receptors is clearly different from that of the enzyme (op cit.).

The probable mechanism of inhibition of the aminopeptidase by bestatin has been relatively well worked out. Since amastatin and bestatin are very much similar in their structure, action, inhibitory spectrum and potency, it is reasonable to assume a similar if not identical mechanism of inhibition. Bryce and Rabin (1964) proposed the reaction mechanism for aminopeptidase. On the basis of their scheme Nishizawa et al. (1977) suggested a mechanism of inhibition, which is outlined in the following discussion. The strong inhibitory activity by bestatin of Leucine-aminopeptidase and aminopeptidase-B is likely

accomplished by binding of bestatin to the active site of these enzymes, as suggested by competitive inhibition. There are three reactive functional groups, NH2, OH and COOH, in the bestatin molecule. The N-terminal amino group of the substrate corresponds to the amino group at C3 of bestatin. However, studies with various sterioisomers indicate that the configuration at C2 but not at C3 is the most important factor for the manifestation of activity and that the stereochemical requirements for the other asymmetric carbon atoms are not very strict with regard to activity. Two important factors are that the S configuration at C2 of bestatin is a requirement for enzyme inhibition, which suggests that in the enzymic reaction the hydration of the carbonyl group is stereospecific. Among bestatin analogs in which L-leucine moiety is substituted by other amino acids, a peptide or an amine containing alpha-, beta- ,gamma- and epsilon-amino acids, or the compound in which the amino group was alpha to the carboxyl group, showed the strongest activity. This suggests that the distance between the amino and carbonyl group also is important for the enzyme inhibition.

The dipeptide Phe-Ala is a selective dipeptidylcarboxypeptidase inhibitor (Ki = 1.0 μM), however it is less potent than thiorphan (where ki=4.0 nM) (Roques et al., 1980). Schwartz et al. (1981) studied the properties of dipeptidyl carboxypeptidase in vitro with respect to: effect of chelating agents, various inhibitors, role of metal ions in reversing the specificity of enzyme by modifying the structure of various substrates (of importance, e.g., at carboxyl terminal), influence of the side chain of the C-terminal amino acid, influence of the side chain of the penultimate amino acid, influence of various

peptide bonds, stereochemical requirements and influence of additional bonding. Based on data from experiments where the just-mentioned phenomenan were studied, Schwartz et al. (1981) proposed a mechanism of inhibitory action for thiorphan and for Phe-Ala. Their concept stemmed from a model of the active site of dipeptidylcarboxypeptidase. This model has been developed by postulating an analogy with the active site of carboxypeptidase-A and angiotensin-converting-enzyme, both $Zn^{++}$ containing peptidases, whose complete three-dimensional structure and catalytic mechanisms at the molecular level have been very well delineated (Quiocho and Lipscomb, 1971). The main features of the model are as follows: (i) a positively-charged residue (analogous to the guanidinium group of Arg145 in carboxypeptidase-A) is responsible for the initial salt-linking of the C-terminal carboxylate group of the peptide structure. (ii) a dead-end hydrophobic pocket accomodates the aromatic (or hydrophobic) side chain of the penultimate amino acid. (iii) unidentified residues in the active site interact with the carbonyl group of the Gly3-Phe4 peptide bond of substrates. (iv) The $Zn^{++}$ atom in the active site polarizes the carbonyl group of the scissile amide bond of substrates (Gly3-Phe4 in enkephalins), making them more susceptible to hydrolytic cleavage.

The hypothetical model is substantiated by the observation that thiorphan (3-mercapto-2-benzylpropanol-glycine) is a potent competitive inhibitor of dipeptidylcarboxypeptidase. In thiorphan most of the active sites are retained and the extent of binding with the $Zn^{++}$ atom is strongly enhanced by the introduction of a thiol group in the proper position. This thiol group is not present in the less potent inhibitor Phe-Ala. Thus, theoretically, the addition of a thiol group to Phe-Ala should increase its potency.

Mechanism of action of the serine proteinase inhibitor phenylmethylsulfonyl fluoride PMSF; see Fig. 1, is characterized by a sulfonyl group directly substituted onto the benzene ring in the position para to the other substitution (Desnuelle, 1960). These are shown to be strong inhibitors of serine proteinases such as trypsin, chymotrypsin, thrombin, plasmin and kallikrein. These inhibitors are bound irreversibly to certain amino acid (serine and histidine) residues of the active centres of proteinases and esterases.

Schwartz et al. (1981) have laid down a total of six criteria for the identification of enkephalin-inactivating neuropeptidases. These criteria are fulfilled at present only by dipeptidylcarboxypeptidase. Schwartz has therefore proposed that, as the only such enzyme, it should be called "enkephalinase". Perusal of existing literature, however, points out that aminopeptidase is likely also to satisfy the criteria laid down by Schwartz. The following are his criteria, along with the evidence in favour of aminopeptidase fulfilling them: (a) The first criterion is that enzyme action should lead to biologically-inactive products. Aminopeptidase cleaves enkephalin at Tyr-Gly bond, and makes it inactive (Hambrook et al., 1976; Meek et al., 1977). Tyr is required for the binding of enkephalins to the active center of opiate receptor (Smythies, 1976; Horn and Rodgers, 1976) and Gly-Gly-Phe-Met/Leu is the resulting inactive tetrapeptide metabolite. Enzymatic protection of the Tyr-Gly bond, as seen synthetic analogs of enkephalin (Pert et al., 1976), or by treatment <u>in vivo</u> with one of the several aminopeptidase inhibitors -- bacitracin (Simmons and Ritzman, 1980) or amastatin (Dua et al., 1981) -- potentiates the effects of enkephalins. It is well-accepted

that aminopeptidase inactivates enkephalins in vitro and in vivo. As Tyr is critically required for the binding of enkephalins to opiate receptors, it seems logical to believe that deletion of Tyr from the rest of the enkephalin molecule will bring about a faster inactivation than would cleavage at any other site. (b) The second criterion laid down by Schwartz et al. is that the neuropeptidase should be preferentially localised strategically to exert its assumed action viz. to hydrolyse the enkephalins immediately after their synaptic or exocytotic release. Ideally one would like to detect the candidate peptidase histochemically within the enkephalinergic synapse complex. This is the only major criterion which is not yet fully met by an aminopeptidase. So far, at least five or more groups of investigators have isolated aminopeptidase (Hayashi and Oshima, 1977; Schnebli et al., 1979; Hersh and McKelvy, 1981; Traficante et al., 1980; Hayashi, 1978). Most of the enzyme was found in soluble form and the distribution of this solublized form was found to be homogeneous throughout the brain, as compared with the heterogenous distribution of enkephalins there (Schwartz et al., 1981). Recently, however, one group (Hui et al., 1981) has isolated a particulate-bound aminopeptidase; the enzyme isolated seems especially to be highly specific for enkephalins. So far no one has achieved uniformity in characterization of the aminopeptidase.

The main argument put forward by Schwartz et al. (1981) against the characterization of the aminopeptidase as an enkephalinase is that there is not a strikingly close correlation between the central distribution of aminopeptidase and that of opiate receptor. It should be kept in mind, however, that these correlations were done with

soluble and not with particulate-bound aminopeptidase, the latter being more specific to enkephalins (Hui et al., 1981). Also, the correlation was made with only one ligand of the opiate receptor, and it is now known that there are many subtypes of opiate receptor (Martin, 1967; Gilbert and Martin, 1976; Lord et al., 1976); the correlation should more properly done with each receptor subtype.

It thus appears that experiments showing the correlation between the distribution of particulate-bound aminopeptidase and that of the opiate receptor are yet to be done. It would be premature to conclude that, because there is no regional correlation between aminopeptidase and the opiate receptor, aminopeptidase has no physiological role to play in opiate receptor/opioid neuropeptide kinetics. Schwartz et al. (1981) in a recent review have themselves pointed out that in view of the probable heterogeneity of aminopeptidase activity one cannot rule out the existence of a more specific enkephalin-hydrolysing neuropeptidase in this group of enzymes (Schwartz et al., 1981). There is growing evidence that enkephalins are stored along with other neurotransmitters e.g. acetylcholine (Kumakura et al., 1980), cathecholamine (Viveros et al., 1979) Substance P (Cuello et al., 1978), ACTH (Weber et al., 1978), neurotensin (Uhl et al., 1978), and vasoactive intestinal peptide (Wharton et al., 1980). So far the physiological significance of this joint storage of neurotransmiters is unknown, possibly one neurotransmitter acts as a neuromodulator for the other. One can speculate that some of this action may not be mediated at classicaly-conceived opiate recognition sites but, instead, via the receptors for the "paired" neurotransmitter. Frederickson and Pinsky (1971) and deJong and Pinsky (1974) have shown, for example, that

opioid narcotics can modulate the action of acetylcholine on the nicotinic receptor. At certain synapses, therefore, enkephalinase might be situated only to the purpose of regulating the amount of enkephalin available for modulation of the companion neurotransmitter. In such a system the location of aminopeptidase might not correlate with that of opiate receptors. The present state of knowledge about aminopeptidase is perhaps in a situation analogous to that of early views on the enzyme catechol-o-methyltransferase. This was at one time thought to be responsible for degradation of peripheral and exogenous catecholamines, but it was later on proved otherwise. (c) The third criterion of Schwartz et al. is that resistance of analogs to the hydrolytic action of a neuropeptidase should account for the increased biological potency of these compounds in vivo (when not explained by increase in potency at the receptor level). Aminopeptidase fits with this criterion very well. For example, certain aminopeptidase-resistant analogs of enkephalin, made by substituting various amino acids at the Gly2 level (Pert et al., 1976; Morley, 1980) are potent in antinociception tests, guinea pig ileum and other biological assays, without an alteration in potency at the receptor level (Morley, 1980). This criterion, however, should not be regarded as critical, since the analogs are made by substituting amino acids, a procedure which is bound to change the spatial configuration of the natural molecule. If one considers the relationship between receptor and ligands as some sort of a lock-and-key relationship, analogs will not represent the true drug:receptor relationship. The retroinverso analogs -- where there is no substitution of amino acid -- show that even such analogs do not agree with the natural peptides in their natural spatial structure as

tested by least-square fit model (Goodman and Chorev, 1981). (d) The fourth criterion is that the neuropeptidase may display adaptive modifications in activity following sustained changes in peptidergic neurotransmission,such changes are noted in dipeptidylcarboxypeptidase and not in other peptidases (Schwartz et al., 1981). As mentioned in discussion on the second criterion, the wrong form of enzyme (soluble, not particulate-bound), probably not a true representative of the in vivo agent, was studied. Hence any conclusion that aminopeptidase is not resposible for physiological degradation of enkephalin would be premature and quite possibly not valid; any judgmental claim on this issue needs more study. (e) The fifth criterion was that selective inhibition of the neuropeptidase should result in a protection of the synaptically-released neuropeptide. Inhibition of dipeptidylcarboxipeptidase by thiorphan or by Phe-Ala enhances the recovery of enkephalins from depolarised brain slices (Roques et al., 1980). A weak aminopeptidase inhibitor, puromycin, fails to do so (Patthy et al., 1977); bacitracin, however, does enhance the recovery of enkephalin (Tsou et al., 1980). Schwartz et al. (1981) mentioned that it remains to be assessed whether his fifth criterion applies to enkephalins released in vivo. He emphasized that experiments need to be done with other potent and selective aminopeptidase inhibitors (Schwartz et al., 1981). Hence, this criterion is partially fulfiled by aminopeptidase; the critical experiments are yet to be done. (f) The last criterion is that selective inhibition of neuropeptidase should result in biological response similar to those elicited by stimulation of neuropeptide receptors. This criterion is very well fulfiled by aminopeptidase. Bacitracin (Patthy et al., 1977; Simmons and Ritzman,

1980; Tsou et al., 1980) and the highly specific aminopeptidase inhibitor amastatin (Barcally et al., 1981; Dua et al., 1982), both produce analgesia and behavioral effects similar to those of opioid peptides. The behavioral effects are reversible by naloxone. Hence, the last criterion of Schwartz et al. (1981) is answered satisfactorily by aminopeptidase.

The foregoing suggests that aminopeptidase fulfills four out of six criteria with certainty, while the remaining two criteria demand further critical experimentation before being validly excluded. As regards the physiological role of aminopeptidase, it be would be improper to name dipeptidylcarboxypeptidase as the only enkephalinase. To avoid such impropriety, a new nomenclature is warranted. This writer would like to propose that we should name the aminopeptidase (or any similar enzyme) which selectively cleaves the Tyr-Gly bond of enkephalins as aminoenkephalinase and that the dipeptidylcarboxypeptidase should be termed carboxy(l)enkephalinase.

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# II. STEREOSPECIFIC PROTECTION OF ENKEPHALIN FROM AMINOPEPTIDASE BY AMASTATIN IN VIVO

## SUMMARY

The aminopeptidase inhibitor amastatin or its stereoepimer, epiamastatin, was administered to rats by intracerebroventricular (i.c.v.) infusion into the lateral ventricle. Amastatin by itself produced analgesia and enhanced analgesic and hyperthermic responses to i.c.v. Met-enkephalin. The responses to amastatin were naloxone-reversible. Epiamastatin did not exert significant pharmacological activity. These results provide the first although indirect evidence for in vivo stereospecific inhibition of a molecular site-selective enkephalin-degrading enzyme in brain.

## 1. Introduction

Enkephalins and other endogenous opioid peptides in brain have been implicated in antinociception and other behavioral responses. The transient nature of responses to centrally-administered enkephalin is apparently due to rapid enzymatic degradation of that peptide (Hughes, 1975; Barclay and Phillipps, 1980). Prolonged pharmacological effects can be achieved *in vivo* by administration of enzyme-resistant enkephalin analogs or by pretreatment of the animal with peptidase inhibitors (Roques et al., 1980; Pinsky et al., 1980, 1982). The inhibitors alone can produce opioidlike responses, presumably by protecting endogenous peptides against degradation. It is widely accepted that aminopeptidase is an important centrally-occurring enkephalin-degrading enzyme that attacks the Tyr-Gly bond of both Leu- and Met-enkephalin (Meek et al., 1977; Malfroy et al., 1978). We now report that the tripeptide, amastatin, a potent inhibitor of aminopeptidase in vitro (Aoyagi et al., 1978; Barclay and Phillipps, 1980; Hui et al., 1981), induces analgesic, hyperthermic and behavioral responses upon intracerebroventricular (i.c.v.) administration in the rat. Amastatin was analgesic by itself and, also, significantly augmented the analgesic and hyperthermic effects of i.c.v. Met-enkephalin. The analgesic and hyperthermic effects were naloxone-reversible. Epiamastatin, a stereoepimer of amastatin, displayed no pharmacological effect. Pharmacological evidence for stereospecific inhibition of peptidases *in vivo* has not been demonstrated previously.

2. Materials and Methods

Amastatin and epiamastatin were obtained from Sigma Chemicals, St. Louis, MO.; Met-enkephalin from Peninsula Laboratories, St. Carlos, CA; naloxone.HCl was a gift from Endo Laboratories, Garden City, NJ. Cannulae were implanted stereotaxically into the right lateral ventricle (Pinsky et al., 1980, 1982) in male Sprague-Dawley albino rats (Canadian Breeding Laboratories, St. Constant, PQ) under pentobarbital-chloral hydrate anesthesia. Rats were tested 8 days after cannula implantation. The degree of analgesia was estimated from the latency to first hindpaw lick or leap upon exposure to a hotplate at 55$^{o}$C. Latency was measured just prior to treatment in each animal. The pretreatment latency in seconds was taken as a baseline value and subtracted from all measurements of latency. Hence, "negative" estimates of analgesia were obtained when latency was less than the pretreatment value, a procedure which permits detection of hyperalgesic states. The rat was removed from hotplate immediately after pretreatment measurement and given intraperitoneal (i.p.) injection of either saline or naloxone.HCl 5.0 mg kg$^{-1}$. This was followed promptly by one or two consecutive i.c.v. infusion(s), each in 10 µl volumes over 5 min, of amastatin 30 µg (58.7 nmol), epiamastatin 30 µg (58.7 nmol) or Met-enkephalin 100 µg (175 nmol). After final infusion the animal was immediately replaced on the hotplate and nine further measurements of analgesia were made until 60 min postinfusion. The "Total Analgesic Response" (Table 1) was measured as the area under the curve for time vs analgesia, (sec min) including and between the measurements at end of first i.c.v. infusion and at 70 min after pretreatment measurement; response areas were obtained by trapezoidal

estimation of area. Core temperature (colonic) was measured in each animal before the pretreatment estimation of analgesia and thereafter immediately after each subsequent estimation. Pretreatment values of core temperature were subtracted from subsequent measurements. Core temperature responses to the different treatments were calculated by finding areas under the curves for time vs difference ($^{o}$C min) from pretreatment temperature over identical limits and by the same algorithm as employed for analgesia. Treatment substances were kept unknown to experimenter during measurement of responses. Placement of cannulae was confirmed for each animal by infusion of dye just prior to sacrifice; data was accepted only from those animals where cannulation had been successful. Statistical inferences were made Student's t-test or by analysis of covariance (ANOVA) with data confirmed by Bartlett's test for homogeneity and compared by Duncan's multiple range test.

## 3. Results

Intracerebroventricular infusion with amastatin, enkephalin, or their combination, produced time vs analgesia response curves which rose rapidly over the first 10 min postinfusion and then displayed very broad, flat, peaks over the subsequent 50-min period of observation (Table 1). Mean latency to first hindpaw lick or leap at the peak of treatment effect with combined infusions of amastatin and enkephalin (Grp G, Table 1) was 44.5 $\pm$ 11.7 S.E. sec. This was significantly greater ($P < 0.02$; Student's t-test) than the mean peak value of 7.3 $\pm$ 1.7 sec found in the saline-infused controls (Grp A).

TABLE 1

Amastatin analgesia and enhancement of enkephalin analgesia.

| Group[a] | Treatment (doses: see text) Injection | Infusions i.c.v. | | | Total Analgesic Response sec min mean ± S.E. | Time to peak effect[b] min | Mean Latency at peak effect[c] sec |
|---|---|---|---|---|---|---|---|
| | IP | 1st | 2nd | n | | | |
| A | Sal | Sal | Sal | 4 | 37 ± 117 | 5 | 7.3 |
| B | Sal | Epi | Sal | 8 | 31 ± 27 | 10 | 11.7 |
| C | Sal | Ama | note[d] | 8 | 332 ± 71 | 10 | 11.1 |
| D | Nlx | Ama | Sal | 4 | -30 ± 108 | 5 | 11.0 |
| E | Sal | Sal | Enk | 4 | 23 ± 65 | 5 | 7.4 |
| F | Sal | Epi | Enk | 8 | -25 ± 39 | 10 | 13.0 |
| G | Sal | Ama | Enk | 8 | 780 ± 357 | 20 | 44.5 |
| H | Nlx | Ama | Enk | 4 | -193 ± 149 | 5 | 6.5 |

Legend, Table 1

Sal = saline, Ama = amastatin, Epi = epiamastatin, Enk = [Met]enkephalin, Nlx = naloxone.HCl

[a]Significant intertreatment differences for Total Analgesic Response, ANOVA: C is significantly different from D,G and H, with $P < 0.05$; G is significantly different from A,B,D,E,F and H, with $P < 0.01$. Student's t-test showed signficant differences for C vs A ($P < 0.05$) and for C vs B ($P < 0.01$).

[b]Taken from curves plotting hindpaw lick latency vs time elapsed after final infusion and smoothed by approximated running-average algorithm. See note[c] below.

[c]Mean latency to 1st hindpaw lick or to leap. Standard errors not shown since this parameter is provided here only to indicate range of observed values.

[d]No second infusion was done in this group; auxiliary experiments have indicated essentially no effect of second infusion of saline on analgesic and hyperthermic responses.

Rats given i.c.v. infusion of amastatin displayed a significantly greater analgesic response than did those given equivolume equimolar infusions of epiamastatin or of equivolume saline (Table 1). The analgesic effect of i.c.v. infusion with Met-enkephalin 100 µg was significantly increased by prior i.c.v. infusion of amastatin but not of epiamastatin. Naloxone.HCl 5.0 mg $kg^{-1}$ i.p. abolished both the analgetic effect of amastatin alone and the enhancement by amastatin of Met-enkephalin-induced analgesia (Table 1). No profound changes in motor behavior were observed with any of the treatments, except for a few leaps and/or wet dog shakes in the rats treated with the combination of amastatin and Met-enkephalin.

Core temperature responses to i.c.v. infusion of amastatin combined with Met-enkephalin showed consistently positive differences with respect to pretreatment temperatures, indicating a hyperthermic effect of the drug combination (Fig. 2). The amastatin/enkephalin combination gave significantly greater hyperthermia than did enkephalin alone or enkephalin combined with epiamastatin. The highest peak temperature response over the 60-min period of observation occurred also with amastatin/enkephalin treatment (Fig. 2). In other experiments (not shown here) neither saline, amastatin 30 µg nor epiamastatin 30 µg produced hyperthermia when given alone. The hyperthermic effect of the enkephalin-amastatin infusion was significantly diminished by pretreatment with naloxone.HCl 5.0 mg $kg^{-1}$ i.p. (Fig. 2).

## 4. Discussion

It seems reasonable to assume that the analgesic and hyperthermic effects of amastatin are due to protection of endogenous opioid peptides from degradation by aminopeptidase (Barclay and Phillips, 1980; Aoyagi et al., 1978). This assumption is substantiated by the observation that amastatin is the most potent among many inhibitors of purified particulate-bound aminopeptidase from brain (Hui et al., 1981). Amastatin enhancement of similar responses to infused Met-enkephalin would be due to protection of the exogenous opioid peptide. Differences between the effects of amastatin and its epimer, epiamastatin, are much greater in potentiation of responses to exogenous Met-enkephalin than in the production of opioidlike responses by the inhibitor congeners themselves (Table 1). This is possibly due to the limited access which the inhibitor might have to enzymes localized at enkephalinergic synapses as compared with the ready accessibility, to amastatin action, of aminopeptidase in cerebrospinal fluid.

Naloxone tended to induce hyperalgesia (e.g. Group H, Table 1), although this never rose to a statically-significant level. The trend may have nevertheless reflected a mild state of withdrawal from single-dose opioid dependency (Pinsky et al., 1980; 1981).

Amastatin enhancement of enkephalin hyperthermia is consistent with the reported hyperthermic response to i.c.v. Met-enkephalin in rats (Ferri et al., 1978). Enhancement of the hyperthermic response to infusion of Met-enkephalin discriminates less clearly between amastatin and epiamastatin than does enhancement of the analgesic response (Fig.

2, Table 1). Thus, opioid effects on temperature regulation may be mediated by a system more rapidly saturated by opiate agonism than is the antinociceptive system, either at the level of the opioid receptor or in subsequent pathways.

Roques et al. (1980) were the first to describe a clear antinociceptive response resulting in vivo from administration of a thoroughly-characterized inhibitor of identified enkephalin-degrading enzymes. They have emphasized the importance, in such studies, of highly specific inhibitors such as thiorphan. The results of this present study have shown that amastatin is significantly more potent in potentiating the analgesic effect of Met-enkephalin than is its stereoepimer, epiamastatin; the same tendency is observed with enkephalin-induced hyperthermia. Epiamastatin differs structurally from amastatin by only a single transposition of -OH with an -H group at $C_2$ (structures provided in Sigma "Catalog on Bioactive Peptides, Aug 1980"). Our in vivo results have shown that epiamastatin is essentially a pharmacologically-inert stereoisomer of amastatin. If a corresponding biochemical inertness can be demonstrated unequivocally in vitro, the observations reported here will have provided the first although indirect evidence for in vivo stereospecific inhibition of a peptide bond-selective enkephalin-degrading enzyme in brain.

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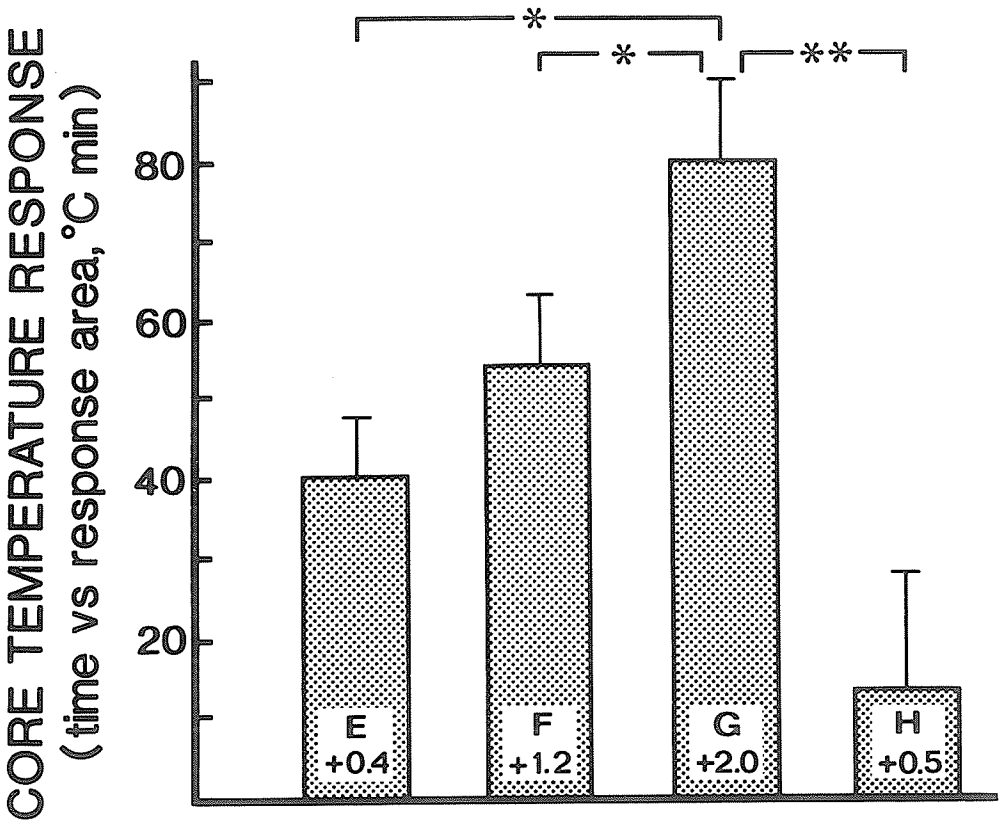
CORE TEMPERATURE RESPONSE
(time vs response area, °C min)
80
60
40
20
*
*
**
E
+0.4
F
+1.2
G
+2.0
H
+0.5

Fig. 2

Legend, Figure 2

Amastatin enhancement of enkephalin hyperthermia. Vertical bars show mean $\pm$ S.E. of the area under the time vs core temperature response curve, in $^{o}$C min, for rats in different treatment groups. Groups correspond to lettered groups in Table 1 and are identified by letters on bars. Figures at base in each bar represent the mean peak change (in $^{o}$C) in core temperature from pretreatment temperature measurements in each group. Horizontal brackets indicate significant differences between groups; * $p \leq 0.05$, ** $p \leq 0.01$.

# III. ENHANCEMENT OF MET-ENKEPHALIN ANALGESIA BY PHE-ALA, A SELECTIVE INHIBITOR OF SPECIFIC ENKEPHALINASE

## SUMMARY

L-Phenylalanyl-L-Alanine (Phe-Ala), a highly selective dipeptidyl carboxypeptidase inhibitor, given intracerebrally in the dose range 30-1000 µg showed an erratic tendency to produce analgesia in rats. Phe-Ala 500 µg given prior to a nonanalgesic dose of Met-enkephalin enhanced the analgesic response of the latter. This enhanced analgesic response was naloxone-reversible. Rats treated with combined Phe-Ala/Met-enkephalin showed neck-twitching and a leftward-lurching phenomenon.

Endogenous opioid peptides have been implicated in antinociception and other behavioral effects (Bloom et al., 1976; Goldstein, 1978). There is evidence to suggest that these peptides are released during certain conditions of stress (Guillemin et al., 1977; Hollt et al., 1978). However, the effects of exogenously-administered and, presumably, of endogenously-released enkephalins are short-lived (Hughes et al., 1975). This evanescence of enkephalin effect is attributed to enzymatic degradation, either by dipeptidyl carboxypeptidase (DCPase) or by aminopeptidase. As Tyr is the first metabolite liberated after intracerebroventricular (ICV) administration of enkephalins, it appears that exogenously-administered enkephalins are more vulnerable to aminopeptidase action at the Tyr-Gly bond (Meek et al., 1977; Malfroy et al., 1978) than to DCPase cleavage at the carboxy end. Anatomical location of DCPase enzyme correlates with that of opiate receptors while that of aminopeptidase does not (Malfroy et al., 1979). It is therefore logical to predict that DCPase action will be predominant in the enzymatic disposal of endogenously-released enkephalins. DCPase has been termed enkephalinase, because it fulfils the several criteria required to qualify characterization as a specific enzyme (Schwartz et al., 1981). DCPase can be inhibited in vivo by specific peptidase inhibitors such as thiorphan (Roques et al., 1980) or L-Phenylalanyl-L-Alanine (Phe- Ala) (Llorens et al., 1980). Phe-Ala has been shown to protect the enkephalin moieties released in vitro from rat striatal slices (Patey et al., 1981); we have previously reported augmentation of Met-enkephalin analgesia and neurobehavioral activity by the stereospecific aminopeptidase inhibitor amastatin (Dua et al., 1982). Site-selective peptidase inhibitors can modify

stress-induced analgesia and serve as pharmacological tools to study endorphinergic mechanisms. We report the in vivo effects of Phe-Ala on antinociception and behavior, when it is given to rats by itself or in combination with Met-enkephalin.

## Materials and Methods

Phe-Ala was obtained from Sigma Chemicals, St.Louis, MO; Met-enkephalin from Peninsula Laboratories, St. Carlos,CA; naloxone.HCl was a gift from Endo Laboratories, Garden City, NJ. Cannulae were implanted stereotaxically into the right lateral ventricle (Pinsky et al., 1980) in male Sprague-Dawley albino rats (Canadian Breeding Laboratories, St. Constant, PQ), 180-200g, under pentobarbital-chloral hydrate anesthesia (Valenstein, 1961). Rats were tested several days after cannula implantation. The degree of analgesia was estimated from the latency to first hindpaw lick, or to first upward leap, upon exposure to a hotplate maintained at $55^0$ C. Latency was measured just prior to drug treatment in each animal. The pretreatment latency in seconds was taken as a baseline value and subtracted from all measured latencies. Hence, "negative" estimates of analgesia were obtained when latency was less than the pretreatment value, a procedure which would permit detection of hyperalgesic responses. The rat was removed from the hotplate immediately after pretreatment measurement and given intraperitoneal (IP) injection of either saline or naloxone.HCl 5 mg $kg^{-1}$, followed promptly by one or two consecutive ICV infusion(s) of appropriate solutions , each in 10 µl volumes over five min. At the end of each infusion the animal was replaced on the hotplate and measurement of analgesia was repeated for a total of 10 times over an

ensuing observation period of 60 min. Areas under the curve for time vs analgesia, including and between the measurements at the end of first ICV infusion and at 20 min after the posttreatment, were obtained by trapezoidal estimation of curve area (Notari, 1971). This gave a value for the total analgesic response integrated over the 20 min period of observation and expressed in units of sec min. Core temperature (colonic) was observed in each animal before the pretreatment estimation of analgesia and immediately after each subsequent estimation. Pretreatment values of core temperature were subtracted from subsequent measurements. Core temperature responses to the different treatments were calculated by finding areas under the curves for time vs difference from pretreatment temperature over identical limits and by the same algorithm as employed for analgesia; this area is expressed in units of $C^0$ min. A blind design was utilized to minimize experimenter bias during measurement of the responses. Correct placement of cannulae was confirmed in each animal by infusion of eosin dye (diluted 1:12 in phosphate buffer), under pentobarbital anesthesia. Data were accepted only from animals with succesful cannulation. Statistical inferences were made by analysis of covariance with data confirmed by Bartlett's test for homogeneity and compared by Duncan's multiple range test.

## Results

Phe - Ala by itself in a dose range of 30 - 1000 μg ICV showed an erratic tendency to produce analgesia, but never to a statistically-significant degree in the stated dose range (e.g., difference between the results in group A and C of Table 2 was not

Table 2

Phe-Ala Analgesia and Enhancement of Enkephalin Analgesia

| Group[a] | Treatment (doses:see text) Injection | Infusions i.c.v. | | | Total Analgesic Response sec min mean ± S.E. | Time to peak effect[b] min | Mean Latency at peak[c] effect sec |
|---|---|---|---|---|---|---|---|
| | IP | 1st | 2nd | n | | | |
| A | Sal | Sal | Sal | 8 | 6 ± 5 | 5 | 7 |
| B | Sal | Sal | Enk | 4 | -26 ± 18 | 5 | 8 |
| C | Sal | Phe-Ala | Sal | 9 | 67 ± 27 | 10 | 13 |
| D | NLX | Phe-Ala | Sal | 4 | -1 ± 49 | 5 | 8 |
| E | Sal | Phe-Ala | Enk | 8 | 123 ± 10 | 15 | 20 |
| F | NLX | Phe-Ala | Enk | 5 | 41 ± 11 | 15 | 12 |

Legend, Table 2

Sal = Saline; Enk = Met enkephalin; NLX = Naloxone.HCl

Doses: I.P. Treatment: Naloxone 5 mg $kg^{-1}$, saline-equivolume to naloxone; ICV Treatment: Phe-Ala 500 µg, Enk 100 µg, Sal 10 µl.

[a] Significant intertreatment differences for Total Analgesic Response; ANOVA: E is significantly different from A, B and D with $P < 0.01$; from C and F with $P < 0.05$; C is significantly different from B and E from F with $P < 0.05$.

[b] Taken from curves plotting hindpaw lick latency vs time elapsed after final infusion and smoothed by approximated running-average alogrithm. See note below.

[c] Mean latency to 1st hindpaw lick or to leap. Standard errors not shown since this parameter is provided here only to indicate range of observed values.

statistically significant). In other experiments done here (data not shown here) we have observed the same erratic analgesic tendency with systemic Phe-Ala given by IP injection over the dose range of 30-1000 mg $kg^{-1}$. Neither with ICV nor IP adminstration of Phe-Ala did the analgesic responses appear to be dose-related.

Met-enkephalin 100 µg ICV was nonanalgesic by itself. However, the combination of Met-enkephalin 100 µg ICV with an nonanalgesic dose of Phe - Ala 500 µg ICV produced significant analgesia as compared to treatment with Met-enkephalin 100 µg or with equivolume saline control (Table 2). This analgesia was evident for 20 min after the second infusion, with maximum analgesia at 15 min. An injection of naloxone 5.0 mg $kg^{-1}$ IP, given prior to ICV infusions of Met-enkephalin or Phe-Ala, blocked the analgesic effects of the combination and eliminated the analgesic tendency of Phe-Ala given ICV by itself ($p \leq 0.005$). The analgesic combination of Phe - Ala and Met-enkephalin increased the core temperature but not to a statistically significant level as compared to treatment with Phe- Ala or Met enkephalin alone, or with saline control infusions (data not shown here).

The analgesic combination of Phe - Ala with Met-enkephalin provoked a pronounced neck-twitching, which often proceeded to frank body-lurching (always to the animal's left), for up to 10 min duration. A typical rat is quiet after receiving Phe - Ala 500 µg infusion alone. By one min after the initiation of the Met-enkephalin 100 µg infusion which followed ICV Phe-Ala, however, rats started showing repetitive neck-twitching or leftward body-lurching movements with an upper frequency of ca. five per min and averaging about three per min. This phenomenon lasted for 10 min. The neck-twitching began with the

appearance of slow, deep, abdominal respiration, the first twitch occuring in the abdomen. Neck-twitching would cease when the rat was held in the hand. Most of the rats showed wet-dog shakes and stiff tail; a few showed teeth-chattering. The neck-twitching phenomenon did not affect the analgesia test on hotplate.

## Discussion

The results presented here indicate that Phe - Ala by itself tends to produce analgesia in the $55^0$C hotplate test, although not in any regular nor statistically-significant fashion. In contrast, it clearly enhances the analgesic and behavioral effects of Met-enkephalin given ICV shortly after infusion with Phe-Ala. The enhancement is naloxone-reversible. We explain these results by presuming that Phe-Ala protects the carboxy terminus of both endogenously-released and exogenously-administered enkephalins from enzymatic cleavage by enkephalinase. These results support the contention by Schwartz et al., 1980, 1981 that Phe-Ala protects the degradation of enkephalins in vitro by inhibiting DCPase.

The short duration of the enkephalin-enhancing effect of Phe-Ala is compatible with the protection of enkephalins at only one site of enzyme attack. It is well-known that the crucial Tyr-Gly bond of enkephalin administered exogenously is more susceptible to aminopeptidase than to DCPase (Malfroy et al., 1978; Schwartz et al., 1981). It is quite possible therefore that exogenously-administered Met-enkephalin is rapidly destroyed by aminopeptidase, leaving very little intact enkephalin to be protected by Phe-Ala. This concept has been substantiated in recent experiments in this laboratory : the same

dose of Met-enkephalin as used in the present study produced a profound analgesia in rats pretreated with a combination of the aminopeptidase inhibitor amastatin and dipeptidylcarboxypeptidase inhibitor Phe-Ala (unpublished). Phe-Ala, a dipeptide, is itself vulnerable to enzymatic destruction . The fact that Phe - Ala produces very little analgesia by itself might indicate that either insufficient enkephalin is released under the conditions of our experiments or that Phe-Ala alone is not enough to protect endogenously-released enkephalins. There is evidence for both possibilities, since (i) Phe-Ala produces greater analgesia in a dose-related fashion on hotplate test at a lower temperature(such as at 44.5$^{0}$C warmplate test, a test for responsivity to mild sustained noxious stimulus (Dua and Pinsky, unpublished) A similar phenomenon has been observed also by other investigators (R. Greenberg, personal communication). (ii) Amastatin, given along with Phe-Ala, produces greater analgesia in the 55$^{0}$C hotplate test than does either of Phe-Ala or amastatin given alone (manuscript in preparation).

The characteristic neck-twitching, leftward body-lurching and wet-dog shakes observed here in rats treated with combined Phe-Ala and Met-enkephalin treatment appears to be a behavioral expression of dyskinetic or, perhaps, seizure activity. Enkephalins have been shown to produce epileptiform activity, at lower doses than those required for antinociception (Lewis et al., 1978). Although neither Phe-Ala nor amastatin produce such behavior by themselves, they will do so when given in combination (Dua and Pinsky, unpublished). This further supports the concept that Phe-Ala given singly does not protect endogenously-released nor exogenously-administered enkephalins to a degree sufficient to produce analgesia. Moreover,the dose of

enkephalin used here has elsewhere been shown to produce hyperthermia (Ferri et al., 1978), the absence of change in core temperature as seen in this study further indicates very little protection of enkephalin by Phe-Ala.

Thus Phe-Ala, a simple compound which is much more readily available for experimentation than another specific enkephalinase inhibitor, thiorphan (use of Phe-Ala for such purpose was graciously suggested to us by Dr. J.C. Schwartz, Paris, to whom we are grateful), should prove a useful agent for studying selective inhibition of enkephalinase in vitro and in vivo.

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# IV. ANALGESIC RESPONSES TO SYNERGISTIC PROTECTION OF MET-ENKEPHALIN SUPPORTS A PHYSIOLOGICAL ROLE FOR OPIOID PENTAPEPTIDES

SUMMARY

Amastatin, a potent aminopeptidase inhibitor, given intracerebrally in combination with Phe-Ala, a highly selective dipeptidyl carboxypeptidase inhibitor, produced a highly significant analgesia in rats. Treatment with either inhibitor alone gave significantly less analgesia. Combined treatment with amastatin/Phe-Ala and Met-enkephalin invoked a many fold potentiation of Met-enkephalin-induced analgesia. The enhanced analgesic effect of Met-enkephalin and amastatin/Phe-Ala combination was naloxone-reversible. These results support the postulate of a physiological role for opioid pentapeptides and suggest that the combined actions of aminopeptidase(s) and dipeptidylcarboxypeptidase(s) are responsible for the rapid termination of enkephalinergic effects at synapses where the pentapeptides are released from nerve terminals.

Soon after the discovery of enkephalin pentapeptides it was recognized that they are subject to degradation by peptidases (Hughes, 1975). It became clear, from a number of studies, that enkephalins are degraded mainly by a dipeptidylcarboxypeptidase (DCPase), and/or by aminopeptidase (APase) (Meek et al., 1977; Malfroy et al., 1978; Schwartz et al., 1981). The DCPase fulfills several criteria required for an enzyme to be regarded as a specific enkephalinase, it has therefore been suggested that DCPase plays a major role in the regulation of endogenous enkephalin activity at opiate receptor sites (Schwartz et al., 1981).The role of APase has not been so well delineated. It is known that Tyr is the first metabolite to appear in tissues after in vivo administration of exogenous enkephalins by various routes, suggesting that APase is important in the enzymatic disposal of exogenously-administered enkephalins (Schwartz et al., 1980). A possible third route of enkephalin degradation could be by enkephalinase-B (a Gly-Gly dipeptidase); the role of this enzyme is as yet uncertain (Gorenstein and Snyder, 1979; Hazato et al., 1982).

It is logical to suspect that the almost instantaneous degradation of enkephalins by peptidases may have obscured the true physiological role of these peptides. This problem might be overcome by the administration of synthetic enzyme-resistant analogs or of specific peptidase inhibitor(s). The enzyme-resistant analog is prepared by modifying the sequence of peptides, thus changing the natural configuration of the endogenous molecule. The actions of such substances may thus not truly invoke the effects of the natural peptides. The use of peptidase inhibitors has an advantage over the use of analogs in that inhibitors may be utilized as pharmacological tools

to modulate and study the physiological role of endogenously-released enkephalins.

We decided to pursue the latter possibility by administering (i) a specific peptidase inhibitor alone and, also, in combination with Met-enkephalin (ME) or (ii) a combination of two different peptidase inhibitors with or without ME. The effects of these various combinations of treatments were studied on behavioral and analgesic responses. We have previously reported behavioral effects of the specific and potent APase inhibitor amastatin (AM), alone or in combination with ME, and compared it with its stereoepimer epiamastatin (EAM) and with saline (Dua et al., 1981). We also observed behavioral effects of the specific DCPase inhibitor Phe-Ala (PA), alone or in combination with ME (unpublished observation). We report here the naloxone-sensitive behavioral and analgesic effects seen after administration of AM and PA together or in combination with ME.

Amastatin (AM), epiamastatin (EAM) and Phe-Ala (PA) were obtained from Sigma Chemicals, St. Louis, MO; Met-enkephalin from Penisula Laboratories, St. Carlos, CA; Naloxone.HCl was a gift from Endo Laboratories, Garden City, NJ. Male Sprague-Dawley rats (Canadian Breeding Laboratories, St.Constant, PQ), 180-200 g, were anesthetized with pentobarbital sodium 60 mg $kg^{-1}$ supplemented with intraperitoneal (IP) chloral hydrate 160 mg $kg^{-1}$ (Valenstein, 1961) and prepared with intracerebral cannulae implanted into the right lateral ventricle (Rezek and Havlicek, 1975). Rats were kept individually in cages, in a 0600 - 1800 h light schedule with food and water ad lib. After several days postoperative recovery the rats were tested between 0800 and 1300 h. The degree of analgesia was estimated from the latency to the first

hindpaw lick or to first leap upon exposure within a large acrylic cylinder to a hotplate at 55°C (Dua et al., 1982). Latency was measured just prior to treatment in each animal. The pretreatment latency in seconds was taken as a baseline value and subtracted from all subsequent measurements of latency. Hence, "negative" estimates of analgesia were obtained when latency was less than the pretreatment value, a procedure which permits detection of hyperalgesic states. The rat was removed from hotplate immediately after pretreatment and given IP injection of either saline or of naloxone.HCl 5.0 mg $kg^{-1}$, followed promptly by three consecutive ICV infusion(s) of appropriate solutions, each in 5.0 µl volumes over 2.5 min.

At the end of the third infusion, the rat was replaced on the hotplate and measurement of analgesia repeated for up to 60 min. Area under the curve for time vs analgesia (min sec), was estimated by the trapezoidal method (Notari, 1971). A blind, random, design was used for drug treatments and observation of responses. Correct placement of cannulae was confirmed in each animal by infusion of 10 µl of eosin dye (diluted in phosphate buffer 1:12) under pentobarbital anesthesia, just prior to sacrifice. Data was accepted only from those animals where cannulation had been successful. Data were checked for homogeneity by Bartlett's homogeneity test, followed by analysis of covariance and intergroup comparisons with Duncan's multiple comparison test (Steel and Torrie, 1960).

The combination of ICV infusions with AM 30 µg, PA 500 µg and sal 5 µl gave a significantly greater analgesic response than that resulting from a combination of EAM 30 µg, PA 500 µg and sal 5 µl, or of three infusions of saline each of 5 µl volume or PA 500 µg or AM 30

µg alone given along with two infusions each of 5 µl saline (Table 3). These doses of AM and PA were analgesic by themselves when administered singly and were chosen from our previous studies (Dua et al., 1982a, and manuscripts prepared for submission). The analgesic response of AM 30 µg, PA 500 µg and sal 5 µl was significantly antagonized by pretreatment with naloxone.Hcl 5.0 mg $kg^{-1}$ IP.

When infusion of a previously-reported nonanalgesic dose of ME 100 µg (Leybin et al., 1976) was added to the analgesic combination of AM 30 µg or PA 500 µg, it produced a significantly greater analgesic response than did combined infusions of AM 30 µg PA 500 µg and sal 5 µl or than did each of AM 30 µg, PA 500 µg, or ME 100 µg, when given along with two infusions of saline each of 5 µl volume (Table 3). Response to the combination of AM 30 µg PA 500 µg ME 100µg also was significantly analgesic compared to the combination of EAM 30 µg PA 500 µg ME 100 µg or to three infusions of saline each of 5 µl volume. This potentiated analgesic response of ME 100 µg in rats pretreated with AM 30 and PA 500 µg was significantly reversed by an injection of naloxone.HCl 5 mg $kg^{-1}$ IP, given prior to ICV infusions ($P < 0.001$ ). The potentiated analgesic response of ME had a mean duration of 35 min and a mean peak latency of 94 sec, whereas, mean duration of analgesic response of AM 30 µg PA 500 µg sal 5 µl was 10 min and the peak latency was 76 sec (Table 3).

Rats treated with AM 30 µg PA 500 µg sal 5 µl were somewhat quiet and showed a typical neck-twitching and lurching to the right; we have observed this phenomenon previously with combination of PA 500 µg/ME 100 µg (prepared for submission). However, rats given combined ICV AM 30 µg, PA 500 µg and ME 100 µg showed no

Table 3

Analgesic Responses to Synergistic Protection of Met-Enkephalin

| Groups[a] (Doses:see text) | n | Injection IP | Infusions i.c.v.[b] 1st | 2nd | 3rd | Analgesia[c] | Time to peak[d] min | Latency at peak[e] sec |
|---|---|---|---|---|---|---|---|---|
| A | 8 | Sal | Sal | Sal | Sal | 6 ± 5 | 5 | 6 |
| B | 4 | Sal | Sal | Sal | Enk | 8 ± 22 | 5 | 7 |
| C | 9 | Sal | Sal | Phe-Ala | Sal | 62 ± 27 | 15 | 13 |
| D | 7 | Sal | Epi | Sal | Sal | 59 ± 30 | 5 | 8 |
| E | 8 | Sal | Am | Sal | Sal | 128 ± 30 | 5 | 14 |
| F | 6 | Sal | EAM | Phe-Ala | Sal | 275 ± 142 | 5 | 20 |
| G | 6 | Sal | Am | Phe-Ala | Sal | 938 ± 365 | 5 | 75 |
| H | 3 | NLX | Am | Phe-Ala | Sal | 12 ± 21 | 10 | 7 |
| J | 5 | Sal | EAM | Sal | Enk | 71 ± 38 | 10 | 13 |
| K | 11 | Sal | Am | Sal | Enk | 512 ± 180 | 20 | 44 |
| L | 8 | Sal | Sal | Phe-Ala | Enk | 123 ± 10 | 15 | 20 |
| M | 4 | Sal | EAM | Phe-Ala | Enk | 75 ± 17 | 5 | 16 |
| N | 8 | Sal | Am | Phe-Ala | Enk | 1764 ± 251 | 5 | 94 |
| O | 4 | NLX | Am | Phe-Ala | Enk | 20 ± 19 | 10 | 7 |

Legend, Table 3

Sal = Saline; Epi = Epiamastatin; Am = Amastatin;
Enk = Enkephalin; NLX = Naloxone.HCl

[a] Significant intertreatment differences for Total Analgesic Response, G is significantly different from A,B,C,D,E,F,H,J,K,L,M,N and O ($P \leq 0.001$, multiple comparisons). Significant differences were noted also with C vs A, E vs A, E vs D, E vs B, A vs K, B vs K, B vs L, J vs K ($P \leq 0.05$, Student's t-test).

[b] In this experiment each rat received three infusions, as preliminary experiments indicated that combination of AM or EAM with ME, yields an inactive solution (possibly due to formation of inactive complex). Our preliminary experiments indicated also that AM given prior to PA resulted in a better response (possibly AM protected PA from degradation) than when the sequence was reversed.

[c] Total analgesic curve area, sec min (mean $\pm$ S.E.).

[d] Taken from curves plotting hindpaw lick latency vs time elapsed after final infusion and smoothed by approximated running-average alogrithm. See note [e] below.

[e] Mean latency to first hindpaw lick or to leap. Standard error not shown since this parameter is provided here only to indicate range of observed values.

neck-twitching, but displayed only wet-dog shakes, head-scratching, occasional gnawing at cylinder, and a slight sedation. There was no catatonia nor loss of righting reflex. Rats treated with naloxone.HCl 5 mg $kg^{-1}$ prior to either AM 30 µg/PA 500 µg and sal 5 µl or AM 30 µg/PA 500 µg/ME 100 µg showed a considerable number of jumps as compared with any other treatment in this set of experiments.

## DISCUSSION

These results indicates that endogenous enkephalins are likely released during the hotplate test at 55°C. These enkephalins must be susceptible to both APase and DCPase, because the combination of AM and PA produced a greater analgesic response than did either of these peptidase inhibitors given alone (Dua et al., 1981). This likely represents an indirect response of endogenously-released enkephalins during hotplate test. The analgesia is apparently mediated through opiate receptors since naloxone.HCl 5.0 mg $kg^{-1}$ given prior to ICV infusion significantly reversed the analgesic response.

The response to a nonanalgesic dose of ME was significantly potentiated by pretreatment with a combination of AM and PA. Response to treatment with combined AM/PA and ME was greater than to treatment with combined AM/PA alone, by more than 800 analgesia units (difference between values in Group N and Group G, Table 3). This provides an estimate of the analgesic effect exerted by the ME in the presence of combined AM/PA and represents a one-hundredfold increase of ME analgesia, by comparison with the effect of treatment with ME by itself (Group B, Table 3). It is a clearcut example of pharmacological

potentiation. The AM/PA-induced potentiation of ME analgesia is presumably due to simultaneous inhibition of the two major enkephalin-degrading enzymes, APase and DCPase. The analgesia observed in the experiments with combined AM/PA and ME often produced hindpaw lick latencies greater than an assigned cutoff time of 120 secs. Analgesia of such profound nature has never been reported for ME at any dose ICV, nor for enzyme-resistant synthetic ME-analogs (Pert, 1976). This observation suggests that enkephalins are in fact highly analgesic substances and that upon release from their nerve terminals they likely initiate analgesic or other opioid agonist responses which is rapidly terminated by the combined action of enkephalin-degrading enzymes localized to the enkephalinergic synapse. This postulate, if confirmed, places the enkephalin opioid pentapeptides in the category of physiologically-important neurotransmitters.

The neck-twitching and rightward-lurching phenomena was seen only with either PA with ME or AM with PA and not with the combination of AM, PA and ME. This may indicate that (i) at a low level of activity on the opiate receptor (e.g. with endogenously released ME in AM/PA treated rats or in rats treated with PA/ME,) enkephalins induce the opioid hyperactive state (Leybin et al., 1976; LaBella et al., 1982) and (ii) at a higher level (e.g. in rats treated with combined AM/PA/ME) they act to suppress the hyperactive state. The 'wet-dog shakes', a behavioral expression of narcotic withdrawal, were seen in the rats were pretreated with naloxone just prior to treatment with AM/PA/ME. The latter may have been an example of the development of opioid dependency withdrawal following a single administration of opioid substances (Pinsky et al., 1982).

In conclusion, this study supports the concept of a physiological role for endogenously released opioid peptide(s). The results also show indirectly that both APase and DCPase are physiologicaly important and act in concert with the DCPase actions proposed by Schwartz et al. (1981).

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# V. ENDORPHIN-LIKE RESPONSES PROVOKED BY INTRACEREBROVENTRICULAR PEPTIDASE INHIBITORS IN MILDLY STRESSED RATS

## Summary

Administration of the peptidase inhibitors aprotinin and bacitracin directly into the rat lateral ventricle resulted in EEG spiking, stiff tail, analgesia to tail pinch and flaccid immobility. EEG spiking could be completely blocked by naloxone.HCl at 20 mg $kg^{-1}$ intraperitoneally; flaccid immobility was enhanced by sustained mild heat/confinement stress and blocked by naloxone. The combination of peptidase inhibition and stress induced a condition resembling acute ("single dose") opiate narcotic dependency in the experimental animals. The results suggest that inhibition of central peptidases promotes the accumulation of central endorphins which are released in response to an appropriate stress.

## Introduction

Evidence that inhibitors of carboxypeptidase-A might prevent the breakdown of central endorphins has been provided by Ehrenpreis (Ehrenpreis et. al., 1978). To pursue this concept we administered peptidase inhibitors directly into the cerebrospinal fluid in order to provoke effects directly at the site of central endorphin release. It seemed reasonable to suppose that mild, sustained, nociception (Pinsky et. al., 1974) would be an optimal stimulus for the central release of endorphin, as suggested originally by Goldstein, 1974.

## Materials and Methods

Preparation and observation. Male Sprague-Dawley rats (Canadian Breeding Laboratories, St. Constant, PQ), 150-175 g, were anesthetized with pentobarbital sodium, 35 mg $kg^{-1}$ intraperitoneally (i.p.) supplemented with chloral hydrate, 160 mg $kg^{-1}$ i.p. (Valenstein, 1961) and implanted with intracerebral cannulae into the right lateral ventricle and with bipolar epidural platinum electrodes bilaterally on sensorimotor cortex (Rezek et. al., 1975). After several days recovery they were tested in experiments where solutions were infused intracerebroventricularly (icv) in volumes of 10 ul over 4 min. The peptidase inhibitors aprotinin (Trasylol, Boehringer) and bacitracin (Mann) or their combination were given intracerebroventricularly (icv) in osmotically-balanced, pH-adjusted solutions and behavioral and EEG responses examined. Control substance infusion was bovine serum albumin (BSA; fraction V, Sigma) adjusted for osmolarity and pH.

After infusion, rats were placed in an open-top, bottom-vented, plastic cylinder 20 cm dia x 30 cm ht on a teflon-surface plate maintained at 22 $\pm$ 2.0 or 44.5 $\pm$ 0.2$^{o}$C (Pinsky et. al., 1975). They were observed for behavioral and EEG responses for 10 or 20 min. Parenteral administration of naloxone.HCl, 20 mg $kg^{-1}$, or of saline (control) was by injection i.p. in volumes of 0.1 ml/100 g wt.

Experimental Design and Analysis. Experiments were designed so that various treatments were spread out randomly throughout the day. Animals were never used more than twice, with a 4-7 day rest period between trials. Some experiments, but all analysis of records, were done blinded fashion. Eosin dye, diluted in phosphate buffer, was infused (10 µl/4 min) in each animal at the end of experimentation, data was accepted only from those animals where dye verified adequacy of cannulation. EEG epileptiform spiking activity was assessed independently by three observers. Statistical inferences were made from parametric or nonparametric tests of significance, as appropriate to the nature of the data being analyzed (Steel and Torrie, 1960).

## Results

Aprotinin icv, over the dose range 20-200 kallikrein-inhibiting units (KIU), provoked clusters of epileptiform spikes (Havlicek et. al., 1978; Pinsky et. al., 1962) within 2-4 min of placement on the 44.5$^{o}$C warmplate (Table 4). This response resembled the spiking activity provoked by beta-endorphin icv and, as reported for endorphin-induced spiking, was blocked by i.p. naloxone (Havlicek et.

Table 4

Induction of Epileptiform Spike Clusters by Peptidase Inhibitors

| | pretreatment, i.p. | Total cluster duration[a] secs (mean ± SE) 44.5°C |
|---|---|---|
| BSA (29) | none | 0 |
| Bacitracin, 5.0µg (1) | none | 0 |
| Aprotinin, | | |
| 20,50,100 KIU (3,3,8) | none | 0 |
| 150 KIU (4) | none | 0.05 ± 0.5 |
| 200 KIU (9) | none (4), saline (5) | 39.0 ± 11.1[b] |
| 200 (9) | naloxone 20 mg $kg^{-1}$ | 0 |
| 200 (4) | naloxone 5.0 mg $kg^{-1}$ | 0 |
| ( ) = no. of rats | | |

[a] Observed on 44.5° warmplate for 10 min immediately after end of infusion.

[b] Significantly different from lower doses of aprotinin and from naloxone-treated, $p \leq 0.01$.

al., 1978). In the present experiments there was complete blockade, in 9 rats, with naloxone 20 mg $kg^{-1}$ i.p. (Table 4). Spiking began and ended abruptly; the essentially all-or-none dose response relationship (abrupt increase at 200 KIU, Table 4) closely resembles the stimulus intensity-epileptiform response seen with electrical stimulation (Pinsky et. al., 1962). There was significantly ($p \leq 0.05$) less hindpaw licking at 44.5°C in the aprotinin-infused animals than in the BSA controls. This analgetic effect was not diminished, but was slightly ($p \leq 0.2$), augmented by naloxone 20 mg $kg^{-1}$ i.p. - a result that may correspond to previous (Pinsky et. al., 1978; Little et. al., 1978) observations of an antinociceptive effect of naloxone in certain forms of nociception.

Combination of aprotinin 50 KIU with bacitracin 5 µg, both ineffective by themselves, caused epileptiform spiking at 22°C after infusion icv. Increased temperature did not seem immediately to enhance this response to the combination. In three experiments, however, replacement on the 44.5°C warm-plate at 2 1/2 hrs after infusion appeared to provoke epileptiform spiking in previously unresponsive rats (Fig. 3D). The same procedure provoked opiate-like behaviors (agitation, pacing, stiff tail, analgesia (tail pinch), frozen stares) in those and in three other animals similarly tested.

Aprotinin 50 KIU combined with bacitracin 2.5 µg failed to produce any epileptiform activity in 10 of 10 rats given the combination icv. This treatment did, however, produce significant scores in various behaviors some of which related to opiate agonism. Animals treated with this inhibitor combination became flaccidly immobile when placed on the 44.5°C warmplate, but displayed no such

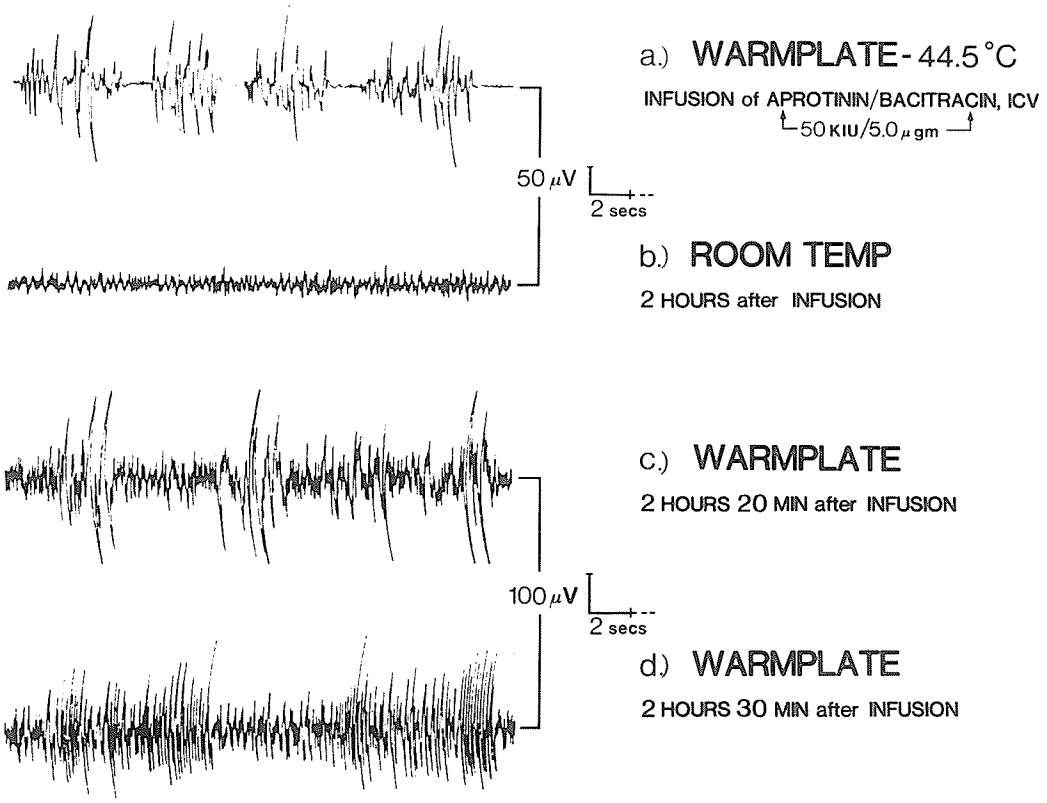
a.) WARMPLATE - 44.5 °C
INFUSION of APROTININ/BACITRACIN, ICV
50 KIU/5.0 μgm
50 μV
2 secs
b.) ROOM TEMP
2 HOURS after INFUSION
c.) WARMPLATE
2 HOURS 20 MIN after INFUSION
100 μV
2 secs
d.) WARMPLATE
2 HOURS 30 MIN after INFUSION

Fig. 3

Fig. 3. Effects of test surface temperature on behavioral and EEG activity in peptidase inhibitor-treated rats. a: Warmplate at 44.5°C, immediately after infusion. Prominent responses were: ataxia, clawing, salivation, wet-dog shakes, backward walking and twitches (large excursions in EEG recordings are movement artefacts). b: Similar plate surface, but at room temperature (22°C), 2.0 hr postinfusion (after 2.0 hr rest in home cage). Behavior was normal, with rat calm, sitting and sniffing or quitely exploring environment in cylinder. c: Response to replacement on 44.5°C warmplate, 20 min after observations in "b". Large EEG potentials coincided with movements, and do not represent epileptiform spiking. There was increased motor activity, with standing, probing vents at bottom of cylinder and pawlicking. d: Rat on same warmplate as in "c", 10 min after observations for "c". EEG recording shows clusters of high-frequency epileptiform spikes, animal was completely motionless (sphinxlike, "frozen stare") during epileptiform activity. Behavior appeared agitated, with increased pawlicks, repetitive standing on hindlegs and stiff tail.

behavior on a 22°C plate. Difference in the number of observations of the immobility, assessed every 11 sec over a 20 min period at each temperature, was statistically significant ($p \leq 0.05$). This response was antagonized significantly ($p \leq 0.05$) by naloxone 20 mg $kg^{-1}$. The same dose of naloxone provoked vigorous upward leaping, spontaneous vocalization and self-mutilation in the inhibitor-infused rats tested at 44.5°C. We have previously observed only limited instances of upward leaping in response to warmplate stress after naloxone treatment in this strain of rat (Pinsky et. al., 1978) and have observed spontaneous vocalization and self-mutilation only in rats withdrawing from morphine habituation.

## Discussion

Infusion of peptidase inhibitors icv to lightly-restrained rats induces several behavioral and EEG responses that resemble opiate-induced activity. Naloxone blockade (e.g. of EEG spiking and of flaccid immobility) supports the possibility that the inhibitors promote the central accumulation of opiomimetic neuropeptides. Mild heat/confinement stress (i.e. the conditions of the 44.5°C warmplate test) appears to enhance the release of endorphins, since some effects of the inhibitors were exaggerated when the rats were placed on the warmplate as compared to activity on an identical surface at room temperature (Fig.). Induction of an opiate withdrawal-like syndrome by naloxone suggests that warmplate stress and peptidase inhibition combine to result in a condition resembling acute opioid dependency, which has been described (Cheney et. al., 1971; Kosersky et. al., 1974;

Smits, 1975) for single large doses of opiate narcotic drugs. Some of the responses to peptidase inhibitors were unlike those expected for opioid agonism (e.g. clawing, backward walking, crawling) and these were not blocked by naloxone (unpublished). This is not a surprising result, since generalized central peptidase inhibition may be expected to result in the accumulation of many neuroactive peptides other than those with affinity for stereospecific opiate receptors.

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# VI. EFFECTS OF BETA-ENDORPHIN GIVEN INTRACEREBRALLY ARE ENHANCED BY APROTININ

## SUMMARY

Intracerebral administration of the serine proteinase inhibitor, aprotinin, enhanced analgesic and behavioral responses to beta-endorphin given intracerebrally in rats. Combination of aprotinin and beta-endorphin, in doses where each substance was inactive by itself, provoked surgical anesthesia, hyperthermia and epileptiform activity. The enhanced analgesic, behavioral and thermic responses were all reversed with naloxone. Death due to beta-endorphin, apparently via aprotinin-enhanced respiratory depression, was observed; this is the first report of a lethal response to beta-endorphin. The observed enhancement of beta-endorphin effects is presumably due to aprotinin protection of the opioid peptide from enzymatic degradation by the endopeptidase which releases endorphin fragments from the parent molecule. Opiate receptor binding studies, done *in vitro* with brain tissue obtained from rats treated with combined aprotinin and beta-endorphin *in vivo*, were consistent with this hypothesis.

Aprotinin (APT) is a serine protease inhibitor capable of inhibiting a wide range of proteases and peptidases (Trautschold et al., 1967, Balldin et al., 1978). Analgesia and other behavioral responses to the parenteral (Novelli et al., 1980 ) or intracerebroventricular (Bertolini et al., 1977, Dua et al.,1979, Pinsky et al., 1980) administration of APT have been described. The responses were attributed to APT -induced protection of endogenous opioid peptides from hydrolysis by brain peptidases. To verify this concept we have administered a combination of APT and beta-endorphin (END) intracerebroventricularly (ICV) in rats. We now report that APT potentiates the behavioral and antinociceptive effects of exogenously-administered END and protects the exogenous opioid peptide from the action of peptidases.

Aprotinin (Trasylol 10,000 KIU/ml) was obtained from Boehringer, Dorval,PQ. Beta-endorphin (Camel) was obtained from Peninsula Laboratories, St. Carlos, CA. Naloxone. HCl was a gift from Endo Laboratories, Garden City, NJ. Drugs were dissolved in sterile pyrogen-free saline, for administration ICV or parenterally.

Experimental design and statistical analysis: Male Sprague-Dawley rats (Canadian Breeding Laboratories, St. Constant PQ), 180-200 g were anesthetized with intraperitoneal (IP) pentobarbital sodium 60 mg $kg^{-1}$ supplemented with chloral hydrate 160 mg $kg^{-1}$ IP (Valenstein, 1964) and prepared with intracerebral cannulae (Rezek and Havlicek, 1975) implanted into the right lateral ventrical and bipolar epidural platinum electrodes bilaterally on sensorimotor cortex (Pinsky et al., 1982). Rats were used in experiments after several days of recovery. On the experimental day rats were first given an IP injection of saline

0.5 ml or naloxone.HCl 5 mg $kg^{-1}$ in equivolume saline. Soon after this rats were given two consecutive ICV infusions of 10 µl volume each over 5 min according to the schedules for infusion, as shown in Table 5. Soon after the end of second infusion rats were placed in an open-top, bottom-vented, plastic cylinder 20cm dia x 30 cm ht on a massive aluminum surface plate maintained at a surface temprature of 55 ± 0.2°C. Latency from the time of placement on the hotplate to first hindpaw lick or upward leap was taken as a measure of antinociception. Soon after the hotplate test, core temperature was recorded by inserting a thermistor probe for a distance of 5 - 6 cm in the rectum of rat, and the temperature was read out from a digital thermometer. This procedure was repeated at various intervals for up to 150 min; rats were placed on the hotplate for a total of eight times. At the end of the experimentation rats were infused with eosin dye (diluted in phosphate buffer 1:12 ) ICV 10 µl over five min under pentobarbital anesthesia, data were accepted only from those rats where dye verified adequacy of cannulation. The pre-infusion values were subtracted from all the post-infusion readings and these normalized observations were utilized for assessing analgesia. Areas under the curves for time vs analgesia and for time vs temperature were obtained by trapezoidal method of estimation of area under the curve (Notari, 1971). Data was checked for homogeneity by Bartlett's homogeneity test, followed by analysis of covariance and intergroup comparisons with Duncan's multiple comparision test (ANOVA) (Steel and Torrie, 1960).

General Behavior: Changes in general behavior due to exposure of rats to the hotplate before and after various treatments were noted over a 150-min experimental period. Behavioral responses noted in

particular were: changes in locomotor activity, catatonia, loss of righting reflex, surgical anesthesia and EEG seizure activity.

EEG Recording: Rats were placed on a plate identical to that used for hotplate test but kept at room temperature. This plate was placed next to the hotplate, to maintain a similar environment. EEG (not shown ) was recorded on an inkwriting polygraph (Grass 5B), after the various treatments as shown in Table 5.

3H-Naloxone Receptor Binding Assay: Cannulated rats were given infusions according to the treatment schedules as in Table 5. After testing for antinociception on a hotplate maintained at 55$^{o}$C at 5 and 20 min postinfusion intervals ( the latter being the time for peak effect of END ), rats were decapitated and the brain removed. Hypothalamus, striatum, hippocampus and cortex were dissected out in less than two min, on ice. Each region of brain was weighed and then homogenized by Polytron in 50mM TRIS buffer with sodium (pH 7.1). The homogenate was centrifuged at 20,000 x g and the supernatant discarded. This process was repeated twice and the precipitate resuspended. The resuspended membranes were incubated in 0.8 ml buffer with 3H-naloxone 1.985 nM/f Ci at 0$^{o}$C for 60 min. At the end of incubation, membranes were centrifuged and the supernatant discarded. The precipitated membranes were dissolved in trichloroacetic acid (TCA) and added to the scintillation medium, Radioactivity was counted in a liquid scintillation counter.

RESULTS

Potentiation of END analgesia: APT 14.5 µg given ICV after sal IP was not significantly more antinociceptive than equivolume control administration of combined sal IP and ICV. END 2.5 and 5 µg ICV given after APT 14.5 µg ICV were significantly analgesic compared to saline control; this latter combination of APT and END, however, was not significantly different in effect from equivolume administration of saline ICV and END 2.5 and 5 µg ICV.

END 10 µg ICV was given after APT 14.5 µg ICV in rats pretreated with saline 0.5 ml IP (Group G, Table 6). This combination produced significantly higher analgesia than an equal amount of END ICV given after saline 10 µl ICV in saline IP-pretreated rats (Group E, Table 6), or rats given only equivolume infusion of saline ICV and an injection of saline 0.5 ml IP (Group A, Table 6). This potentiation of END 10 µg with APT 14.5 µg was reversed by an injection of naloxone 5mg $kg^{-1}$ IP given just prior to ICV infusion (Group J, Table 6).

END 15 µg given ICV to rats pretreated with APT 18 µg ICV produced a significantly higher analgesia than did rats given equidose END after equivolume ICV saline or with rats given equivolume ICV saline alone (Table 6).

Behavioral, Motor, Electrocerebral, Thermic and Respiratory Responses:- Several behavioral responses were noted following the various treatments summarized in Table 5. The responses were most prominent in rats given ICV infusion of APT 14.5 µg followed by END 10 µg, and in those infused with APT 18 µg followed by END 15µg. Infusions of the same doses of END or APT administered singly, or of equivolume saline, produced much less behavioral change than either combination. The major observations were of sniffing, licking, forward

Table 5

Schedule of Injection and Infusions

| Group | Treatments (doses:see legend) Injection[a] IP | Infusions[b] ICV 1st | 2nd |
|---|---|---|---|
| A | Sal | Sal[d] | Sal[d] |
| B | Sal | Sal | APT[e] |
| C | Sal | Sal | End[g] |
| D | Sal | Sal | End[h] |
| E | Sal | Sal | End[j] |
| F | Sal | Sal | End[k] |
| G | Sal | APT[e] | End[j] |
| H | Sal | APT[f] | End[k] |
| J | NLX[c] | APT[e] | End[j] |

Legend, Table 5

Sal = Normal saline; NLX = Naloxone.HCl; APT = Aprotinin;

End = beta-endorphin

[a] All injections 0.5 ml volume; [b] All infusions 10 µl volume over 5 min;

[c] Naloxone.HCl 5 mg $kg^{-1}$; [d] Inert control; [e] Aprotinin, 14.5 µg;

[f] Aprotinin, 18 µg; [g] beta-endorphin, 2.5 µg; [h] beta-endorphin, 5 µg;

[j] beta-endorphin, 10 µg; [k] beta-endorphin, 15 µg.

Table 6

Potentiation of B-END Analgesia by APT

| Groups (Doses:see Table 5) | n | Total analgesic curve area sec min mean ± S.E. | Time to peak effect[b] min | Mean latency at peak effect[c] sec |
|---|---|---|---|---|
| A | 6 | 157 ± 70 | 60 | 6 |
| B | 3 | 1078 ± 1010 | 60 | 11 |
| C | 2 | - 20 ± 258 | 60 | 11 |
| D | 5 | 5427 ± 4191 | 60 | 55 |
| E | 7 | 2834 ± 963 | 60 | 66 |
| F | 4 | 2569 ± 2282 | 60 | 64 |
| G | 6 | 17124 ± 3634 | 30 | 120 |
| H | 4 | 13155 ± 3544 | 30 | 120 |
| J | 4 | 168 ± 163 | 30 | 6 |

Legend, Table 6

[a] Significant intertreatment differences for total analgesic response, ANOVA: G vs A,B,C,D,E,F,H,J and H vs A,B,C,D,E,F,J, ($P \leq 0.001$); Students 't' test: A vs G ($P \leq 0.05$).

[b] Taken from curves plotting hindpaw lick latency vs time elapsed after final infusion; smoothed by approximated running-average alogrithm. See note [c] below.

[c] Mean latency to first hindpaw lick or to leap. Standard errors not shown since this parameter is provided here only to indicate range of observed values.

and /or backward walking in circle around the interior periphery of the cylinder, leaning on the cylinder, grooming and teeth-gnashing. Stiff tail was observed in most of the rats; in these rats the tail could be shaped into any form, a phenomenon similar to cerea flexibilitas or "waxy flexibility" observed in catatonic patients. Alternately, the tail could stand erect spontaneously, as is seen in the Straub tail response with morphine in mice. The behavioral elements were very prominent in those rats treated with APT 18 ug and END 15 µg; loss of motor coordination, inability to maintain a normal standing posture, ataxia and deep catatonia were also observed. The core temperature of rats treated with APT and END was significantly higher than in rats given infusion of END followed by saline or of saline alone (Table 7).

Two out of eight rats in group G, Table 8, showed a loss of righting reflex. These animals appeared to be in a state resembling surgical anesthesia. The pinna could be cut through with scissors without production of struggling or vocalization. Pinching the tail or poking the rats with a needle caused an increase in respiratory activity but evoked no flinch or vocalization. This state of profound indifference to highly noxious stimuli was never observed in rats pretreated with naloxone prior to identical infusions with APT and END (Group J, Table 8).

Epileptiform activity (not shown ) was observed in rats given infusions of APT 14.5 µg and END 10.0 µg (Group G, Table 8) or of APT 18 µg and END 15 µg (Group H, Table 8). This was not seen in rats pretreated with naloxone.HCl 5 mg $kg^{-1}$ IP (Group J, Table 8). Cyanosis and periods of apnea were noted in five rats treated with APT 18 µg and

Table 7

Thermic Responses to beta-Endorphin,
Enhancement by APT and Sensitivity to Naloxone

| Group[a] (Doses:see Table 5) | n | Total thermic curve area °C min mean ± S.E. | Time to[b] peak effect min | Mean core[c] temperature at peak °C |
|---|---|---|---|---|
| A | 6 | 31 ± 5 | 5 | 37.5 |
| C | 2 | 238 ± 121 | 60 | 40.0 |
| D | 5 | 220 ± 36 | 60 | 39.0 |
| E | 2 | 177 ± 65 | 60 | 40.0 |
| F | 2 | 177 ± 17 | 60 | 40.0 |
| G | 6 | 369 ± 42 | 60 | 40.0 |
| H | 4 | 465 ± 135 | 60 | 40.0 |
| J | 4 | 155 ± 21 | 60 | 37.0 |

Legend, Table 7

[a] Significant intertreatment differences for total core temperature response (areas under time vs thermic response curve, $^{o}$C min), ANOVA: G vs A, G vs E, G vs J ($P < 0.001$), F vs A, F vs H ($P < 0.05$); Student 't' test: J vs A, D vs A, F vs A, G vs A ($P < 0.001$), H vs A, G vs J ($P$ 0.05).

[b] Taken from curves plotting core temperature vs time elapsed after final infusion and smoothed by approximated running-average alogrithm. See note[c] below.

[c] Mean core temperature. Standard errors not shown since this parameter is provided here only to indicate range of observed values.

Table 8

Behavioral Responses Due to B-END and its Enhancement by APT

| Behavioral Responses | Groups (Doses:see Table 5) | | | | |
|---|---|---|---|---|---|
| | D | E | G | H | J |
| Stereotypies | + | ++ | - | - | - |
| Stiff tail | - | ++ | ++ | +++ | - |
| Motor Incoordination | - | + | ++ | +++ | - |
| Catatonia (bar test) | - | + | ++ | +++ | - |
| Surgical Anaesthesia | - | - | ++++ | ++ | - |
| EEG Spiking | - | - | + | ++ | - |
| Respiratory Arrest | - | - | - | death (2/5) | - |

Table 9

$^{3}$H-Naloxone Binding in Vitro After Beta-END Protection in Vivo

| Group (see:Table 5) | $^{3}$H-naloxone mean final count (procedure:see text; n-2) | | | |
|---|---|---|---|---|
| | Ctx | Str | Hip | Hyp |
| A | 2116 | 2013 | 1872 | 1532 |
| E | 1503 | 1665 | 1147 | 955 |
| G | 1114 | 1648 | 913 | 776 |

Ctx = cortex; Str = striatum; Hip = hippocampus;

Hyp = hypothalamus

END 15 µg (Group H, Table 8), the apnea could be overcome temporarily by gentle handling. Two of the five rats were found dead in their home cages, with their noses buried in the wooden shavings bedding, at 45 min after infusions. These observations mark the first reported instance of death caused by beta-endorphin.

Opiate receptor binding assay: The 3H-naloxone receptor binding assay showed a low naloxone binding in rats treated with combined APT 14.5 µg and END 10 µg (Group G, Table 9). The effect was least evident in striatal region. Unprotected END 10 µg had less effect than its combination with APT, in all regions (Group E, Table 9).

## DISCUSSION

The carboxyl and amino ends of beta-END are enzyme-resistant, whereas its central portion is vulnerable to endopeptidase (Austen and Smyth, 1977). We tested APT as a putative antagonist of *in vivo* endopeptidase action on beta-END, since APT is a peptidase inhibitor with a broad range of inhibitory activity (Trautschold, 1967) while the substrate specificity of beta-END-cleaving endopeptidase is low (Austen and Smyth, 1977). Our results indicate that APT significantly potentiates beta-END effects on antinociception, behavioral responses and opiate receptor binding. This view is also supported by previous reports of analgesia with APT given by various routes of administration (Bertolini et al., 1977; Dua et al., 1979; Pinsky et al., 1980; Novelli et al., 1980). Moreover, the analgesia induced with parenteral APT was seen here to be reversed by naloxone, thereby strongly implicating the involvement of opiate receptors in the observed responses.

It is likely that the endorphinomimetic and endorphin-potentiating effects of APT seen here in vivo are due to inhibition of the beta-END-degrading endopeptidase that has been reported by Burbach et al. (1981), from studies done in vitro. The responses observed with APT in this study resemble beta-END agonist action on opiate receptors of the naloxone-sensitive mu subtype; e.g. analgesia, respiratory depression, surgical anesthesia (Havlicek et al.,1978, 1980), EEG spiking, hyperthermia and catatonia. A contribution from the alpha- and gamma-END fragments of beta-END (Burbach et al., 1981; Austen and Smyth, 1977) cannot be ruled out in our present experiments. Such endorphin fragments might contribute to both the in vivo responses and to the changes in opiate receptor binding that were seen here after in vivo treatment with APT. The question of relative changes in brain content of beta-END and its fragments could be resolved by radioimmunological measurements. Ogawa et al. (1981) were able to demonstrate enzymatic protection in vitro of beta-END with APT treatment in rat brain homogenate, using radioimmunoassay. Their observations support our own conclusion that beta-END is the prime species of endogenous opioid peptide involved in the endorphin-mimicking and -potentiating effects of APT which we have seen in this study.

The death due to END, reported here for the first time, suggests that in certain conditions there might be a syndrome of END overactivity. This possibility might eventually prove to be of clinical interest.

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# VII. PHENYLMETHYLSULFONYL FLUORIDE (PMSF) GIVEN SYSTEMICALLY PRODUCES NALOXONE-REVERSIBLE ANALGESIA AND POTENTIATES EFFECTS OF BETA-ENDORPHIN GIVEN CENTRALLY

## SUMMARY

Intraperitoneal (IP) injection of the serine proteinase inhibitor phenylmethylsulfonyl fluoride (PMSF) produced dose-dependent analgesia in Sprague-Dawley rats. AD50 was 2.9 $\pm$ 1.5 (S.E.) mg $kg^{-1}$, the analgesia was antagonized by naloxone but unaffected by atropine. PMSF significantly enhanced the analgesic effect of beta-endorphin (END) given by intracerebroventricular (ICV) infusion in rats, the enhanced END analgesia was naloxone-reversible. In Swiss-Webster mice the 24-hr LD50 value for PMSF was 215 $\pm$ 55 mg $kg^{-1}$ IP; autonomic and behavioral responses were similar to those seen in rats with ICV END. These results indicate that systemic PMSF can protect central endorphin(s) from enzymatic destruction. The significant analgesia, low toxicity, naloxone reversibility and minimal anticholinesterase effects suggest the use of PMSF as a parenteral analgesic.

## Introduction

The organophosphate cholinesterase inhibitor, diisopropyl phosphofluoridate (DFP) produces analgesia in rats ( Koehn and Karczmar, 1978); the analgesic action is antagonized by atropine and by naloxone (Koehen et al., 1980) . It was proposed that DFP analgesia is exerted via a cholinergically-mediated release of endogenous opioid peptides (Koehen et al., 1980). It is known also, however, that inhibitors of proteolytic enzymes can produce analgesia in rats, presumably by protection of endorphins from enzymatic cleavage (Pinsky et al, 1980; Bradbury et al, 1978). In addition to its anticholinesterase action DFP is also an effective inhibitor of serine peptidases (Desnuelle, 1960; Jensen and Balls, 1952; Fahrney and Gold, 1963). The observation of Karczmar and his colleagues prompted us to search the literature for other lipid-soluble organic substances which have inhibitory action on serine peptidases in some fashion similar to DFP. The sulfonyl fluoridated aromatic alkane, phenylmethylsulfonyl fluoride (PMSF; phenylmethane sulfonyl fluoride; toluene-p-sulphonyl fluoride) has been shown to inhibit bovine trypsin and chymotrypsin as well as several species of acetylcholinesterase in rodents and humans (Myers and Kemp, 1954; Fahrney and Gold, 1963; Turini et al, 1969; Klocking et al., 1975). PMSF has been shown to cross the blood-brain barrier in rodents (Turini et al., 1969) as does DFP. Because of these reported similarities between the pharmacological actions of PMSF and DFP on proteolytic and esteratic enzymes we decided to examine PMSF for comparative analgesic and toxic effects in rats and mice.

## Material and Methods

Animals and Material: Male Swiss-Webster mice (18-20 g) and Sprague-Dawley rats (180-200 g) were obtained from Canadian Breeding Laboratories, St. Constant, PQ. PMSF and atropine sulfate were obtained from Sigma Laboratories, St. Louis, MO; beta-Endorphin (camel) (END) from Peninsula Laboratories, St. Carlos, CA; peanut oil (PO) (Planter's, COR quality) from domestic supplier. Naloxone.HCl was a gift from Endo Laboratories, Garden City, N.J. PMSF was dissolved in PO; beta-Endorphin, atropine sulfate and naloxone in sterile injectable saline.

Analgesia test in Rats: For assessment of analgesia, rats were placed in an open-top, bottom-vented plastic cylinder 20 cm dia x 30 cm ht on a massive aluminum plate maintained at a surface temperature of 55 $\pm$ 0.2$^{o}$C. Latency from the time of placement on hotplate to first hindpaw lick or upward leap was taken as a measure of analgesia. Analgesia was measured prior to any drug or vehicle control treatment, measurement was followed immediately by an intraperitoneal (IP) injection of PO 0.2 ml or of equivolume PMSF in doses ranging from 0.3 to 30 mg $kg^{-1}$. Estimation of the dose for production of 50 % maximal analgesia (AD50) was made from a log-probit plot (Miller and Tainter, 1944) of dose-response data.

Pharmacological antagonism of PMSF analgesia : Rats were tested on hotplate before administration of any drug or vehicle control and treated immediately thereafter with either PO or PMSF 4.0 mg $kg^{-1}$ IP. Rats then received i.p. injections of either saline 0.5 ml IP or equivolume atropine sulfate 4.0 mg $kg^{-1}$ at 30 min posttreatment, or of

naloxone.HCl 5.0 mg $kg^{-1}$ at 40 min posttreatment. These times of injection accounted for respective times to peak effects for the antagonists. Rats were tested on hotplate for analgesia at 60 min posttreatment, and any change in behavior was noted.

PMSF potentiation of END analgesia : Rats were anesthetized with pentobarbital sodium, 35 mg $kg^{-1}$ IP supplemented with chloral hydrate 160 mg $kg^{-1}$ IP (Valenstein, 1964). Intracerebral cannulae (Rezek and Havlicek 1977) were implanted stereotaxically into the right lateral ventricle (Konig and Klippel, 1963; coordinates A+5.7, V-8.4, L+1.8) of each anesthetized animal. Experiments were done after several days of recovery. Analgesia was measured before any drug or vehicle control treatment and IP injections of either PO 0.2 ml or PMSF 4.0 mg $kg^{-1}$ were given immediately thereafter. An injection of saline 0.5 ml IP or of equivolume naloxone HCl 5.0 mg $kg^{-1}$ IP was given 40 min after PO or PMSF treatment. Intracerebroventricular (ICV) infusion of END in volumes of 10 µl over 5 min was done promptly after saline or naloxone injection. Analgesia was measured at the end of END infusion and thereafter at various intervals until 210 min after END infusion. Any changes in behavior were recorded during the postinfusion period.

Core Temperature Responses : Core temperature was recorded by inserting a probe 5-6 cm in the rectum. This probe was attached to a digital thermometer and temperature was recorded before all treatments and after every hotplate test.

Experimental design and analysis of data: A blinded design was utilized to minimize effects of experimenter bias. Rats were anesthetized with pentobarbital at the end of the experiment and given ICV infusions of eosin dye ( diluted 1:12 in phosphate buffer ) 10ul

over 5 min. Data were accepted only from those rats where dye verified adequacy of cannulation. Area under the curve for time vs analgesia (sec min) was measured by an iterative trapezoidal method (Notari, 1971). Data were checked for homogeneity by Bartlett's homogeneity test,followed by analysis of covariance (ANOVA) and intergroup comparison with Duncan's multiple range test (Steel and Torrie, 1960).

PMSF Toxicity in mice: Mice in groups of ten were given IP injections of either 0.2 ml of PO or equivolume PMSF in various doses (Table 10). They were then observed for changes in behavior and for general signs of toxicity immediately after injection and at various intervals until 48 hr postinjection. LD50 was calculated from data plotted on log-probit paper. PMSF- treated mice which died over the 48-hr period of observation were examined postmortem for gross pathological changes.

## Results

PMSF analgesia and dose-response relationship in rats: PMSF produced a dose-dependent analgesia ; the dose-response relationship was of sigmoid shape and showed an AD50 of 2.9 $\pm$ 1.5 (S.E. mg $kg^{-1}$ (Fig. 4). Naloxone.HCl 5.0 mg $kg^{-1}$ IP given 20 min prior to testing, significantly reversed the analgesia exerted by PMSF 4.0 mg $kg^{-1}$ (Fig 5). Atropine sulfate, injected in a separate group of rats at 30 min prior to injection of PMSF, had no effect on PMSF antinociception (Fig. 5).

Potentiation of END analgesia in rats : PMSF 4.0 mg $kg^{-1}$ IP significantly potentiated the analgesic activity of ICV END as compared

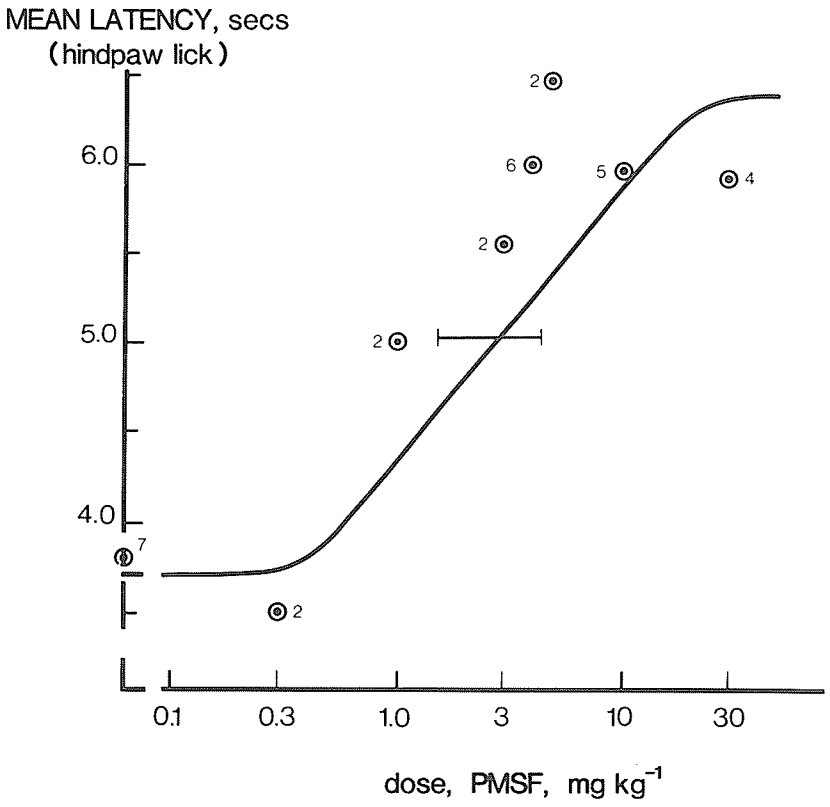
MEAN LATENCY, secs
(hindpaw lick)
6.0
5.0
4.0
2
6
5
4
2
2
7
2
0.1
0.3
1.0
3
10
30
dose, PMSF, mg kg$^{-1}$

Fig. 4

Fig. 4. Log (dose)-response curve for analgesia in rats with systemic PMSF. Curve determined from log-probit plots of data. Plotted curve passes through AD16, AD50 and AD84. Estimated AD50 = $2.9 \pm 1.5$ (S.E.) mg $kg^{-1}$.

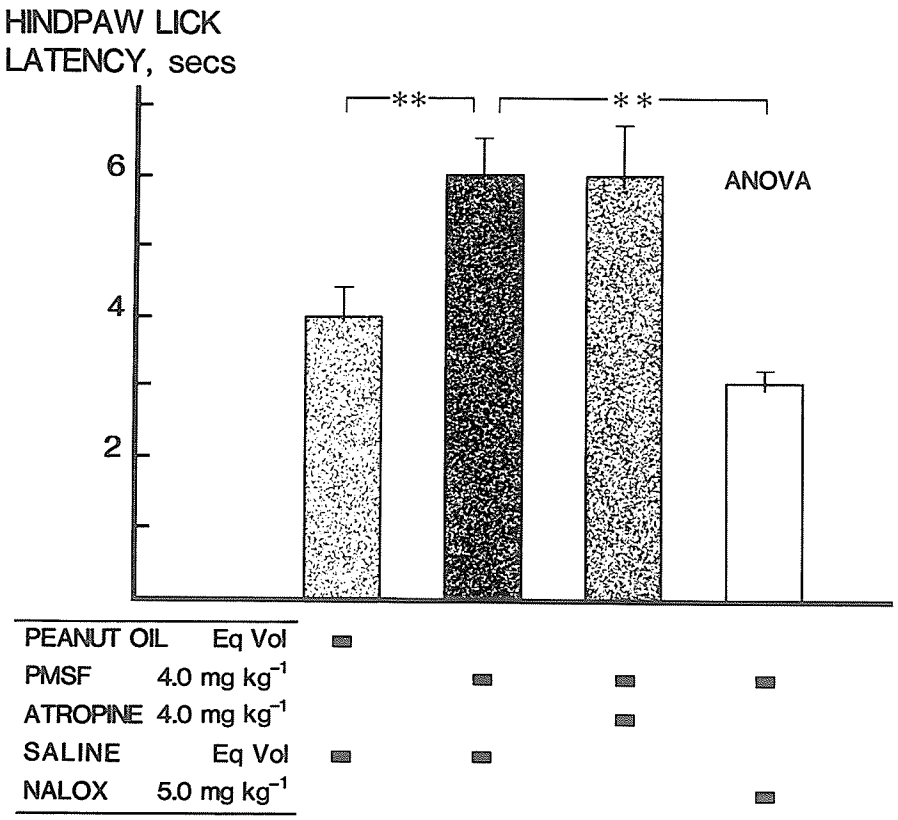
HINDPAW LICK
LATENCY, secs
6
4
2
**
**
ANOVA
PEANUT OIL Eq Vol
PMSF 4.0 mg kg-1
ATROPINE 4.0 mg kg-1
SALINE Eq Vol
NALOX 5.0 mg kg-1

Fig. 5

Fig. 5. Analgesia with systemic PMSF and responses to pharmacological antagonists.

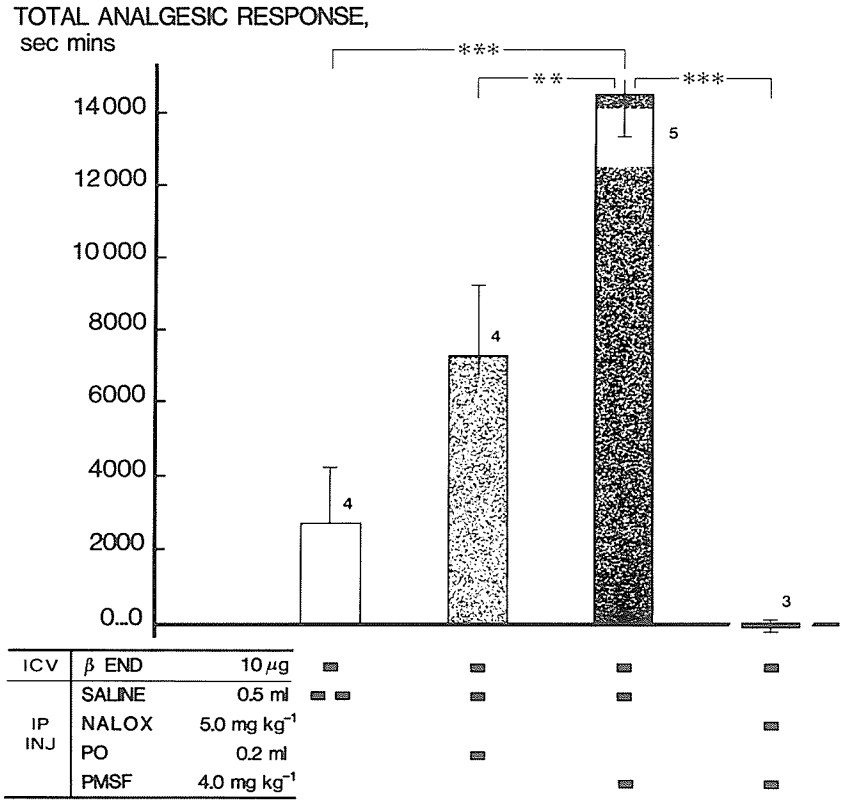
TOTAL ANALGESIC RESPONSE,
sec mins
14000
12000
10000
8000
6000
4000
2000
0...0
***
**
***
4
4
5
3
ICV
β END
10 μg
SALINE
0.5 ml
IP INJ
NALOX
5.0 mg kg⁻¹
PO
0.2 ml
PMSF
4.0 mg kg⁻¹

Fig. 6

Fig. 6. Total analgesic curve areas, showing potentiation by systemic PMSF of beta-endorphin analgesia, and demonstration of naloxone sensitivity. Details as in text.

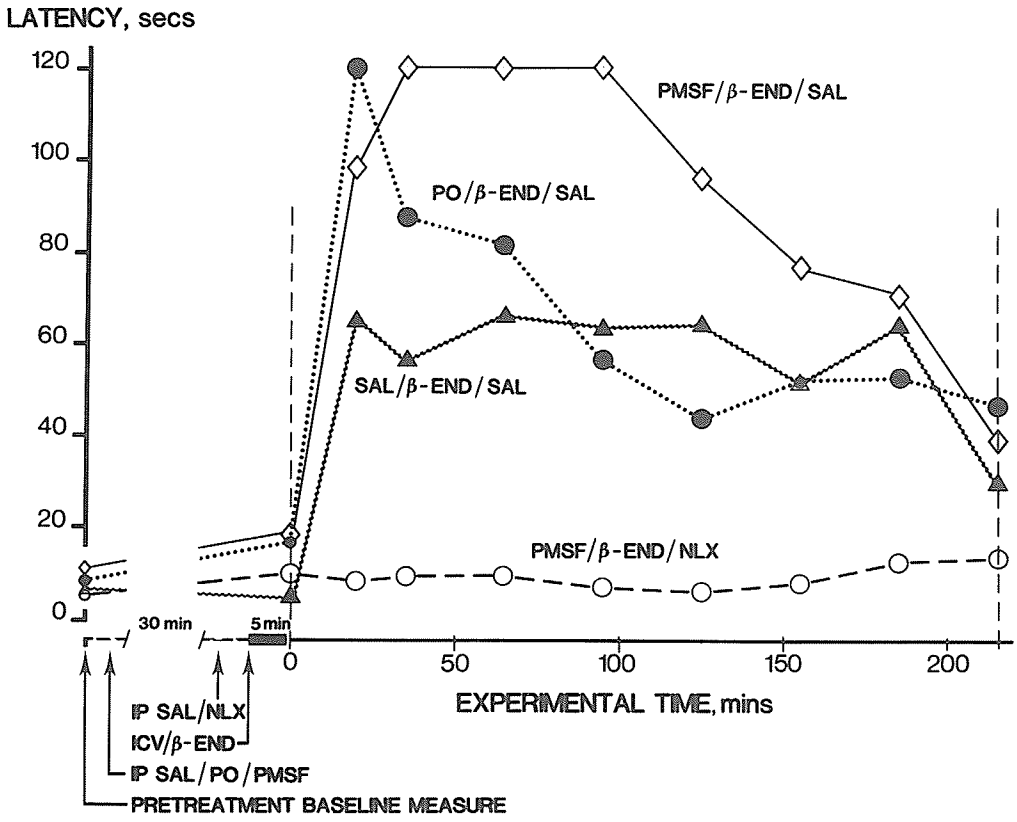
LATENCY, secs
120
100
80
60
40
20
0
PMSF/β-END/SAL
PO/β-END/SAL
SAL/β-END/SAL
PMSF/β-END/NLX
30 min
5 min
0
50
100
150
200
EXPERIMENTAL TIME, mins
IP SAL/NLX
ICV/β-END
IP SAL/PO/PMSF
PRETREATMENT BASELINE MEASURE

Fig. 7

Fig. 7. Curves of analgesia (hindpaw lick latency in secs) vs time of experimental observation for demonstration of potentiation of beta-endorphin effects by systemic PMSF, and demonstration of naloxone sensitivity. Areas under the curves were found within the boundaries of the vertical dashed lines; other details as in text.

with equivolume PO IP (Fig. 6). Naloxone.HCl 5.0 mg $kg^{-1}$ IP given just prior to END significantly reversed the analgesia induced by the combined effect of PMSF 4.0 mg $kg^{-1}$ IP with ICV END 10 µg (Fig 6). The time course of PMSF enhancement of END-induced analgesia, and of its blockade with naloxone is shown in Fig. 7.

General behavior and Core Temperature Responses in Rats : Core temperature measurements, made after each test for analgesia, showed that PMSF at 4.0 mg $kg^{-1}$ IP did not potentiate the hyperthermic response to END 10 µg ICV (data not shown). Rats treated with PO appeared very slightly sedated but were otherwise normal. One of eight rats treated with PMSF 4.0 mg $kg^{-1}$ IP showed circular movement during the hotplate test. A few rats treated with the combination of PO IP and END ICV showed stiff tail, slight catatonia (hypomotility, limb stiffness, frozen stance) and head jerks. Upon return to their cages one animal remained frozen and another tilted to the left with contralateral hindlimb elevation. Another of the rats treated with combined PO/END fell suddenly to the hotplate surface after briefly standing on its hindlegs along the cylinder wall. Rats treated with PMSF IP and END ICV showed wet-dog shakes, moderate catatonia, jumps and frozen stance in cage. One other rat in the PMSF/END group exhibited backward walking on hotplate.

Mortality and toxicity in mice : The 24-hr LD50 value for PO-dissolved PMSF in mice was 215 $\pm$ 55 mg $kg^{-1}$, calculated from the data shown in Table 10. Deaths were usually preceded by brief tonic-clonic convulsions. Most animals that survived longer than 4 hr postinjection were still alive by 48 hr. Many of the autonomic and behavioral responses to PMSF seen in these mice (legend, Table 10) were

Table 10. Mortality and toxicity with PMSF in mice.

| Treatment | Deaths Observation Periods (hrs) 0-1 | 1-2 | 2-24 | 24-48 | Proportion Deaths/ Total |
|---|---|---|---|---|---|
| PO 0.2 ml i.p. | 1 | 0 | 0 | 0 | 1/10 |
| PMSF mg $kg^{-1}$ i.p. | | | | | |
| 50 | 1[a] | 0[a] | 0[b] | 0 | 1/10 |
| 100 | 0 | 0 | 0[c] | 0 | 0/10 |
| 200 | 1[d] | 2[e] | 1[f] | 0 | 4/10 |
| 500 | 7[g] | 1[h] | 0[i] | 0 | 8/10 |

Legend, Table 10.

Notes[a-i] indicate behavioral and autonomic responses that were prominent, as follows: [a]Increased urination in most mice. [b]Depressed motor activity in 2/9 mice. [c]Depressed motor activity in 1/10 mice. [d]Depressed motor activity in most mice. Ocular mucous exudate in most mice. [e]In various of 9 surviving mice: increased ocular exudate ptosis (most mice); Straub tail; air hunger, coarse tremor, clonic convulsion. [f]Depressed motor activity in 4/7 survivors; incoordination, slight ataxia; very coarse tremor; air hunger; slight cyanosis; Straub tail; recovery from most signs by about 24 hr postinjection, with depressed motor activity still evident in 2/6 survivors at that time. [g]Increased urination; increased ocular exudate; tonic-clonic convulsions preceding most deaths. [h]Increased urination; increased ocular exudate; ptosis in 2 surviving mice. [i]Marked cyanosis with eventual recovery by 24 hr postinjection.

very similar to those reported for the rat with ICV END (Bloom et al, 1976; Havlicek et al., 1978, 1980). These responses had worn off by about 24 hr postinjection, and were not evident at all in animals still surviving at 48 hr (Table 10).

Postmortem examination for gross pathology was made in three mice that succumbed to IP PMSF 200 mg $kg^{-1}$ and in three that died after 500 mg $kg^{-1}$. None showed pathological changes except for a slight peritoneal congestion at the site of injection. A single death occured within 1 hr of IP injection in the PO-treated group (Table 10). No previous or subsequent PO-related deaths have been observed after several dozen similar PO injections given to mice and rats in this laboratory; a faulty injection is therefore suspected in the instance observed here.

## DISCUSSION

PMSF in rats produces a dose-related analgesia which is antagonized by naloxone and is totally unaffected by atropine in doses where the two different antagonists can be expected to exert their respective pharmacological effects. It thus appears that inhibition of serine proteinases, which would normally cleave endorphins into inactive fragments, must be largly responsible for the analgesic effects observed with PMSF in this study. This postulate is supported by the nature of behavioral and autonomic signs observed with PMSF toxicity in mice, those signs were strikingly reminiscent of responses to ICV adminstration of END in rats.

Second-order rate constants for inactivation of trypsin and chymotrypsin; have shown PMSF to be from two-to-sixfold more potent than is DFP for inhibition of trypsin or chymotrypsin (Fahrney and Gold, 1963; Klocking et al., 1975); a calculation for pseudo-first-order rate constants has implied that the true ratios may be much higher (Fahrney and Gold, 1963). As well, IC50 values show DFP to be 350-fold more potent than PMSF as an inhibitor of rat brain true cholinesterase (Myers and Kemp, 1954). It is therefore unlikely that inhibition of serine proteases contributes more than a small proportion of the analgesic effect which Karczmar and associates (Koehen et al., 1980) have observed with DFP. Hence, although we have not tested DFP directly, our present results, and the in vitro work reported by previous investigators, support the hypothesis put forward by the Karczmar group regarding a cholinergic basis for DFP analgesia.

The analgesic effect of parentral PMSF, its low toxicity in rodents (this study; Myers and Kemp, 1954; Turini et al, 1969; Klocking et al., 1975) and its ready susceptibility to naloxone blockade, all suggest that PMSF or some of its congeners might be potentially useful as parenteral analgesics in animals or humans. In this regard the PMSF congeners and other compounds listed in the report of Klocking et al. (1975) should be of considerable interest.

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# VIII. PEPTIDASE INHIBITORS REDUCE OPIATE NARCOTIC WITHDRAWAL SIGNS, INCLUDING SEIZURE ACTIVITY, IN THE RAT

## SUMMARY

Narcotic withdrawal was precipitated by administration of naloxone in a low dose at 2 hr after the final dose of morphine in a 9-day dependency-inducing schedule. Withdrawal was characterized by leaps, increased nocifensor activity and by cerebral cortical epileptiform activity, the latter not generally reported to be prominent in narcotic withdrawal. Single large doses of morphine did not provoke epileptiform activity at 2 hr postinjection but did induce an acute opioid dependency wherein a moderately high dose of naloxone, ineffective in nondependent rats, provoked upward leaping and electrocortical epileptiform activity. Pretreatment of the 9-day dependent rats with peptidase inhibitors, administered intracerebroventricularly, significantly reduced withdrawal severity including the epileptiform activity. We propose that peptidase inhibitors protect certain species of endogenous opioids and/or other neuropeptides that tend to suppress expression of the narcotic withdrawal syndrome. Furthermore, our findings suggest that epileptiform activity is a nascent form of cerebral activity hitherto largely unnoticed in narcotic withdrawal and that neuropeptides may be involved in certain epileptic states.

Discovery of the endorphins - endogenous opiomimetics (Goldstein, 1976; Hughes et al., 1975) - has led to speculation on their role in opiate narcotic dependency. Kosterlitz and Hughes (Kosterlitz and Hughes, 1975; 1976) postulated that deprivation of endorphinergic tone at stereospecific opiate receptors during opiate narcotic withdrawal contributes to the characteristic abstinence syndrome. Thus, prevention of enzymatic degradation of endorphins during opiate withdrawal should ameliorate the severity of that syndrome. Ehrenpreis and his group (Ehrenpreis et al., 1979; 1980) have claimed that parenteral administration of D-phenylalanine produces naloxone-reversible analgesia in both mice and humans; they attribute this analgesia to the protection of enkephalins from enzymatic degradation. Tsou and co-workers (Tsou et al., 1979; 1980) found that the aminopeptidase inhibitor, bacitracin (Desbuguois et al., 1974; Miller et al., 1977; Smyth and Snell, 1977), produced a naloxone-reversible enhancement of the analgesic responses to intracerebroventricular (ICV) $Met^5$-enkephalin (Tsou et al., 1979) and to acupuncture (Tsou et al., 1980) in rats. They reported also that ICV bacitracin increased the level of enkephalins in rat striatum and hypothalamus (Tsou et al., 1980). Pinsky et al. (1980) administered peptidase inhibitors to rats by ICV infusion; many of the responses were beta-endorphin-like and naloxone-sensitive, suggesting that the inhibitors had preserved brain levels of endorphin(s). We now report that antagonist-precipitated narcotic withdrawal is obtunded in morphine- dependent rats by the ICV administration of peptidase inhibitors.

METHODS

Chronically-treated rats. Twenty-eight male Sprague-Dawley albino rats, 155-175 g at start of experiment, were implanted with ICV cannulae into the right lateral ventricle and with bipolar epidural platinum electrodes bilaterally on sensorimotor cortex (Rezek and Havlicek, 1975). After 7 days of recovery they were placed on a morphine dependency-inducing schedule consisting of intraperitoneal (IP) injection of morphine sulphate given twice-daily (9:00 a.m. & 9:00 p.m.) at increasing doses over a 9-day schedule which began with a morning injection (Table 11). Rats were assigned by randomized selection into batches of four and injected according to the schedule in Table I; initial injections were staggered so that day 9 of schedule fell on different days for each batch. This permitted withdrawal experiments to be done over the same 2 hrs each day, minimizing diurnal variation. Animal quarters were on a 6:00 a.m. - 6:00 p.m. light, 6:00 p.m. - 6:00 a.m. dark, schedule; holding and testing environments were temperature- and humidity-controlled. On the mornings of schedule days 3,6 and 9 two rats from each batch, designated to be in the control group, received ICV infusion of bovine serum albumin (BSA, Sigma) 1.3 nmoles. The two others, designated for the experimental group (Table 12), received ICV infusion of the peptidase inhibitor aprotinin (Trasylol, Boehringer) (Trautschold et al., 1967), 1.3 nmoles, or a mixture of aprotinin 656 pmoles with bacitracin (Upjohn) 1.7 nmoles. Infusion was done 2 hr after morning dose of morphine, the four infusions were sequenced randomly. All solutions were in Krebs-Henseleit medium at pH 7.4, infused in 10 µl volume over 5 min.

Acutely-treated rats. A group of nondependent rats was tested with morphine to distinguish whether epileptiform activity observed in the

morphine-dependent animals had been provoked by the acute epileptogenic effects of morphine (Pinto Corrado and Longo, 1961; Verdeaux and Marty, 1954) administered to maintain dependency, or was a feature of the withdrawal state. Seven drug-naive rats were prepared with bilateral epidural electrodes and given 9:00 A.M. morning injections of morphine sulphate IP, five of these at 300 mg $kg^{-1}$ and two at 150 mg $kg^{-1}$. ECoG recordings were observed in these animals for the first 1/2-hr postinjection, to correspond to the ECoG studies in the morphine-dependent animals. All recordings were made in duplicate, at first with the animals at room temperature and then on the warmplate at 44.5°C.

Precipitation and quantification of morphine withdrawal. IP naloxone.HCl 150 µg $kg^{-1}$ was used to precipitate the opiate narcotic withdrawal syndrome in each rat, chosen in random order, five min after the ICV infusion on morning of day 9. This low dose of opiate antagonist (approximately 1/2000 the final morphine dose on a molar basis) was found to be effective when combined with the stress of warmplate confinement (rat held on 44.5° warmplate for 10 min period observation within acrylic cylinder 20 cm dia x 49 cm ht, open at top, rimmed at base with 1 cm holes which permitted ventilation and minimized heat rise inside the cylinder (Ho et al., 1978; Leybin et al., 1976; Pinsky et al., 1980; Pinsky et al., 1975). Four withdrawal responses were selected for quantitative evaluation of withdrawal severity (Table 12): (i) "leaps" (an upward leap with all four legs and tail in air, directed toward escaping from the cylinder); (ii) "hole probes" (each single episode of sniffing with snout pushed through a hole at cylinder base; (iii) "stands" (each incident of rat standing on

hind limbs while exploring cylinder wall; (iv) "hindpaw licks" (each incident of rat lifting and then licking either hindpaw with no subsequent grooming activity). These were chosen because they are readily quantitated and are seen only infrequently in drug-naive animals tested in the same cylinder-warmplate apparatus; they are prominent in and characteristic of opiate narcotic withdrawal as observed in numerous experiments on morphine dependence done in this laboratory. The upward leaping activity is almost strictly pathognomic of opiate withdrawal in this strain of rat (Pinsky et al., 1980).

Electrocorticographic (ECoG) recording and assessment of epileptiform ECoG activity. The bilateral ECoG was led off from the epidural electrodes and recorded (Fig. 8) on two channels of a pen recorder (Grass, P5, 6db down at 1.0 and 60 hz). Recordings were obtained from all rats during the 5-min period of ICV infusion on day 9 and the 10-min observation period which followed the injection of naloxone given immediately thereafter. ECoG recordings were taken from a smaller number of rats during the 5-min periods of infusion on days 3 and 6. Criteria for a positive assessment of epileptiform activity in the ECoG were: rapid nonsymmetrical excursion of the trace with peak amplitude at least 2X an estimated root mean-square amplitude of the 1.0-sec preceding activity; sharp spike-like points of inflection at maximum amplitude, estimated to be less than 50 msec duration at 90% spike maximum.

Analysis of data. All recording of data and subsequent analysis were done in blinded fashion; statistical procedures are described in the legends for Table 12 and Fig. 8. At the end of these experiments ICV infusions of dye were made into all animals tested; data were

accepted only from those rats in which histological confirmation of dye penetration into ventricles and therefore of successful cannulation was obtained.

## RESULTS AND DISCUSSION

Within 2-3 minutes of naloxone injection, each rat tested on the warmplate began to display the galaxy of signs characteristic of opiate narcotic withdrawal in that species (Blasig et al., 1973; Pinsky et al., 1973). Prominent among these were: salivation, urination, piloerection, teeth chatter, body shakes, twitches, upward leaps, hyperalgesia (expressed here as hindpaw licks), exploratory standing and hole probing. Table 12 shows the effects of peptidase inhibitors, infused intermittently during the induction of morphine dependency on severity of the precipitated withdrawal reaction as assessed by four primary expressions of that syndrome. Three infusions of inhibitor significantly diminished withdrawal severity, in comparison with withdrawing animals infused with bovine serum albumin (BSA). In separate experiments (Pinsky et al., 1980 and unpublished) identical infusion of BSA into drug-naive rats ($n > 60$) caused no significant changes in behaviour from previously established (Ho et al., 1978; Leybin et al., 1976; Pinsky et al., 1978) saline controls. Presumably, the ameliorating effect of the inhibitors derives from protection against enzymatic destruction of endorphins and, perhaps, other peptides which can suppress (Kosterlitz and Hughes, 1975; 1976) the signs of narcotic withdrawal. Ogawa et al. (1979) reported that aprotinin is an effective inhibitor of beta-endorphin degradation in

Table 11

Morphine administration schedule for induction of dependency

| | day of schedule | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| | dose, morphine sulphate, mg $kg^{-1}$, IP | | | | | | | | |
| morning | 5 | 15 | 30 | 50 | 80 | 120 | 180 | 240 | 300 |
| evening | 10 | 25 | 50 | 80 | 120 | 180 | 240 | 300 | |

Table 12 Effects of ICV peptidase inhibitors on withdrawal severity

| Response | Control | Experimental | |
|---|---|---|---|
| | BSA | aprotinin | aprotinin/bacitracin |
| | (n = 15) | (n = 10) | (n = 4) |
| leaps | 7.3 | 2.8[a] | 6.8 |
| hole probes | 59.4 | 34.3[b] | 29.8 |
| exploratory standing | 27.5 | 18.7 | 21.8 |
| hindpaw licks | 2.6 | 2.3 | 0.0 |
| total severity[c] | 6.84 ± 0.72 | 4.95 ± 0.66[d] | |

Legend, Table 12.

Values for each response indicate mean number of observations per rat per 10 min observation period during precipitated withdrawal.

[a]Significantly different ($p \leq 0.05$) from control by square-root transform two-tailed t-test (Steel and Torrie, 1960).

[b]Significantly different ($p \leq 0.05$) from control by Wilcoxon's two-sample rank test for unpaired data (Steel and Torrie, 1960).

[c]Mean $\pm$ S.E. of sq. rt. transform (Steel and Torrie, 1960) data, weighted as follows:
leap = 5, hole probe = 4, stand = 2, hindpaw lick = 1. Data for Experimental mean obtained by pooling data from rats receiving peptidase inhibitors aprotinin and aprotinin/bacitracin mixture.

[d]Significantly different ($p \leq 0.05$) from control by single-tailed t-test.

homogenates of rat whole brain, this accords with both the wide spectrum of activity of the inhibitor (Sullivan et al., 1980) and the variety of enzymes (Burbach et al., 1981; Grynbaum et al., 1977; Smyth and Snell, 1977; Sullivan et al., 1980) likely to be involved in the destruction of the opioid peptides. Bacitracin potentiates the binding of $Leu^5$- and $Met^5$-enkephalin to brain opiate receptors (Miller et al., 1977) and enhances the effects of those peptides on cyclic GMP accumulation in rat striatum (Minneman and Iversen, 1976). The specific nature of the ability of these peptidase inhibitors to suppress opiate withdrawal distress, as observed in this study, is apparent from those reports (Pinsky et al., 1980; Tsou et al., 1979; 1980) in which aprotinin- and bacitracin-induced antinociception and suppression of activity were shown to be naloxone-sensitive.

An unexpected result in our study was the observation of epileptiform spiking in the ECoG recorded during the development of morphine dependency and in naloxone-precipitated withdrawal (Fig. 8a, 8b). Epileptiform activity observed at 2 hr post-morphine injection on days 3 and 6 of the dependency schedule was characterized by intermittent bursts of spiking activity; burst durations on those days ranged from 0.5 to 2.0 secs in the inhibitor-treated group. Significantly less total epileptiform activity, assessed from the number of bursts observed over the entire 9-day period of dependency induction and precipitated withdrawal, was emitted by the inhibitor-treated rats than by the BSA-treated group (Fig. 8c).

No epileptiform activity was observed in the 14 inhibitor-treated rats during naloxone-precipitated withdrawal but a total of 34 epileptiform bursts, with mean burst duration $\pm$ S.E. = 5.7

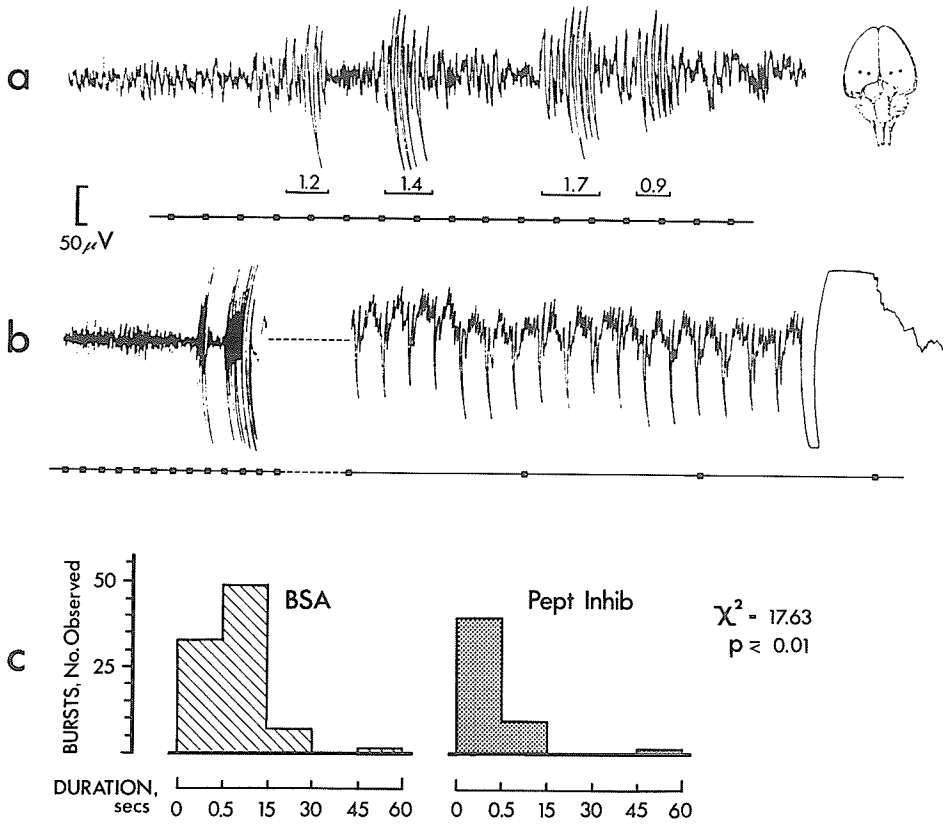
a
1.2
1.4
1.7
0.9
50μV
b
c
BURSTS, No. Observed
50
25
BSA
Pept Inhib
$\chi^2$ - 17.63
p ≤ 0.01
DURATION, secs
0 0.5 15 30 45 60
0 0.5 15 30 45 60

Fig. 8

FIGURE LEGEND

Fig. 8. Epileptiform activity in morphine-dependent rats. a, epileptiform burst activity, right hemisphere, in BSA-treated (Table 12) rat undergoing precipitated withdrawal. Occurrence of bursts and their duration in sec indicated by horizontal markers beneath pen recording. Rat was sitting quietly during occurrence of indicated burst activity. Time marks 1.0 sec. b, Two epileptiform bursts, left hemisphere, different BSA-treated rat during precipitated withdrawal. Recording was partly at high paper speed to confirm burst criteria. Record was broken (----) at speed change for approximately 3.0 sec, total duration (not displayed) of the second burst was 52 sec. Time marks 1.0 sec. Rat exhibited exploratory standing during the longer burst. Inset at right in a shows placement of electrodes on dorsal surface of brain, for a and b a positive-going signal at the lateral electrode yields an upward deflection. c, Summary of epileptiform burst data pooled from 43 observations on 15 BSA-treated rats and 42 observations on 14 peptidase inhibitor-treated rats tested during the 5-min infusion period on days 3,6 and 9 of the morphine-dependency schedule (Table 11) and during the 10-min observation period of naloxone-precipitated withdrawal on day 9. Data for a & c represent the mean of estimations made by three observers working from coded records to ensure a "blinded" analysis.

$\pm$ 0.93 sec, was seen in recordings from 5 of 12 rats pretreated with BSA during the induction of dependency. The difference between proportions of rats displaying epileptiform activity during precipitated withdrawal in the BSA-treated and peptidase inhibitor-treated groups (5/12 vs 0/14) was significant at the level of $p \leq 0.02$. Epileptiform activity during precipitated withdrawal was more stereotyped and rhythmic than that recorded during induction of dependency and often terminated with a large positive-negative ECoG deflection resembling slow waves of spreading depression (Grafstein, 1956; Leao, 1944) that are seen at the end of electrically-induced epileptiform afterdischarges (Perlstein, 1947) (Fig. 8b).

In the 7 rats given single injections of morphine sulphate at high doses, epileptiform spiking was observed 15 min after injection in one of the rats given 300 mg $kg^{-1}$ but in neither of those at the lower dose of 150 mg $kg^{-1}$. No spiking was seen in any of the seven rats at two hours after injection. In contrast, the epileptiform activity observed in those rats made dependent with multiple injections of morphine could be seen at two hours after their last injection of morphine sulphate at 30 and at 120 mg $kg^{-1}$ (on days 3 and 6 of dependency schedule, see Table 12), doses much below that needed to provoke epileptic activity soon after a single injection in one of seven nondependent animals. Naloxone.HCl 5.0 mg $kg^{-1}$ was administered at 2 hr post-morphine injection to all seven rats given the single morphine injections. This provoked salivation, urination, piloerection, teeth chatter, body shakes and twitches in all seven rats and upward leaping in the 5 rats that had been given the higher single dose of morphine sulphate. We have consistently observed, in other studies (Pinsky et al., 1980 and

unpublished), that IP naloxone at the dose given here will provoke neither upward leaping nor electrocortical epileptic activity in the nondependent rat. Epileptiform spiking was seen after naloxone administration in the same rat that had shown spiking 15 min after a single dose of morphine sulphate 300 mg $kg^{-1}$ and in one of the rats that had received 150 mg $kg^{-1}$ of the narcotic. Placement on the warmplate at various times after morphine injection and just after the injection of naloxone failed to provoke epileptiform spiking, in all seven rats.

The results with single large doses of morphine are consistent with the occurrence of cerebral epileptiform activity as a withdrawal response along with other cardinal expressions of opioid dependence. In this instance the dependent state was induced by a single high-dose injection of an opioid agonist; similar induction of acute opioid dependency has been previously reported (Kosersky et al., 1974; Pinsky et al., 1980; Smits, 1975). Epileptiform activity, therefore, appears to be a feature of the morphine withdrawal syndrome in rats made dependent on the narcotic by either multiple or single injections and provoked into withdrawal by naloxone precipitation or by withholding the narcotic. The results argue against the possibility that the epileptiform discharges recorded from rats given multiple doses of morphine sulphate were due to the direct opioid agonist action of the 2-hr precedent injection of any single dose of morphine in the dependency-inducing regimen. Differences in epileptic activity between BSA- and peptidase inhibitor-treated groups cannot be attributed to an epileptogenic effect of BSA, since we have previously shown that ICV infusion of saline (Herzlinger et al., 1977; Pinsky et al., 1978) or of

BSA (Pinsky et al., 1980) by itself causes no epileptiform activity. Hence, in the present study, treatment with peptidase inhibitors appears to have suppressed both epileptogenic and behavioral expressions of morphine withdrawal.

Our observation of epileptiform activity appears to contradict the generally held view (Jaffe, 1980) that narcotic dependency and withdrawal are not associated with convulsive activity. However, human neonates born to opioid-dependent mothers often display a withdrawal syndrome which includes both motor (Behrman, 1979; Burnett, 1970; Herzlinger et al., 1977; Perlstein, 1947; Rosen and Pippenger, 1976; Rothstein and Gound, 1974; Tow, 1937) and EEG (Herzlinger, 1977; Rosen and Pippenger, 1976) seizure activity, as do human adults dependent on very high doses of heroin (Mendelson and Mello, 1978) and rodents dependent on high doses of morphine (Hanna, 1960). The neonatal narcotic-dependent seizure activity responds well to opiates but not to conventional anticonvulsant agents (Herzlinger et al., 1977; Rosen and Pippenger, 1976).

Seizure activity may be a nascent form of cerebral excitability that has gone largely unnoticed in narcotic withdrawal, since in many of our experiments there was spiking and epileptiform activity in the ECoG with no overt convulsive or other motor hyperactivity (Fig. 8). There might be a relationship between these results and the seizure activity often seen in withdrawal from alcohol, a drug of abuse which appears to interact with central opioid mechanisms (Blum et al., 1976; Pinsky et al., 1981; Ross et al., 1974). Parenteral opioid narcotics (Pinto Corrado and Longo, 1961; Verdeaux and Marty, 1954) as well as ICV endorphins can be excitant and epileptogenic (Cowan et al., 1979;

Frenk et al., 1978a, 1978b; Kosersky et al., 1974; Urca et al., 1977), suggesting the presence of opiate receptors which mediate epileptogenesis. Conversely, there appears to be another population of opiate receptors which mediate an anticonvulsant action (Adler et al., 1976; Blum et al., 1976; Cowan et al., 1979). It is noteworthy that parenteral naloxone (4-5 mg $kg^{-1}$) can enhance audiogenic seizures in mice (Schreiber, 1979) and facilitate amygdaloid-kindled seizures in rats (Hardy et al., 1980); epileptiform spiking in rat sensorimotor cortex is provoked by ICV naloxone in dose-related fashion (Pinsky et al., 1978). An intriguing possibility suggested by the present results is that an anticonvulsant endorphin or other peptide species is released during naloxone-precipitated withdrawal, in amounts which oppose the excitant and proconvulsant actions of certain other endorphins or neuropeptides. Suppression of ECoG spiking would thus be enhanced in rats treated with appropriate peptidase inhibitors. The effects of peptidase inhibitors in certain epileptic or convulsant states might be usefully considered.

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# IX. PHARMACOLOGICAL INDUCTION AND BLOCKADE OF LONG-AXIS ROTATION IN RATS SUGGEST INVOLVEMENT OF NEUROPEPTIDES IN MINIMAL BRAIN DYSFUNCTION

## SUMMARY

Rotation around the longitudinal body axis was observed in rats treated intracerebrally with bacitracin. The phenomenon appeared to be all-or-none, with a threshold dose of 200 µg bacitracin. The response was blocked by, naloxone, diazepam, phenytoin and amphetamine. A review of literature, going back 150 years, revealed that long-axis rotation may be a distinct clinical syndrome that has remained inadequately characterized. The involvement of neuropeptides in the clinical syndrome, and in other examples of experimentally-induced long-axis rotation, is supported by work from other laboratories. We propose that experimentally-induced long-axis rotation in rats may be a model for the hyperkinetic syndrome said to be associated with minimal brain dysfunction.

The phenomenon of rotation around the longitudinal axis in animals and human beings is a rare one. A search of literature indicates that this phenomenon can occur due to changes in four different areas of the brain, or can result from the administration of peptides. Auditory nerve irritation, due either to direct stimulation of the nerve as shown in rabbit by Brown-Sequard (1859) or, in humans, to instillation of hot water in either ear can lead to rotation around the longitudinal axis (Bert, 1869). In patients a tumor in the left cerebellar hemisphere has been implicated in causing the rotational symptom (Minchin, 1859). A cerebral origin of this phenomenan has been observed after experimental lesions in dogs. Bechterew (1882), from his experiments in dogs, arrived at the conclusion that injuries not only to middle and posterior cerebellar crura can produce such rotation, but so also can lesion of the inner part of the crura in its whole course (from thalamus to pons) and deep injury to medulla oblongata. When the inner part of the crura cerebri is injured the rotation occurs about the contralateral uninjured side, whilst a lesion of the external layer of the crux cerebri causes rotation about the injured side. Rotation about the longitudinal axis is produced through a lesion of those fibers which go from the cerebellum through the upper part of the crura cerebri to the corpora quadrigemina.

Longitudinal axis rotation has also been observed in alcoholic black Americans (Parker et al., 1939), where it was attributed to alcoholic psychosis. In Faxen's syndrome in schizophrenics (a subclassification of the adversive syndrome; Durham, 1960) such a phenomenon has been described in detail; the symptom appears to be peculiar to catatonic schizophrenia . Apart from any pathological

expression a group of people, called Darvish, voluntarily exhibit longitudinal rotation as a part of their religious rituals (Clemens, 1911).

This phenomenon has been described recently in laboratory animals after intracerebroventricular (ICV) infusions of vasopressin (Kruse et al., 1977; Burnard et al., 1982), oxytocin (Kruse et al., 1977), Substance P (Rondeau et al., 1977), cholecystokinin (Mann et al., 1980), dynorphin (Herman et al., 1980), luteinizing hormone releasing hormone (Cohn et al., 1978), somatostatin (Cohn and Cohn, 1975) and bradykinin (personal communication, Bose, R.). The phenomenon observed with dynorphin and Substance P were not naloxone-reversible, while the somatostatin-induced rotational activity was reversed by atropine. d-Amphetamine failed to reverse the somatostatin- and substance P -induced barrel rolling (Rondeau et al., 1978; Cohn and Cohn, 1975).

We have now observed this phenomenon in rats after administration of bacitracin. During a course of studies on behavioral effects of peptidase inhibitors in rats, bacitracin in doses of 200 ug and higher was unexpectedly seen to produce long-axis rotation. It could be prevented by treatment postinduction with naloxone, diazepam, d-amphetamine and by pretreatment with phenytoin.

Sterile bacitracin (50,000 units) was procured from Upjohn of Canada, Don Mills, Ont. Naloxone.HCl was a gift from Endo Laboratories, Garden City, NJ. Diphenylhydantoin was obtained from Sigma Chemicals, St. Louis, MO. Sprague-Dawley rats (Canadian Breeding Laboratories, St.Constant, PQ), 180-200g, were prepared with indwelling cannulae implanted stereotaxically in the right lateral ventricles (Pinsky et al., 1980). After several days of recovery, the rats were given ICV

infusions of various doses of bacitracin ranging from 5-500 µg in 10 µl volume over 5 min.

Rats given bacitracin infusions in the dose range of 5-150 µg did not show any change in normal behavior. Just before or at the end of bacitracin 200 µg infusion most of the rats began to exhibit barrel-rolling (rotation along the longitudinal axis) in the cage. When placed on a flat surface, they usually rolled up to 70 cm away. At the start of rolling the rat is typically quiet, then its head turns upward halfway, followed by upward extension of contralateral forelimb; at this point the animal starts rolling. The roll initiates from the upper half of the torso, which twists, while the rigid extended lower half of the body and hind limbs follow. The twisting motion of the body thus leads to the rolling of the entire body, like a barrel. There are periods of rest between bouts of rolling. During such rest intervals the animal is quiet and appears to be exhausted. The eyes are fixed during all stages and there is no sign of nystagmus. An intraperitoneal (IP) injection of naloxone.HCl 5.0 mg $kg^{-1}$ prior to the infusion of bacitracin abolishes the barrel-rolling (Table 13). The same dose of naloxone administered after the rolling has started is ineffective. However, phenytoin 1.0 µg/ 10 µl ICV given after the onset of barrel-rolling abolishes the response completely (Table 13). Diazepam 2.5 and 5.0 mg $kg^{-1}$ IP was also effective in blocking the response after its onset (Table 13). Pretreatment with d-amphetamine.HCl 5.0 and 7.5 mg $kg^{-1}$ IP also prevented the onset of barrel-rolling in rats, which had been pretested for their response to bacitracin several days earlier (Table 13).

Table 13

Pharmacological Induction and Blockade of Long-Axis (LAR) Rotation

| Group[a] | Treatments (doses:see legend) Injection IP | Infusion(s) i.c.v. 1st | 2nd | LAR Index (Respondent) (Treated) | Mortality Index (Died) (Treated) |
|---|---|---|---|---|---|
| A | Sal[b] | Bac[d] | - | 0/11 | 0/11 |
| B | Sal | Bac[e] | - | 23/32 | 7/32 |
| C | Sal | Bac[f] | - | 6/16 | 2/6 |
| D | Sal | Bac[g] | - | 2/2 | 2/2 |
| E | Nlx[c] | Bac[e] | - | 2/12 | 2/12 |
| F | Amph[c] | Bac[e] | - | 0/8 | 0/8 |
| G | Diaz[c] | Bac[e] | - | 0/4 | 0/4 |
| H | Sal | Bac[e] | DPH[h] | 0/2 | 0/2 |

Legend, Table 13

Sal = Saline; BAC = Bacitracin; NLX = Naloxone.HCl;
DPH = Phenytoin; Amph = d-Amphetamine; Diaz = Diazepam;
LAR = Long Axis Rotation

[a] Significant intertreatment differences for Long-Axis Rotation (chi-square test): B vs E, B vs F ($p \leq 0.01$); B vs G ($p \leq 0.02$);
[b] Normal saline, 0.5 ml; [c] Naloxone.HCl, d-Amphetamine.HCl, Diazepam, all at 5 mg $kg^{-1}$; [d] Bacitracin, 5-150 µg; [e] Bacitracin, 200 µg;
[f] Bacitracin, 500 µg; [g] Bacitracin, 5000 µg; [h] Phenytoin, 1 µg in 10 µl.

It is evident from the previous literature that alternative cerebellar or cerebral origins of rotation along the longitudinal axis can be differentiated on the basis of nystagmus. Nystagmus will be present whenever the cerebellum or labyrinth is involved. Cerebellar or labyrinthine origins of the phenomenon are usually of a localized neuropathological nature, e.g. hyperactivity of nerve fibres, as compared with a cerebral origin which is usually global and biochemical in nature. The long-axis rotation provoked by bacitracin in our rats was unaccompanied by nystagmus and was consequently likely due to an upset in cerebral neuropeptide activity.

Alcohol, whose overconsumption can apparently provoke long-axis rotation in humans, is known to interact with opiate receptors (Pinsky et al., 1981), particularly the delta subgroup (Hiller et al., 1982). It is quite possible also that hallucinations in alcoholics are closely related to those which occur in schizophrenia, especially in the catatonic form of the latter (Alpers, 1963). A possibility is that alcoholic hallucinosis is a schizophrenic reaction, released by alcohol (Parker et al., 1939). Neuropeptides, especially opioid peptides, produce catatonia (Bloom et al., 1978) and have been implicated also in the causation of schizophrenia (ibid.). Furthermore, relatively high doses of naloxone have been found to reduce the frequency of auditory hallucinations in chronic schizophrenic patients (Akil et al., 1978; Berger, 1979). We have shown here that naloxone blocks the phenomenon of long-axis rotation if given prior to bacitracin, a known peptidase inhibitor (Desbuquois et al., 1974). This suggests that at least a part of the phenomenon could involve opioid mechanisms. There is no known common site of interaction nor a known common structural moiety among

those peptides which produce rotation along the axis, hence it seems probable that excessive release of neuropeptides in alcoholics and in schizophrenics might somehow interact with a number of central mechanisms to produce such rotation. These mechanisms could be mediated either by opioid or cholinergic mechanisms, as both naloxone and atropine block the rotational response and the cholinergic and opiate systems are somehow interrelated (Frederickson and Pinsky, 1971; de Jong and Pinsky, 1974; Harris et al., 1969).

The prevention shown here of bacitracin-induced barrel-rolling by amphetamine has several interesting implications. The rolling could be an expression of hyperkinesia in animals and this model might therefore prove useful in exploring hyperkinesia in minimal brain dysfunction for which no good animal model has as yet been described. Lesions of the nigrostriatal dopaminergic system or administration of the dopamine neurolytic agent 6-hydroxydopamine can induce a similar rotational syndrome. The ameliorative effect exerted by amphetamine therefore suggests that dopaminergic or dopaminelike (Cohn et al., 1978) mechanisms are coupled to the postulated neuropeptide system involved in the long-axis rotational syndrome. The rationale behind the use here of phenytoin and diazepam was that if the phenomenon involved convulsive activity it should be blocked by these two anticonvulsants. Diphenylhydantoin inhibits convulsive activity either via its membrane stabilizing effect or perhaps by direct action on its own proposed receptors (Burnham et al., 1981). Diazepam acts as an anticonvulsant either via its general inhibitory property or by direct action on the benzodiazepine receptors (Squires and Braestrup, 1977; Mohler and

Okada, 1977). The blockade of bacitracin-induced barrel-rolling by phenytoin and by diazepam indicates that a convulsive component may participate in the overall behavioral response. The intriguing possibility that the hyperkinesia seen in the minimal brain dysfunction syndrome may involve cerebroconvulsive activity is also brought forward by our results, since amphetamine is known sometimes to have a salutary effect in hypsarrythmia (Toman and Davis, 1949; Weiner, 1981) a condition postulated to result from minimal brain damage in children (Gross and Wilson, 1974).

The foregoing suggests that the phenomenon of long-axis rotation has clinical relevance and may prove to be a specific neurological entity. It should therefore be further explored to help shed light on the aetio-pathogenesis and pharmacotherapy of the minimal brain dysfunction syndrome and on the possible involvement of neuropeptides in schizophrenia, catatonia and other severe disorders of mentation and neuromotor ability.

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# X. APPARENT CENTRAL ACTIONS OF NALOXONE IN THE UNRESTRAINED RAT

## SUMMARY

Morphine sulphate (MS) and naloxone hydrochloride (NLX) appear to exert a common excitant action on nervous tissue accessible to substances infused into the rat lateral ventricle. With infusion there was reversal of NLX-induced excitation by calcium ion, known to diminish many specific opiate actions. Very large concentrations of dextrorphan and of NaCl were inactive. Hence, at certain CNS sites responsible for electrocortical hypersynchrony in the rat, there may exist a unique receptor for which MS is a full agonist with relatively low affinity while NLX behaves as a weak or partial agonist with low intrinsic activity. Whether this situation represents the existence of yet another class of CNS opiate receptor or a specialized opiate-sensitive CNS excitant receptor has yet to be determined. An apparent antinociceptive effect of parenteral NLX was observed with mild, prolonged noxious stimulus (44.5$^{o}$C warmplate). This may be mediated at least in part by some mechanism other than that suggested for our ICV results. NLX blockade of the high-affinity stereospecific central opiate receptor might be involved in provoking an over-compensating release of endorphin, which could result in antinociception under the particular conditions of our experiment. This latter possibility might explain the variable and sometimes seemingly contradictory results that have been reported for the effects of NLX on nociception.

## Introduction

Opioids exibit considerable agonist and antagonist stereospecifity at very low concentrations (Kosterlitz et al, 1973). At higher concentrations they exibit "nonspecific" actions. These may be mediated by an opiate receptor which can distinguish between opioid and non-opioid chemical structure, but not as readily between opioid agonists and antagonists.

## Methods and Results:

Electrocortical and motor behavioral responses to intracerebroventricular (ICV) infusion. Male Sprague-Dawley albino rats, 150-250g at operation, were implanted with cannulas into the right lateral ventricle and with bipolar epidural electrodes on right and left sensorimotor cortices. Substances were infused in vehicle volumes of 10.0 µl over 5 min. ICV morphine sulfate (MS) in saline provoked electrocorticogram (ECoG) spikes, ECoG seizures and motor seizures. Severity and incidence of such responses were threshold at 30 µg and clearly related to dose (10,30,50,100 and 200 µg; tested in total of 13 rats). Naloxone.HCl (NLX) produced very similar results, but with a threshold dose of 100 µg. Infusion of 5000 µg NLX along with 400 µg calcium chloride (n=3) resulted in activity almost identical to that observed in vehicle-infused controls (n=10) as did dextrorphan.HCl (DEX) at 3000 µg (n=2), DEX at 5000 µg (n=1) and NaCl at 3000 µg (n=3) and at 5000 µg (n=3).

Effects of parenteral MS and NLX in rats tested on 44.5°C warmplate. Rats were injected with saline, MS or NLX, and tested 5 min later on a warmplate at 44.5°C. MS, 0.5-16.0 mg $kg^{-1}$, significantly diminished forepaw licking (FPL), hindpaw licking (HPL), exploratory rearing (ER) and leaping (L) in dose-related fashion. NLX, in the same dose range, increased the incidence of ER ($p < 0.05$ at 4.0 and 8.0 mg $kg^{-1}$) and of L ($p < 0.005$ at 4.0 mg $kg^{-1}$). Unexpectedly, NLX produced an apparent antinociceptive effect, showing significant supression of FPL at 0.5, 1.0 and 16.0 mg $kg^{-1}$. NLX also diminished HPL activity, but not to a statistically significant level.

## Discussion

The foregoing results suggest that MS and NLX exert a common excitant action on nervous tissue accessible to substances infused into the rat lateral ventricle. With ICV infusion there was reversal of NLX-induced excitation by calcium ion, known to diminish many specific opiate actions (Radouco-Thomas, 1971; Sanfacon and LaBrecque, 1977). Very large concentrations of DEX and of NaCl were inactive. Hence, at certain CNS sites responsible for electrocortical hypersynchrony in the rat, there may exist a unique receptor for which MS is a full agonist with relatively low affinity while NLX behaves as a weak or partial agonist with low interensic activity. A peripheral model of such a receptor has already been demonstrated by Frank (Frank, 1975). Whether this situation represents the existence of yet another class of CNS opiate receptor or a specialized opiate-sensitive CNS excitant receptor (Jacquet et al., 1977; LaBella et al., 1978 ), has yet to be

determined. The apparent antinociceptive effect of parentral NLX may be mediated at least in part by some mechanism other than that suggested for our ICV results. A NLX blokade of the high-affinity stereospecific central opiate receptor might be involved in provoking an over-compensating release of endorphin, which could result in antinociception under the particular conditions of our experiment. This latter possibility might explain the variable and sometimes seemingly contradictory results that have been reported for the effects of NLX on nociception (Grevert and Goldstein, 1978; Jacob et al., 1974; Pinsky et al., 1976).

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# XI. PERINATAL ROLE OF OPIOID AGONISTS IN PLANT AND ANIMAL KINGDOM: A PROPOSED HYPOTHESIS

A decade has gone by since the original postulation, isolation and characterization of endorphins. Yet, despite a vastly available knowledge of endorphin-structure, metabolism, responses to external stimuli, central distribution and actions, we still know very little about the precise physiological role and importance of endogenous opioid peptides. An even vaster array of knowledge is available describing the actions of the alkaloid opiate narcotic agonist, morphine. It is undeniably logical to compare the action and effects of endorphins with those of morphine in animal experiments where responses to both chemical classes of opiate agonists can be observed. In such experiments the opioid peptide is considered to be an endogenous substance, the opioid alkaloid an exogenous substance, with specific actions on a common receptor. This writer now suggests that an artificial dichotomy has been made in establishing this "endogenous/exogenous" distinction. Indeed, if living organisms of all kingdoms are taken as representing a natural continuum, both kinds of opioid agonist may be considered as being "endogenous" to living organisms in general, beginning perhaps at the level of grasses in the plant kingdom. It is further ventured that opioid agonists have a positive survival value at a most fundamental level -- that of propagation of the species -- with many functional similarities and analogies between the processes for such a value in plants and animals. The basis for these assertions is drawn from data in the literature which show a striking similarity, hitherto not commented on, between the pattern of variation in opioid agonist levels in reproductive organs of the opium poppy and of mammalian females (in the argument to follow the term "female" shall refer to the mammalian species, "poppy" to the opium poppy).

In the poppy plant, levels of opioid alkaloid(s) are highest in the opium capsule as compared with other parts of the plant. Important to the following exposition is the well-known (and economically-important) fact that the highest alkaloid levels are found during the flowering season (True and Stockburger, 1916; Manske, 1950; James, 1950). This portion of the life cycle of the plant corresponds with pregnancy in the female. The poppy's capsule may be considered as being roughly analogous to the uteroplacental system of the pregnant female. In the poppy plant morphine is present as an inert precursor in the latex. This inert substance is converted into the active form in the presence of enzymes and cofactors. The level of latex and the activity of converting enzyme is high in the capsule (True and Stockburger, 1916; Claus et al., 1970). There are remarkable similarities between variations in levels of endorphin and levels of morphine alkaloid in the reproductive organs of the poppy and of mammals. Endorphins are present in the placenta, along with their inactive precursor beta-LPH. In one study (Wardlaw et al., 1979) beta-LPH levels in the placenta were found to be higher, on a molar basis, than that of beta-endorphin (a review of the detailed evidence for profound changes of endorphin levels in pregnant females is provided as a footnote to this section). Importantly, the level of beta-endorphin in serum/placenta rises continuously during pregnancy and is highest on the day that immediately precedes delivery in humans (Baldi et al., 1979; Salmon et al., 1979; Csontos et al., 1979). Corresponding with these observations we find that an inactive precursor of morphine is present within the opium poppy capsule in the form of latex. However, in the presence of enzyme and cofactors, the

latex is converted to the active opioid agonist, morphine (True and Stockburger, 1916; Claus et al., 1970). The level and activity of the enzyme which converts latex into morphine is high during the flowering season (True and Stockburger, 1916; Claus et al., 1970) it would be interesting to study the level and activity of enzymes which converts beta-LPH into beta-endorphin during the gestation period in mammals). There is a diurnal variation in the content of alkaloid in the latex of opium plants. Presumably, the content is higher in the early morning, as it is collected quite early in the morning (Claus et al., 1970). Frederickson et al. (1979) have reported that in mice the baseline latency to hindpaw lick in the hotplate test is higher in the early morning than in the late afternoon. That study indirectly indicates that levels of opioid peptide were highest in the morning. Although this correlation provides only marginal support for a possible evolutionary memory trace between plants and animals, it is nevertheless consistent with the concept of analogous mechanisms for the regulation of endogenous opioid activity within the two kingdoms.

Let us now consider the postulate that the presence of peptide opioid agonists in the mammalian placenta, and the fluctuation of such opioids therein, is somehow analogous to natural variations in the level of opioid alkaloids in the poppy. An important first question to ask in such consideration would be concerned with the possible sources for high levels of certain peptide mediators that have been found in placenta and umbilical cord blood (Wardlaw et al., 1979; Houck et al., 1979; Fraioli and Genazzani, 1980; Csontos et al., 1979; Nakai et al., 1978). It has been reported that the opioid peptide endorphin and ACTH are released simultaneously from the anterior pituitary into the blood

in response to various stressor treatments in rats (Winters et al., 1971; Allen et al., 1973; Kaupilla et al., 1974). It is doubtful, however, whether peptide output from the pituitary could, after dilution and enzymatic degradation in the plasma and other tissues, account for the high levels of ACTH, endorphin(s) and enkephalin(s) (Csontos et al., 1979; Houck et al., 1979; Fraioli and Genazzani, 1980; Wardlaw et al., 1979; Baldi et al., 1979; Ramasastry et al., 1980) that have been observed in placenta and umbilical cord blood at term. It has therefore been suggested that some of the opioid (and other peptides) may be synthesised in the placenta itself (Csontos et al., 1979; Genazzani et al., 1980). Evidence for the in vitro synthesis of endorphins, ACTH and MSH in the placenta has been provided by several workers (Genazzani et al., 1974, 1975, 1980; Rees et al., 1975; Liotta et al., 1977). It is known that placenta is not innervated (Fujiyama et al., 1971). Hence the placental synthesis of opioid peptides would have to be regulated by local and/or circulating neurohumoral factors of central origin (the non-innervated status of placenta would make it a useful object for studies on the regulation of endorphin synthesis).

Further instances of correspondence between peptide and alkaloid opioids in the propagatory process may be noted. In the poppy, the major latex is present in the lactiferous ducts surrounding the endocarp (Claus et al., 1970; James et al., 1950). In both kingdoms, during gestation, endorphin levels are high in the placenta (which may be regarded as equivalent to the nourishing ducts) but not in the endocarp (equivalent to the amniotic fluid environment). Seeds of the opium poppy contain only a negligible to nil quantity of morphine alkaloid; the human neonate (Csontos et al., 1979) and rat pups (Patey

et al., 1980; Bayon et al., 1979) have been reported as having only very low levels of endorphins. The critical time for the collection of economic quantities of morphine alkaloid is just before the poppy ripens (changes from bluish green to yellowish in color) -- a procedure established by millennia of empirical observation. The critical harvesting period for opioid alkaloids of plant origin is analogous to the time just before parturition in the mammals, when the level of beta-endorphin in placenta, maternal blood and cord blood is at its highest. This temporal correspondence evokes the corollary example that an experimenter in need of large quantities of natural endorphins should best obtain blood samples from pregnant females just prior to parturition.

The foregoing account of the natural similarities between the perinatal patterns of endogenous opioids (of whatever chemical nature) in an important morphine-yielding plant and in mammals prompts a question seeking to unify the biological implications of the observed similarities. Do opioid agonists of birth cavity origin -- alkaloid(s) in the plant capsule and peptide(s) in the uteroplacental system in mammals -- play a functional role in the perinatal physiology of their respective biological sources? The most prominent concept concerning the possible function of uteroplacental endorphins in pregnancy is the suggestion that they act to induce a salutary analgesia or euphoria (Csontos et al., 1979; Houck et al., 1979). This is highly unlikely since the endorphins would have to exit from the birth cavity, cross several cellular barriers, be submitted to dilution and enzymatic destruction in the circulation and then cross a highly-resistant blood-brain barrier in order to exert an analgesic or euphoriant effect

on the mother. A more parsimonius theory is that MSH, ACTH and endorphins are all synthesized in the placenta (Genazzani et al., 1974; 1975; 1980; Rees et al,. 1975; Liotta et al., 1977) and that they act locally -- on the fetus and on the uterus -- alone or under the influence of circulating neurohumoral factors of central or peripheral origin, or both.

If neither analgesia nor mood-elevation result from the liberation of placental endorphins, what then might be the function of such intrauterine opioids? They might very well exert a protective action on the fetus, by suppression of neural activity in medullary and pontine respiratory centres. Respiratory movement of the chest wall or diaphragm are almost totally absent in the normal fetus (Dawes, 1971); such movements would indeed be purposeless if not dangerous to the fetus or neonate, since they could lead to inspiration of amniotic fluid into the lung. It is not unreasonable to propose that high levels of placental endorphin(s) could suppress such incipient fetal respiratory activity, via the well-known respiratory depressent effect of opioid agonists. Chernick et, al. (1980) and others (Fisher and Cook, 1980; Wardlaw et al., 1979) have reported that the opiate antagonist naloxone can reduce apnea and stimulate respiration in the newborn, this suggests that antenatal and neonatal respiratory activity is considerably under the control of endorphins. In addition to protection against amniotic drowning, endorphinergic suppression of fetal respiratory efforts might play an important paradoxical role in the initiation of spontaneous respiration. Supposing that fetal endorphin levels originate from the placenta, birth would remove the fetal respiratory neurones from endorphinergic inhibition. Hence, the

neonatal respiratory centres might enter newborn life in a state of supersensitivity, due to removal of a precedent antenatal suppression of activity. Hyperventilation is a well-known feature, for example, of withdrawal from opiate narcotic dependency, especially in the newborn of narcotic-dependent mothers (Rosen and Pippenger, 1976; Rothstein and Gold, 1974; James, 1977). The uteroplacental origin of fetal endorphinergic tone, therefore, would constitute an elegant system for protection of the fetal lung and for "priming" of respiratory network in the neonatal brain.

Another locus of putative salutary perinatal action for placental endorphins might be the uterus itself. Morphine is known to restore the activity of the oxytocin-stimulated uterus toward normal levels of tone, frequency and amplitude of contraction (Jaffe and Martin, 1980). Hence opioid peptides of placental origin may be part of a protective mechanism which helps prevent premature labor. It could equally protect the fetus from uterine contracture which might otherwise occur under the influence of excessive oxytocic stimulation during labor.

What might be the corresponding "perinatal function" of morphine in the opium poppy life cycle? It is unlikely that the toxic effects of the poppy alkaloids are sufficient to hold off herbivores from eating the flowering plants, although the bitter alkaloid taste might do so to some extent. It has been suggested that the function of morphine is part of a detoxification process whereby the plant is protected from the buildup of substances that would interfere with several metabolic processes (Claus et al., 1970; James, 1950). It was, however, pointed out that such a mechanism would be thermodynamically

unsound, since the end product of such a detoxification process -- morphine -- is a more complex molecule than the presumed toxic precursor (James, 1950). Nor would morphine serve as a good storage form of nitrogenous material for plant metabolism, by virtue of similar thermodynamic constraints (James, 1950). The most attractive hypothesis to explain the selective evolutionary history which must have gone into the formation of plant-originated morphine is one which ascribes a growth-factor regulatory role to the morphine (Claus et al., 1970; James, 1950). Although the evidence is mainly circumstantial, it has been suggested (James, 1950; Claus et al., 1970) that the alkaloids help regulate the times for flowering and seeding in the opium poppy. Once the seeds have been shed, the capsule falls off in a manner analogous to the placenta after parturition in mammals. Hence an intriguing, if admittedly speculative, comparison is complete; plants and animals seem to have in common a system of endogenous opioids, albeit of two differing chemical families, which seems to be intimately related to the propagatory cycle. Indeed, even the chemical dichotomy may soon disappear, as a morphinelike alkaloid has recently been found in grass and in mammals (Hazum et al., 1981) and opioid peptides (or their precursors) in cereals (Zioudrou and Klee, 1978).

The hypothesis presented in the foregoing presents all naturally-occuring opioid agonists as fundamentally important in the life cycle process of plants as well as of animals. If this is so, one would expect to find specific opiate receptors in plants. Although these have never been sought, their existence can be predicted from the fact that such receptors are found in filter papers of plant origin. This does not suggest that such receptors are functional (nor were

they, in the original living material) but the arguments presented here make it possible to suggest that if such receptors are found in plant tissues a tentative function could be assigned to them. This might make the search logical and worthwhile.

Agonist substances for which the opiate receptor has affinity seem to occur in the most primitive life form and tissues (e.g. in grasses and in amoeba; Hazum et al., 1981; Josefsson and Johansson, 1979). The receptors appear to occur ubiquitously in excitable tissues, although at times with what appears to be a primitive or vestigial efficacy (Frank, 1975; Hunter and Frank, 1979). Could it be that the opiate receptor was the first pharmacological receptor to evolve in biological history? Such a possibility is reinforced if we consider that an opioid peptide could have been generated by prebiotic mechanism which selected different chains of amino acids ("proteinoids") for survival largely on the basis of minimal free energy requirements (Lehninger, 1976). In the list of possible polypeptides that could have arisen quasi-randomly in this way, the tyrosine residue, cardinal to the opiate action of any peptide (Smythies, 1976; Horn and Rodgers, 1976), figures prominently (Lehninger, 1976). Once a peptide with appropriate configuration had arisen nonbiotically, it certainly could have selected primitive membranes with acceptor sites for the peptide structure, to produce a membrane-bound agonist/acceptor system that had positive survival value by some process of natural selection at the macromolecular level. The genetic memory of such an event must be very persistent if this hypothesis is correct, but it does seem to be so. No disease based on an error in endorphin metabolism is as yet known, but errors in opiate receptor metabolism may prove to be responsible for

opiate narcotic addiction patterns or for the congenital condition where the sensation of pain is absent (Dehen et al., 1978). The genetic machinery which produces endorphins and enkephalins appears to be highly resistant to mutation, the leucine-enkephalin sequence is never found within the beta-endorphin molecule, although the genetic coding for methionine (AUG) is different by only one nucleotide residue from the genetic codon (UUG) which will incorporate leucine. Hence the opioid ligand:acceptor (receptor) site system may indeed represent a fundamental control system whose heritage spans the most primordial cells up to the most sophisticated products of the human brain -- pain, pleasure, joy, sorrow and the divine gift of self-awareness.

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FOOTNOTE:

Evidence for the Presence of Endorphins in the Pregnant Female: The first evidence for the presence of endorphins in the pregnant female was provided by Gautray et al. in 1977. They showed the presence of significantly high levels of endorphins in the amniotic fluid, as judged by radioimmunoassay (RIA) techniques. Evidence for presence of endorphins in the human and animal placenta came from the studies of Odagiri et al. (1979). Baldi et al. (1979), Salmon et al. (1979, Nakai

et al. (1978), Csontos et al. (1979) and of Houck et al. (1980). The presence of Met-enkephalin in the placenta(?) was reported by Ramasastry et al. in 1980. It has been reported that beta-endorphinlike immunoreactivity keeps rising every month during pregnancy until it reaches high levels (up to 5-10 times normal) on the day that immediately precedes delivery in humans (Baldi et al., 1979; Salmon et al., 1979; Csontos et al., 1979).

Julliard et al. (1980) reported that high molecular weight immunoreactive beta-endorphin in extracts of human placenta is a fragment of immunoglobulin-G. They cautioned that results from the immunological studies should not be taken as the sole criterion for chemical identity. This caution, and the report, requires further critical experimentation, as other people have used endorphin antibodies which differ from that used by Julliard et al.

The presence of opioid peptides in placenta has been suggested by the radioreceptor technique (Houck et al., 1980) and by chromatographic techniques for separating beta-LPH and beta-endorphin (Fraioli and Genazzani, 1980) (to differentiate the two). Opiate receptors, especially the kappa subgroup, have been reported in the human placenta (Porthe et al., 1981).