Kirk-Othmer Encyclopedia of Chemical Technology.

OPIOIDS, ENDOGENOUS

Decades of research in opioid analgesia culminated in the discovery of the endogenous opioid peptides (see Analgesics, antipyretics, and antiinflammatory agents; Neuroregulators). Early studies of the structure–activity relationships of opiate alkaloids (qv) had provided evidence of the stereospecificity and antagonist reversibility of opiate action, suggesting that these drugs acted through specific receptors. However, pioneering attempts to demonstrate specific opiate receptors in the brain met with only marginal success, largely because researchers were limited to high ligand concentrations resulting from the low specific activity of opioid ligands available at that time (1). In 1973, stereospecific opioid binding in rat brain was independently demonstrated in three separate laboratories (2–4). These demonstrations relied on comparison of binding by stereoisomers, eg, levorphanol [77-07-6] and its inactive enantiomer, dextrorphan [125-73-5], which differed by four orders of magnitude in their ability to bind to opiate receptors. Radioligands having high specific activity ($3.7 - 14.8 \times 10^{11}$ Bq/mmol (10–40 Ci/mmol)) were essential to these studies (see Radioactive tracers).

The presence of specific opioid receptors in the vertebrate central nervous system suggested the existence of endogenous ligands for these receptors, a hypothesis which received considerable support from the finding that electrical stimulation of specific sites in the rat brain elicited profound analgesia (5). This stimulation-produced analgesia was naloxone [465-65-6] reversible (6), and was subject to tolerance development and to cross-tolerance to morphine [57-27-2] (7). Moreover, a close correlation existed between those brain areas most sensitive to stimulation-produced analgesia and regions containing a high density of opioid receptors (8). These results were most readily explained by the electrically induced release of endogenous substances having morphine-like properties.

Evidence soon emerged that the endogenous opioids were peptides rather than simple morphine-like molecules (9). The first direct evidence for endogenous opioids in brain extracts was provided in 1975 when two pentapeptides were purified that differed only in the carboxyl terminal amino acids (10) (Table 1). These peptides were called methionine- (Met-) and leucine- (Leu-) enkephalin, from the Greek term meaning "in the head."

At the time of the discovery of Met-enkephalin, its sequence was observed to be identical to that of residues 61–65 contained in the C-fragment of the pituitary hormone β-lipotropin [12584-99-5] (β-LPH) (see Hormones), first isolated in 1964 (11). In 1976, the isolation of a larger peptide fragment, β-endorphin [60617-12-1], that also displayed opiate-like activity was reported (12). This peptide's 31-amino-acid sequence comprised residues 61–91 of β-LPH. Subsequently, another potent opioid peptide, dynorphin [72957-38-1], was isolated from pituitary (13). The first five amino acids (qv) of this 17-amino-acid peptide are identical to the Leu-enkephalin sequence (see Table 1).

The three principal classes of endogenous opioid peptides share one common characteristic: the pentapeptide structure of enkephalin, either the Met- or Leu-derivatives. Loss of any portion of that structure significantly reduces the affinity of β-endorphin, dynorphin, or enkephalin in binding to opioid receptors. Although at first glance the structures of these pentapeptides do not resemble that of stereotypical opiate alkaloids like morphine, a large number of structure–activity studies have clearly established the structural similarities between enkephalin and morphine (14, 15). These similarities are illustrated in Figure 1. For

Table 1. Structures of Endogenous Opioid Peptides

Compound	CAS Registry Number	Structure
		Pro-opiomelanocortin-derived
β-endorphin	[60617-12-1]	H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Val-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln-OH
		Pro-enkephalin-derived
Leu-enkephalin	[58822-25-6]	H-Tyr-Gly-Gly-Phe-Leu-OH
Met-enkephalin	[58589-55-4]	H-Tyr-Gly-Gly-Phe-Met-OH
octapeptide		H-Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu-OH
heptapeptide	[73024-95-0]	H-Tyr-Gly-Gly-Phe-Met-Arg-Phe-OH
		Pro-dynorphin-derived
dynorphin A	[80448-90-4]	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH
dynorphin B	[85006-82-2]	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr-OH
α-neoendorphin	[77739-20-9]	H-Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys-OH

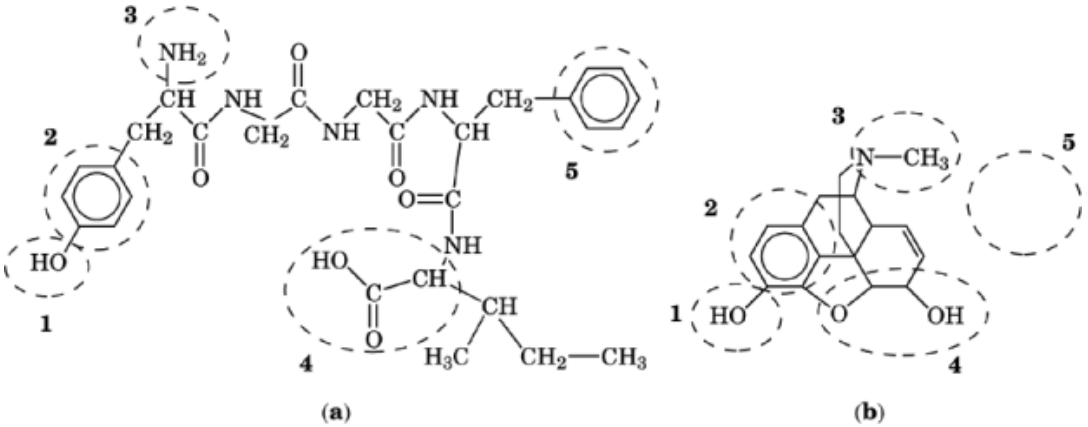

Fig. 1. Structures of two types of opioid agonists where dotted circles surround structural elements common to both compounds: (**a**) Leu-enkephalin and (**b**) morphine.

example, the phenolic aromatic ring A of morphine corresponds to the tyrosine residue on enkephalin (regions 1 and 2), whereas the N-terminus of enkephalin corresponds to the *N*-methyl group on morphine (region 3). The C-terminus of enkephalin most closely corresponds to the hydroxyl group on ring C and the ether bridge on morphine (region 4). There is no aromatic moiety on morphine that corresponds to the phenylalanine residue on enkephalin (region 5); however, addition of hydrophobic groups to the corresponding region on morphine (region 5) greatly increases its binding at opioid receptors. These findings suggest that opiate alkaloids and opioid peptides share common structural features which are crucial for high affinity binding at their receptors.

A number of peptides have been discovered that are related to the classical opioid peptides. FMRFamide [64190-70-1], which contains the first four amino acids of enkephalin, is biologically active in various invertebrates (16), and FMRFamide-like peptides have also been isolated from mammalian brain. Although these peptides are structurally similar to the enkephalins, they do not bind with appreciable affinities to the opioid receptors. In contrast, the casomorphins, a group of peptides originally isolated from milk, do not contain the enkephalin sequence, yet bind with relatively high affinity to opioid receptors (see Milk and milk products) (17).

Similarly, a group of unusual D-amino acid-containing peptides isolated from frog skin and termed dermorphins and deltorphins (18) have appreciable affinity for μ- and δ-type opioid receptors, respectively. Another group of peptides that do not contain the enkephalin sequence, but do share some sequence homology with the enkephalins, such as Tyr-MIF-1(H-Tyr-Pro-Leu-Gly-NH_2), have been isolated from mammalian brain and show both opioid-like and antiopioid activity (19).

1. Biosynthesis of the Opioid Peptides

1.1. Opioid Precursors

The sequence homology between β-LPH, β-endorphin, and Met-enkephalin suggested that Met-enkephalin might be formed by the proteolytic cleavage of β-endorphin or β-LPH. Likewise, some researchers assumed that dynorphin was a precursor for Leu-enkephalin. Such assumptions were incorrect, however, as demonstrated by experiments showing that the anatomical distributions of β-endorphin and dynorphin were different from that of Met- and Leu-enkephalin (20). Subsequently, the precursor relationships of the various opioid peptides were clarified by the use of cloned complimentary deoxyribonucleic acid (cDNA) techniques (see Biotechnology; Genetic engineering). Each opioid peptide is formed by the cleavage of one of three precursor proteins, each of which is encoded by a separate gene (Fig. 2).

Group I contains β-endorphin and its fragments, which arise from the 31,000 mol wt (265 amino acid) pro-opiomelanocortin [66796-54-1] (POMC) (21). POMC is cleaved to yield several peptide products, including an N-terminal fragment, γ-melanocyte-stimulating hormone (γ-MSH) (adrenocorticotrophic hormone [9002-60-2] (ACTH), and β-LPH at the carboxyl terminus (Fig. 2). Cleavage of POMC occurs between pairs of basic amino acids by a trypsin-like proteolytic enzyme. Further processing of POMC is species-dependent. In some systems $ACTH_{1--39}$ is the final product, whereas in others it is further cleaved to yield α-melanocyte-stimulating hormone [9002-79-3] and corticotrophin-like intermediate peptide [53917-42-3] (CLIP). β-LPH is cleaved to produce γ-lipotropin hormone [78065-47-1] and β-endorphin. There is little evidence that further cleavage of β-endorphin to Met-enkephalin occurs. However, tryptic digestion of β-endorphin produces the biologically active intermediates α-endorphin [59004-96-5] (β-$endorphin_{1--16}$) and γ-endorphin [61512-77-4] (β-$endorphin_{1--17}$). The predominant POMC peptide products in the hypothalamus are β-endorphin and α-MSH, neither of which appears to be acetylated to any significant degree (22). Although α-MSH and β-endorphin are also the primary POMC products in the medulla, more than 50% of the peptides are *N*-acetylated in this region (23). The acetylated form of β-endorphin does not bind to opioid receptors and has no significant analgesic activity (24).

Group II consists of the enkephalins which come from the 267-amino acid precursor pro-enkephalin A [88402-54-4] (Fig. 2). This protein contains four copies of Met-enkephalin, one copy of Leu-enkephalin, and the extended peptides Met-enkephalin-Arg^6-Phe^7 (the last Met-enkephalin sequence in Fig. 2) and Met-enkephalin-Arg^6-Gly^7-Leu^8 (the fourth Met-enkephalin sequence in Fig. 2) (25, 26). All of these products are formed by trypsin-like cleavage between pairs of basic residues. The extended enkephalin peptides are further cleaved by carboxypeptidase E (27) to form authentic Met-enkephalin.

The Group III peptides come from the 256-amino acid precursor, pro-dynorphin [88402-55-5] (pro-enkephalin B). This group contains dynorphin A [80448-90-4] and B [85006-82-2] as well as α-neoendorphin [77739-20-9] (Fig. 2), all of which can be further cleaved to form biologically active intermediates, eg, dynorphin A_{1--8} and β-neoendorphin [77739-21-0] (α-$neoendorphin_{1--9}$) (28). The longer of these peptides are relatively basic because of the number of Lys and Arg residues.

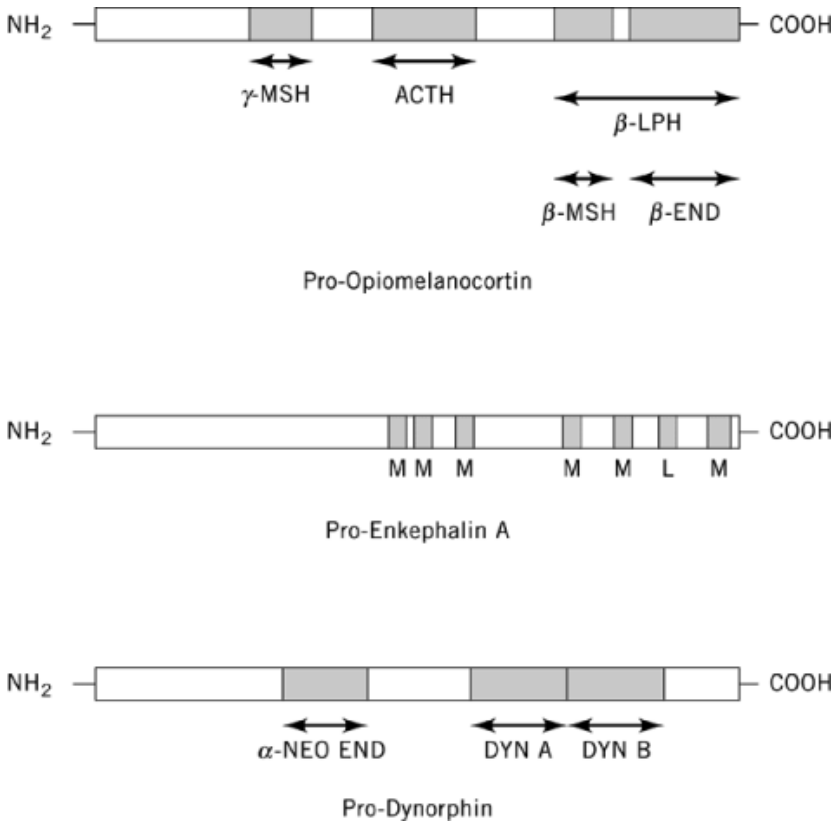

Fig. 2. Schematic drawing of the precursors for opioid peptides. Shaded areas represent the location of sequences of active peptide products which are normally released by trypsin-like enzymes acting on pairs of basic amino acid residues. Precursors are not necessarily drawn to scale. β–END=β–endorphin, L=Leu–e, M=Met–enkephalin, α-NEO END=α–neoendorphin, and DYN=dynorphin. See Table 1 and text.

1.2. Regulation of Biosynthesis and Post-Translational Processing

The biosynthesis of opioid peptides, like that of other neuropeptides, is regulated by factors that influence messenger ribonucleic acid (mRNA) synthesis. A number of studies have examined regulation of POMC synthesis. Both cyclic adenosine monophosphate (cAMP) and calcium have been shown to increase POMC expression in pituitary cultures (29), and corticotrophin-releasing factor [9015-71-8] (CRF) stimulates POMC synthesis in the pituitary (30). This effect of CRF on POMC may be mediated by the immediate early gene *c-fos* (31), a gene product which regulates the expression of other genes. The fact that the CRF response is mimicked by forskolin, 8-bromo-cAMP, and phorbol ester suggests that this effect is elicited via second messenger regulation of the POMC gene (30). Glucocorticoids inhibit POMC transcription in the pituitary anterior lobe and hypothalamus, and removal of endogenous glucocorticoids by adrenalectomy increases POMC mRNA levels (32, 33). These effects are mediated by a negative glucocorticoid response element (nGRE) located on the promoter of the POMC gene (34). A region of the POMC promoter has been identified which binds several regulatory elements that act synergistically to regulate POMC transcription. This region includes the nGRE binding site and an AP-1 site, which binds to immediate-early gene products (35).

Pro-enkephalin mRNA is also under the positive influence of cAMP through the cAMP-responsive promoter element (CRE) (36). In contrast to the POMC system, glucocorticoids increase pro-enkephalin mRNA

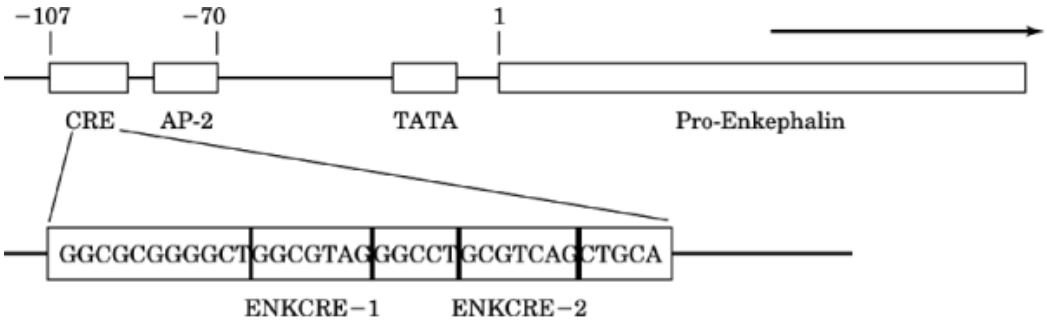

Fig. 3. Representation of promoter sites on the pro-enkephalin gene. The numbers represent the distance in nucleotides from the pro-enkephalin initiation codon; the arrow indicates the direction of transcription. The TATA promoter box occurs immediately before the pro-enkephalin initiation site; the AP-2 site, which binds immediate-early gene products, is 70 nucleotides upstream, and the CRE site, which binds a regulatory protein involved in cAMP induction of mRNA synthesis, is 107 nucleotides upstream from the initiation codon. The expanded section shows that the CRE site actually consists of two elements, ENKCRE-1 and ENKCRE-2, which separately confer cAMP sensitivity to pro-enkephalin mRNA synthesis.

levels in the adrenal medulla (37). Nicotine also increases adrenal pro-enkephalin, presumably through increased calcium influx (38). Protein kinase C may also regulate proenkephalin expression, probably via multiple mechanisms that include calcium and phosphatidyl inositol pathways (39). The promoter region of the pro-enkephalin gene has a CRE-2 site necessary for cAMP and phorbol ester induction, as well as CRE-1 and AP-2 sites which are necessary for maximal CRE-2 effects (Fig. 3) (40). In addition, immediate-early gene components of the AP-1 complex may regulate CRE. Jun D binds to the CRE sequence either as a homodimer or heterodimer with Fos whereas Jun B inhibits transcription by Jun D (41). *In vivo*, seizures have been correlated with increased levels of Fos and Jun, in the hippocampus, that apparently regulate pro-enkephalin transcription (42). Several AP-1-like sites are also found on the pro-dynorphin gene, although only one functional AP-1 site has been identified (43). There is also evidence that noxious stimuli that increase *c-fos* in spinal neurons also increase pro-dynorphin levels, indicating a potential link between immediate early genes and the dynorphin system (43).

As for many neuropeptides, post-translational modifications are important for opioid peptide function. Such modifications are particularly important for POMC. POMC is glycosylated at the N-terminal portion of the protein prior to proteolytic cleavage, although the number of glycosylation sites differ among species (44). These cleavages produce ACTH (which may or may not be glycosylated), a glycosylated N-terminal fragment, and β-LPH. Tunicamycin, a glycosylation inhibitor, has been used to determine the importance of glycosylation to normal POMC processing. Pituitary tumor cells treated with tunicamycin are able to process POMC into unglycosylated ACTH and β-LPH, and secrete unglycosylated ACTH and β-endorphin (45). In contrast, tunicamycin treatment of cells from the toad intermediate lobe disrupts POMC processing by the formation of unstable intermediates (46).

2. Anatomical Distribution and Colocalization of Opioid Peptides

The anatomical distribution of the opioid peptides and their precursors has been mapped in the brain using immunocytochemistry and *in situ* hybridization of corresponding mRNA. The various opioid peptides exhibit different anatomical distributions in brain, and the widespread distribution of opioid immunoreactive fibers suggests opioid involvement in many functional systems. Neurons containing β-endorphin and related POMC-derived peptides, as well as POMC mRNA, are primarily located in the hypothalamic arcuate nucleus (47). A

second smaller group is in the nucleus tractus solitarius (NTS) in the medulla (48). Fibers from the hypothalamic neuronal group project extensively throughout the telencephalon, diencephalon, and medial brainstem, whereas NTS-derived fibers are confined primarily to the lateral brainstem (49).

In contrast to the confined localization of β-endorphin neurons, enkephalin and dynorphin cell bodies have a ubiquitous distribution throughout the brain, with both local projections and long fiber pathways (50). Enkephalin and pro-enkephalin mRNA-containing neurons are found in the hippocampus, amygdala, striatum, septum, hypothalamus, thalamus, interpeduncular nucleus, parabrachial nucleus, nucleus locus coeruleus, periaqueductal gray, brainstem raphe and reticular nuclei, NTS, and spinal cord (51). Enkephalin is often co-localized in the same neurons with other peptides and neurotransmitters, including catecholamines, acetylcholine, epinephrine, γ-aminobutyric acid (GABA), serotonin, and substance P (52, 53). Although enkephalin has been primarily localized in neurons, it has also been identified in cultured astrocytes, where it may be important in development (54).

Dynorphin is widely distributed throughout the brain, and overlaps in many regions with enkephalin. Dynorphin-containing neurons are found in the hippocampus, central amygdala, striatum, cortex, hypothalamus, periaqueductal gray, brainstem reticular nuclei, NTS, and spinal cord (55). Colocalization of dynorphin with other neurotransmitters and hormones, such as vasopressin in the posterior pituitary and hypothalamic magnocellular neurons (56) and leutinizing hormone and follicle-stimulating hormone in the anterior pituitary (57), has also been reported. Few studies have colocalized the opioid peptides to the same neurons, although enkephalin and dynorphin have been colocalized in neurons in the spinal cord (58).

In addition to the well-defined opioid systems in the central nervous system, the three opioid peptides and their precursor mRNA have also been identified in peripheral tissues. β-Endorphin is most abundant in the pituitary, where it exists in corticotroph cells with ACTH in the anterior lobe and in melanotroph cells with MSH in the intermediate lobe (59). Enkephalin and pre-pro-enkephalin mRNA have been identified in the adrenal medulla (60) and this has been the source of material for many studies of pro-enkephalin synthesis and regulation. Pre-pro-enkephalin mRNA has also been identified in the anterior and posterior lobes of the pituitary (61). mRNA for all three opioid precursors has been identified in the reproductive system (62–64). POMC mRNA and peptide products have been found in the digestive system, kidney, liver, lung, and spleen (62) and pro-enkephalin mRNA has been identified in the heart (65). Pro-enkephalin has also been found in lymphocytes (66), generating interest in possible opioid effects on the immune system.

3. Receptors for Opioid Peptides

3.1. Multiple Opioid Receptors

The concept of multiple opioid receptors was first postulated in 1976 (67). Three distinct opioid receptors were postulated: mu (μ), kappa (κ), and sigma (σ). A fourth type of opioid receptor, the delta (δ) receptor, was postulated in 1977 (68) after discovery of the endogenous opioid peptides. Originally, the prototype agonists for these receptors were morphine [16206-77-2] (μ), ketazocine [36292-69-0] (κ), *N*-allylnormetazocine (SKF-10,047) [14198-28-8] (σ), and Met- and Leu-enkephalin (δ), although more selective compounds for each receptor type are available. The σ-receptor is no longer thought to be a receptor for the endogenous opioids and is therefore not discussed further herein. The original confusion was in reference to the cross-reactivity of σ-ligands with μ- and κ-opioid receptors. The classification of opioid receptor types is primarily based on the specific affinities displayed by various opioid drugs and peptides in radioligand-binding assays and on the potency of these compounds to inhibit smooth muscle contractions, or to block opioid inhibition in the case of antagonists, in isolated organ preparations such as the guinea pig ileum (μ- and κ-receptors) or the mouse vas deferens (δ-receptors). Confirmation of the original discoveries of multiple opioid receptor types is being

obtained by molecular cloning studies. A more accurate reclassification scheme is expected to arise from these studies.

The opioid peptides vary in their binding affinities for the multiple opioid receptor types. Leu- and Met-enkephalin have a higher affinity for δ-receptors than for the other opioid receptor types (68), whereas the dynorphin peptides have a higher affinity for κ-sites (69). β-Endorphin binds with equal affinity to both μ- and δ-receptors, but binds with lower affinity to κ-sites (70). The existence of a β-endorphin-selective receptor, the ϵ-receptor, has been postulated; whether this site is actually a separate β-endorphin-selective receptor or is a subtype of a classical opioid receptor is a matter of controversy (71, 72). The existence of opioid receptor subtypes in general is quite controversial although there is some evidence for subtypes of μ- (73), δ- (74), and κ-receptors (72, 75), confirmation of which may be obtained by future molecular cloning studies.

3.2. Opioid Peptide Analogues and Their Receptor Affinities

In an effort to develop nonaddictive and nontolerance-producing opioid analgesics numerous metabolically stable enkephalin analogues have been synthesized (see Psychopharmacological agents). The most successful stability-enhancing techniques have included the replacement of naturally occurring L-amino acids with the D-isomer and amidation of the carboxyl terminal residue, to form compounds such as D-Ala^2,Met^5-enkephalinamide [61090-95-7] (76). These derivatives show little promise as nonaddictive analgesics, because they share the tolerance and dependence liabilities of the endogenous opioids (77). However, many enkephalin analogues show remarkable receptor selectivity compared to the naturally occurring peptides. This observation has led to the design of hundreds of analogues having increased selectivity for the multiple opioid receptor types. The principal design strategies include (*1*) substitution, addition, or deletion of amino acid residues; (*2*) introduction of conformational restrictions; and (*3*) modification of peptide bonds. As of this writing, there are a number of commonly used peptide and synthetic opioid ligands that are among the most highly selective agonists and antagonists available for the multiple opioid receptor types. Compared to the native enkephalins, the modified peptide analogues can display increased receptor selectivity for one of three reasons: (*1*) decreased affinity for other sites along with unchanged affinity for the target site; (*2*) increased affinity for the target site with no change in affinity for other sites; or (*3*) a combination of the above.

Among the peptide derivatives that are agonists, D-Ala^2, *N*-Me-Phe^4, Gly-$(CH_2OH)^5$-enkephalin [78123-71-4] (DAMGO), where Me represents methyl, is highly μ-selective (70). Similar compounds having increased μ-selectivity have been achieved by replacement of residues 4 and 5 of DAMGO with an aliphatic chain (78). Some of the atypical (nonenkephalin-containing), naturally occurring opioid-like peptides also show some degree of μ-selectivity. β-Casomorphin (H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH) [102029-74-3], for example, is moderately μ-selective, and deletions of residues 5–7 produces morphiceptin [74135-04-9], a compound with improved μ-selectivity (17). Further modification of this tetrapeptide to H-Tyr-Pro-*N*-Me-Phe-D-Pro-NH_2 (PLO17) (79) leads to μ-selectivity which is somewhat greater than that of DAMGO. Dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) [77614-16-5] is unusual in that it naturally contains D-Ala in the 2-position and a C-terminal carboxamide (80). This compound, as well as many of its tetra- and tripeptide derivatives, including DALDA(H-Tyr-D-Arg-Phe-Lys-NH_2), is relatively μ-selective (81). A series of dermorphin analogues with increasing positive charge was synthesized to test the hypothesis that δ-receptors are in a cationic membrane environment from which positively charged ligands are electrostatically excluded (82). Results showed that the μ-selectivity of these analogues increased with increasing number of positive charges so that the peptide with the highest positive charge, [D-Arg^2,Lys^4]dermorphinamide, was 10 times more selective than DAMGO. Other experiments examined the relationship between the Phe^3 residue in dermorphin and the Phe^4 in enkephalin by synthesizing hybrid analogues with Phe in both the 3- and 4-position (83). The prototype Phe^{3,4} analogue H-Tyr-D-Ala-Phe-Phe-NH_2 (TAPP) displayed high affinity and selectivity for μ-receptors (82). Nitration in the para position of the aromatic moiety of Phe^3 decreased the affinity of TAPP for μ-receptors, whereas similar

nitration of the Phe^4 residue produced an increase in affinity, supporting the contention that the Phe^4 residue of the enkephalins interacts with the μ-receptor in a different fashion than the Phe^3 residue of dermorphin.

The enkephalins are structurally flexible and capable of assuming a number of energetically favorable conformations in aqueous solution. A successful approach to increase receptor selectivity has been to introduce conformational constraints by cyclization of peptides. An example of this approach is the peptide H-Tyr-cyclo[-D-A_2bu-Gly-Phe-Leu-], where A_2bu is D-α,γ-diaminobutyric acid (84). Cyclization of the γ-amino group of A_2bu to the carboxyl terminus conferred significant μ-selectivity compared to the corresponding nonselective linear analogue containing α-aminobutyric acid (85). This comparison provided the first direct demonstration of conformational selectivity among peptide receptor subtypes. Studies have demonstrated a lack of receptor-selectivity when more than one low energy conformation of a constrained, cyclized peptide can be assumed, as has been shown with [D-$AlaL^2$,L-$AlaL^5$] enkephalinamide, where AlaL represents lanthionine-containing residues linked by a monosulfide bridge, which displays equal potency in bioassays for μ- and δ-activity (86). Another series of μ-selective cyclized peptides are the cyclic dermorphin tetrapeptides, such as H-Tyr-cyclo[-D-Orn-Phe-Asp]-NH_2 and its derivatives (87), which have been used to characterize the importance of the arrangement of the Tyr and Phe aromatic rings to μ-receptor affinity.

The cyclization approach has been extremely successful in the synthesis of highly selective δ-agonists. Substitution of D- or L-penicillamine moieties in the 2 and 5 position of enkephalin has led to compounds having a high degree of δ-selectivity (88). Two of the most selective analogues that have been used extensively as δ-receptor agonists are H-Tyr-cyclo[-D-Pen-Gly-Phe-D-Pen]-OH (DPDPE) and DPLPE (with L-Pen in the 5 position). The δ-selectivity of these compounds was shown to result from steric interference at the μ-site, caused by the presence of the *gem* dimethyl groups in the 2-position side chain (89). Although DPDPE and DPLPE are extremely δ-selective, their absolute affinity for the δ-receptor is low compared to some the δ-selective linear enkephalin analogues. However, this problem has been solved by replacement of Phe^4 with *p*-chlorophenylalanine, which improves the δ-selectivity of the compound by fivefold over DPDPE because of an increase in affinity at the δ-site (90).

Although the natural enkephalins are somewhat δ-selective, a number of linear enkephalin analogues have been synthesized with improved selectivity. One of the earliest peptides analogues employed as a δ-selective ligand was H-Tyr-D-Ala-Gly-Phe-D-Leu-OH (DADLE) (91). Although DADLE is only slightly more selective than the enkephalins, replacement of D-Ala in the 2 position with D-Ser or D-Thr combined with the addition of a Thr residue at the C-terminus to form the analogues H-Tyr-D-Ser (or D-Thr)-Gly-Phe-Leu-Thr-OH (DSLET and DTLET) led to marked improvement in δ-selectivity by reducing the affinity for μ receptors (92). Because improved δ-selectivity was assumed to be due to steric interference at the μ-site by the side chains of residues 2 and 6, compounds were designed with increased bulk in these positions by the addition of *tert*-butyl, *t*-C_4H_9, groups to the hydroxyl moieties of Ser or Thr. This strategy produced compounds with greatly increased δ-selectivity, such as H-Tyr-D-Ser(O*t*-C_4H_9)-Gly-Phe-Leu-Thr (or O*t*-C_4H_9Thr)-OH (DSTBUTLET and BUBU) (93). Replacement of D-Ser(O*t*-C_4H_9) with D-Cys(S*t*-C_4H_9) to form the compound BUBUC has been shown to increase δ-selectivity to a level comparable to that of the cyclic Pen-containing analogues DPD(L)PE (94).

Another class of δ-selective peptides, isolated from extracts of frog skin, is the deltorphins. These compounds are based on the structure H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2 [119975-64-3] and are approximately equal in δ-selectivity to DPDPE (95). Two analogues, H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 (D-Ala^2-deltorphin I) and H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 (D-Ala^2-deltorphin II) display greater δ-selectivity than DPDPE owing to their higher δ-receptor affinity (96). These compounds both contain the same N-terminal tripeptide sequence as the μ-selective dermorphins, which underscores the importance of the C-terminal tetrapeptide sequence in conferring δ-selectivity.

The endogenous peptide dynorphin A_{1--17} and its C-terminally degraded fragments dynorphin A_{1--13} and dynorphin A_{1--9} are somewhat κ-selective (70). Several substituted analogues of dynorphin have shown moderate improvement in κ-selectivity, including Ala^8-dynorphin A_{1--13}, Trp^8-dynorphin A_{1--13}, and

D-Pro^{10}-dynorphin A_{1--13} (97). Analogues with C-terminal deletions, such as D-Pro^{10}-dynorphin A_{1--11}, have been found to display further improvement in κ-selectivity (98). Replacement of residues 7–15 of dynorphin A with an alternating Lys and Val sequence, along with the substitution of Ser in positions 16 and 17, has produced moderate increases in κ-selectivity (99). Although peptide cyclization has been a successful technique in the development of ligands with improved μ- and δ-selectivity, cyclized dynorphin analogues have proven to be relatively nonselective (100), or more μ- or δ- than κ-selective (101). Thus, the effect of cyclization tends to produce peptide conformations that are not compatible with the κ-receptor binding site.

Among the peptide analogues that are opioid antagonists, the most highly μ-selective are derived from somatostatin [38916-34-6]. These cyclic compounds, based on H-D-Phe-cyclo[-Cys-Phe-D-Trp-Lys-Thr-Cys]Thr-ol (SMS-201995) [83150-76-9] (102), bear no obvious structural resemblance to the opioid peptides. Most interesting is the lack of an N-terminal Tyr residue, which is common among all the opioid peptides and the atypical opioid-like peptides including β-casomorphin, the dermorphins, and the deltorphins. The presence of another aryl-containing residue, Phe, in place of Tyr may account for the antagonist properties of the somatostatin-based analogues, though this has not been proven. The most μ-selective of these analogues H-D-Tic-cyclo[-Cys-Tyr-D-Trp-Orn-Thr-Pen]-Thr-NH_2 (TCTOP) contains another aromatic group, Tic (tetrahydroisoquinoline-3-carboxylic acid), in the 1 position and retains its full antagonist properties (103). The two most commonly used analogues H-D-Phe-cyclo[-Cys-Tyr-D-Trp-Lys-Thr-Pen]-Thr-NH_2 (CTP) [103335-28-0] and H-D-Phe-cyclo[-Cys-Tyr-D-Trp-Orn-Thr-Pen]-Thr-NH_2 (CTOP) [103429-31-8] (104) are slightly less μ-selective than TCTOP. However, all of these compounds display reduced affinity for somatostatin receptors compared to the parent compound SMS-201995.

Enkephalin-based antagonists having high δ-selectivity but low δ-affinity have been synthesized by diallylation of the N-terminal α-amino group along with modification of the peptide bond at the 3 to 4 position (105). Replacement of the Gly^2-Gly^3 sequence with α-aminoisobutyric acid (Aib) to form the compound *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174864) [89352-67-0] led to improvement in both δ-receptor affinity and selectivity (106). Conformationally restricted analogues of this compound have shown similar results. Similar modifications of truncated dynorphin peptides, such as *N,N*-diallyl-Tyr^1,Alb^{2,3},D-Pro^{10}-dynorphin A_{1--11} have produced analogues that are antagonists but are not significantly κ-selective (107). To date there have been no highly κ-selective peptide antagonists developed.

Another recently developed class of δ-antagonists are short-chain (3–4 residues) peptides consisting entirely of aromatic amino acids, and having Tic in the 2 position (108). The most potent and selective of these analogues is H-Tyr-Tic-Phe-Phe-OH (TIPP) which displayed extreme δ-selectivity and improved potency compared to reported values for ICI 174864. Interestingly, replacement of L-Tic with the D-isomer changed the compound to a μ-selective agonist, whereas the amino derivative TIPP-NH_2 was a moderately potent μ-agonist with δ-antagonist properties. However, the corresponding tripeptides H-Tyr-Tic-Phe-OH or -NH_2 were both δ-selective antagonists. These results provide compelling evidence that intrinsic activity as well as receptor-binding affinity and selectivity can be affected by opioid peptide conformation. Pseudopeptide analogues of the Tic-containing antagonists have been developed which contain a reduced peptide bond between the Tic^2 and Phe^3 residues (109). The compound with the highest potency, H-Tyr-TicΨ[CH_2NH]Phe-Phe-OH (TIPP[Ψ]) displayed subnanomolar affinity and the greatest degree of selectivity for the δ-receptor of any ligand yet known.

Although the emphasis of this article is on the opioid peptides, a brief discussion of nonpeptide (alkaloid and synthetic) ligands is appropriate. Among the nonpeptide agonists, the opiate alkaloids, such as morphine, and their synthetic derivatives, such as fentanyl, are relatively μ-preferring. Though not as selective as DAMGO, these have generally equal or greater potency. This is also true of the opiate antagonists, such as naloxone (Fig. 4), which are not as selective as the cyclic somatostatin analogues but tend to be more potent. For δ-receptors, there are few selective nonpeptide ligands available. Naltrindole [111555-53-4] (NTI) (Fig. 4) is a δ-selective nonpeptide antagonist (110) that is more potent but less selective than TIPP. Naltrindole

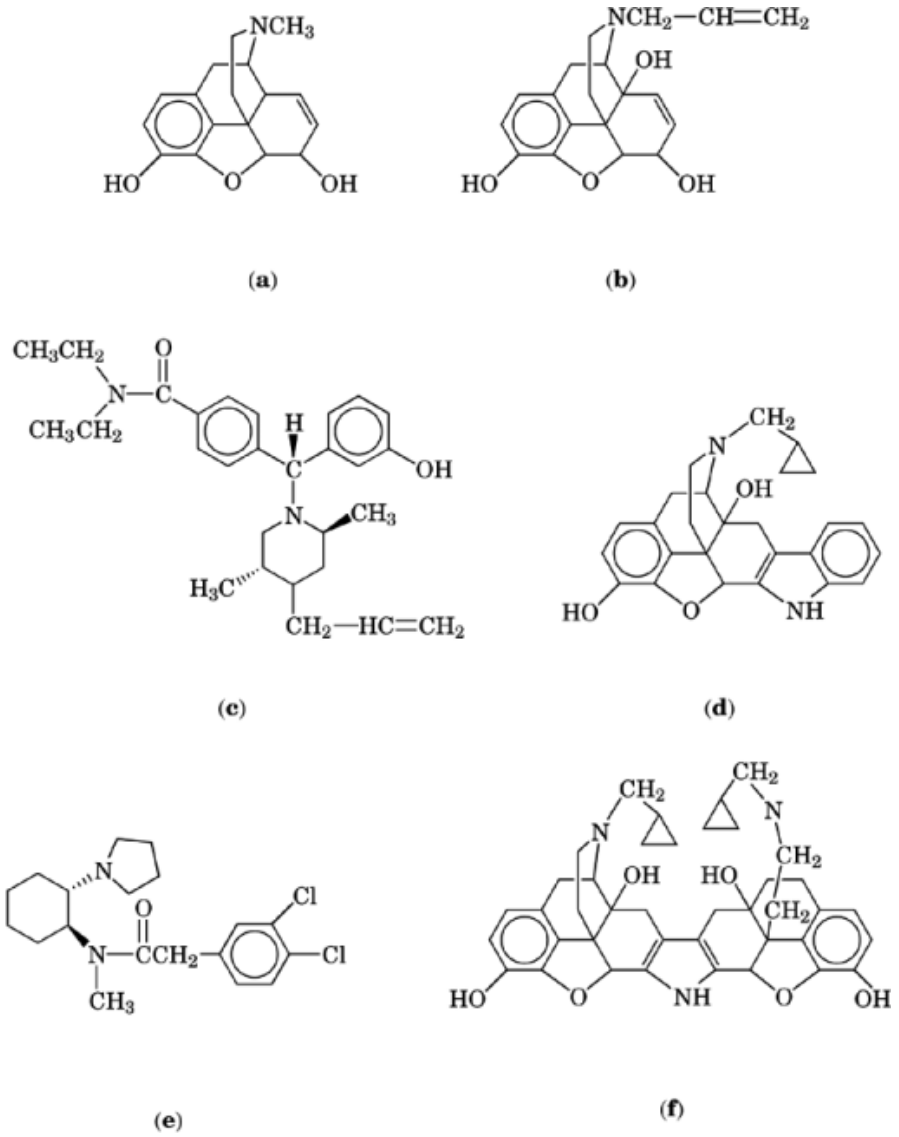

Fig. 4. Structures of several nonpeptide opioid agonists, (**a**) morphine, (**c**) BW373U86, (**e**) U50488 and antagonists, (**b**) naloxone, (**d**) naltrindole, and (**f**) norbinaltorphimine, with specificities at μ-, δ-, κ-opioid receptors.

has approximately the same affinity as ICI 174864 for non-δ sites, but its affinity for the δ-receptor is orders of magnitude greater (111). The benzofuran analogue of NTI (NTB) and 7-benzylidenenaltrexone (BNTX) discriminate between the putative δ-receptor subtypes, δ_2 and δ_1, respectively (112). The recently developed BW373U86 (Fig. 4) is a δ-selective agonist (113) that has less selectivity but higher affinity for the δ-receptor than DPDPE has. Compared to the linear δ-selective analogue DSLET, BW373U86 is approximately equal in binding affinity but is more potent in functional assays owing to the antagonist-like binding properties of this full agonist (114).

The most highly κ-selective ligands are not of peptide origin. Selective nonpeptide κ-agonists include U50,488δ [67198-13-4] (Fig. 4) and related compounds (70, 115). One of the most highly selective κ-antagonists is norbinaltorphimine (nor-BNI) (Fig. 4), a dimeric derivative of the opiate antagonist naltrexone (116). In general, the original ketazocine-based benzomorphan ligands are no longer considered to be sufficiently selective for examining κ-sites.

3.3. Receptor Structure and Function

All of the known opioid receptor types belong to the superfamily of G protein-coupled receptors. These receptors reside on the plasma membrane and affect cell physiology by interacting with the signal-transducing guanosine triphosphate (GTP)-binding regulatory proteins (G proteins) (117). In most cells, opioid receptors are coupled to G_i and G_o, a class of G proteins that are adenosine diphosphate (ADP)-ribosylated by pertussis toxin. It is the G protein, rather than the receptor itself, that determines which effector(s), an enzyme or ion channel, are affected by receptor activation. The effector activity can be stimulated or inhibited by the receptor, depending on the G protein involved. For example, all opioid receptor types are known to inhibit the activity of adenylyl cyclase (118), the enzyme which converts adenosine triphosphate (ATP) into cyclic-AMP. These receptors also decrease calcium conductance (119) and increase potassium conductance by direct actions of G proteins on the corresponding channels (120). All of these opioid-induced responses tend to decrease neuronal activity by hyperpolarization or to inhibit neurotransmitter release by blocking depolarization-induced calcium influx. Thus, the opioid peptides are generally considered to be inhibitory neurotransmitters, although excitatory actions have been reported (121, 122). Moreover, opioids inhibit the activity of cAMP-dependent protein kinase through their effect on adenylyl cyclase (123). Reviews of opioid receptor-mediated effects on cell biochemistry and physiology are available (124, 125).

Despite the knowledge of sequence homology obtained by the cloning of many other G protein-coupled receptors, attempts at cloning the opioid receptors remained unsuccessful until 1992. In that year, two independent reports emerged on the expression cloning of a δ-opioid receptor from NG108-15 cells (126, 127), a cell line known to express a high density of δ-opioid receptors (see Cell culture technology). The cloned receptors, when expressed in COS cells, showed a binding profile expected of a δ-receptor and mediated opioid inhibition of adenylyl cyclase. The cloned δ-receptor contained 371–372 amino acids and showed significant homology to other G protein-coupled receptors, with the characteristic seven transmembrane domains, three intracellular and three extracellular loops, and multiple glycosylation sites on the amino terminal domain. Soon after the reported cloning of the δ-opioid receptor, cloning of the μ- (129–131) and κ- (132–136) opioid receptors, as well as multiple opioid receptor types, were reported (137–139). The multiple opioid receptors share extensive sequence homology with each other, as well as with the somatostatin receptor (Fig. 5). Whether there are multiple subtypes of opioid receptors remains unclear as of this writing.

4. Biological Activities

Soon after the identification of endogenous opioid peptides, studies were conducted to determine their contribution to physiological function. Morphine was a well-established analgesic drug with central actions mediated by an endogenous anatomical substrate (140). Intracerebral (icv) injection of β-endorphin in mice also elicited naloxone-reversible analgesia (141). β-Endorphin elicited other opiate effects including shivering, pinnae vasodilation, mydriasis, tachypnea (rapid breathing), vocalization, hyperexcitability, and catelepsy (142). Further studies on analgesia demonstrated that β-endorphin was more potent than morphine in eliciting analgesia in a variety of species, including the human (143). Moreover, evidence accumulated to implicate endogenous opioid peptides in mediating stimulation-produced analgesia, especially when stimulation was applied to the periaqueductal gray (PAG), a region rich in opioid peptides and receptors (5). This evidence included (*1*) partial

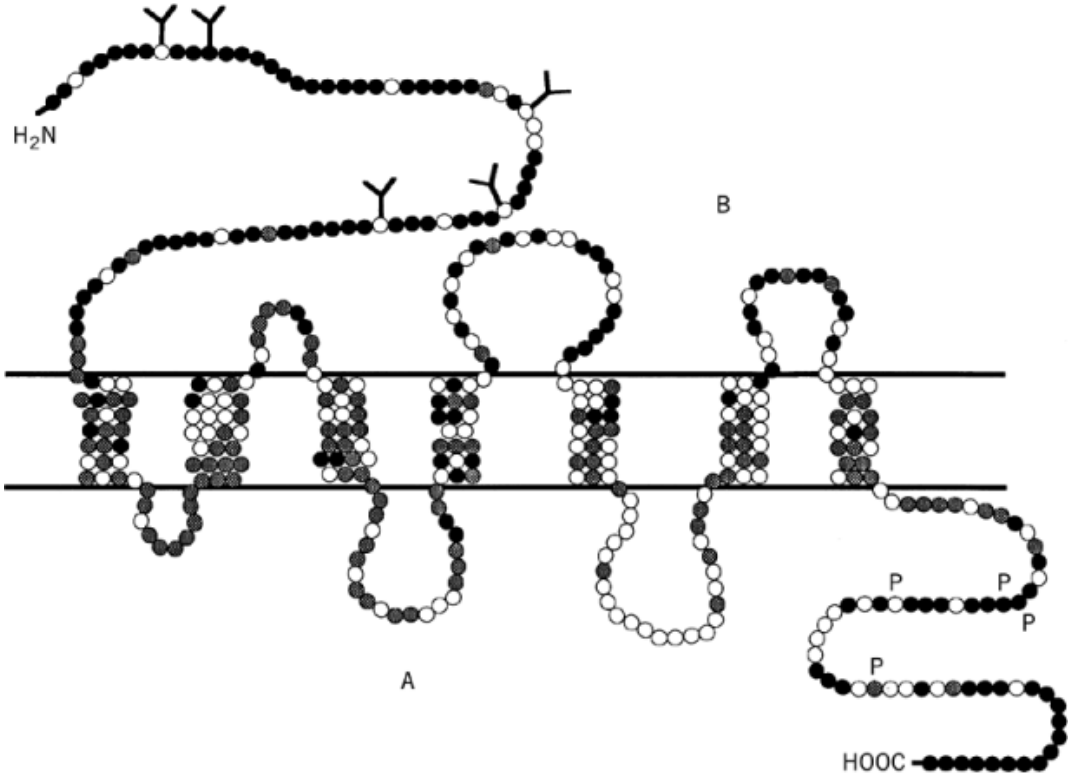

Fig. 5. Schematic diagram of the presumed arrangement of the amino acid sequence for the δ-opioid receptor, showing seven putative transmembrane segments; three intracellular loops, A; three extracellular loops, B; the extracellular N-terminus; and the intracellular C-terminus, where (•) represents amino acid residues common to μ-, δ-, and κ-receptors; (●), amino acid residues common to all three opioid receptors and other neuropeptide receptors; and (○), other amino acids. Branches on the N-terminal region indicate possible glycosylation sites, whereas P symbols in the C-terminal region indicate possible phosphorylation sites. Adapted from Ref. 128.

reversal by naloxone of stimulation-produced analgesia (144), (*2*) tolerance to repeated stimulation, and (*3*) cross-tolerance with morphine (145). Another potential analgesic effect of opioid peptides may be stress-induced analgesia, in which noxious or stressful stimuli elicit an analgesic response. This is a complex phenomenon with several neural, including nonopioid, components. However, the findings of partial naloxone reversibility, development of tolerance, and an increase in endogenous opioid levels during stress-induced analgesia suggest at least some involvement of opioid peptides (146).

In contrast to the potent, long-lasting analgesic effects of β-endorphin (147), the enkephalins are extremely weak analgesics in laboratory tests. This difference is likely a result of the relatively short (2–3 min) biological half-life of the enkephalins vs the long (2–3 h) half-life of β-endorphin (148). Thus, only transient analgesia has been found in rats, mice, and cats (149) with Met-enkephalin even when administered by icv injection. Not surprisingly, massive doses (320 mg/kg) of Leu-enkephalin administered intravenously produced only weak analgesic activity in mice (150). Nonetheless, the enkephalins may be involved in the physiological response to sensory input from noxious stimuli (nociception) by acting at the spinal level. Enkephalin (151) and opioid receptors (152) have been localized in regions of the spinal cord associated with processing of noxious stimuli. Lesions affecting afferent nociceptive input decrease opioid receptor binding (152, 153), indicating that opioid receptors are involved in this system. Enkephalin levels in the spinal cord increase in response to noxious

stimuli (154), and increasing enkephalin levels by administration of enkephalinase inhibitors leads to dose-dependent, naloxone-reversible analgesia (155). Application of enkephalin to spinal neurons has also been reported to decrease cell firing in response to noxious stimuli (156). These actions of enkephalin at the spinal level may actually result from modulation of substance P release from primary afferent fibers (157).

Dynorphin may also influence nociception at the spinal level. The levels of prodynorphin mRNA and immunoreactive dynorphin increase in the chronic inflammatory arthritic model (158). Dynorphin$_{1--13}$ also inhibits morphine or β-endorphin-induced analgesia in naive animals and enhances analgesia in tolerant animals, indicating that this peptide may have a regulatory role in opioid analgesia (159). This effect does not appear to be mediated by a classical opioid receptor, since des-tyrosine dynorphin, which does not bind to opioid receptors, also antagonizes morphine analgesia (160).

The finding of analgesic activity for the endogenous opioids created a renewed but short-lived hope that these or related peptides might lead to an analgesic devoid of dependence liability. However, Met-enkephalin and β-endorphin produce symptoms of physical dependence (161) and evidence of tolerance and morphine cross-tolerance in animals and *in vitro* (162). Furthermore, β-endorphin (163, 164) and the enkephalins (165) are reinforcing stimuli in behavioral experiments. The effects of these peptides may be mediated in part by disinhibition of mesolimbic dopaminergic neurons (166), which have been implicated in mediating the reinforcing effects of morphine (167). Moreover, drug discrimination experiments in rats indicate that Met-enkephalin is generalized to the narcotic fentanyl (168). Thus, the evidence indicates that the opioid peptides, including at least β-endorphin and the enkephalins, are similar to the opiate alkaloids in their reinforcing properties as well as in their ability to produce tolerance and dependence.

Although many studies have focused on the analgesic effects of opioids, the endogenous opioid peptides have been found to influence a wide range of physiological functions. Opioid peptides and receptors are found in brain areas that influence respiratory and cardiovascular function. Injection of β-endorphin into the NTS results in dose-dependent and naloxone-reversible decreases in mean arterial pressure and heart rate (169). Intracisternal β-endorphin also depresses respiration in a naloxone-reversible manner (170). One aspect of opioid function that has received a great deal of interest is the effect of endogenous opioid systems on immune function (171). Both β-endorphin and Met-enkephalin enhance the cytotoxicity of natural killer cells in a manner that is inhibited by naloxone (172). In contrast, the C-terminal fragment of β-endorphin reduces the activity of natural killer cells; however, this activity is not affected by naloxone (173). Endogenous opioid peptides may also influence reproductive behavior. Studies in rodents with β-endorphin (174) and an enkephalin analogue (175) have demonstrated inhibition of copulatory behavior. POMC mRNA levels are also decreased by both estrogen and testosterone (176). In contrast, estradiol has been shown to increase proenkephalin mRNA levels in the hypothalamus in a manner that coincided with the display of lordosis (177). Another hypothalamic action of opioid peptides is thermoregulation. Hyperthermia occurs after the injection of a μ-agonist, whereas dynorphin decreases temperature by decreasing metabolic rate (178).

5. Metabolic Inactivation of Opioid Peptides

Several enzymes, none of which are completely specific for the enkephalins, are known to cleave Leu- and Met-enkephalin at various peptide bonds. The main enzymes that degrade enkephalin are zinc metallopeptidases. The first enkephalin-degrading enzyme to be identified, an aminopeptidase which cleaves the amino terminal Tyr-Gly bond (179), has been shown to be aminopeptidase-N (APN) (180). It is a cytoplasmic enzyme which is uniformly distributed throughout the brain. The increased analgesic activity of synthetic enkephalins substituted by D-amino acids at position 2, eg, [D-Ala^2]-Met-enkephalin, is probably the result of increased stability toward this aminopeptidase (181). A second enkephalin-degrading enzyme, enkephalinase B, is a dipeptidylaminopeptidase (DAP) which cleaves the Gly–Gly bond of enkephalin (182). This membrane-bound enzyme has the least overall enkephalin-degrading activity in crude brain homogenates and is uniformly distributed

throughout the brain. Enkephalinase A, a dipeptidylcarboxypeptidase which cleaves the Gly–Phe bond of enkephalin (182, 183), has been identified as neutral endopeptidase 24.11 (NEP) (184). This membrane-bound enzyme has a K_m for the enkephalins of approximately 20 μM (182, 185), and its distribution parallels that of the opioid receptors (186) as well as the enkephalins (187). The finding that administration of thiorphan, a synthetic inhibitor of NEP, produces naloxone-reversible analgesia has provided support for the suggestion that NEP is largely responsible for *in vivo* inactivation of the enkephalins (188) (see Enzyme inhibitors). Enkephalin analogues with increased stability toward NEP have been synthesized; the modifications used in making these analogues include *N*-methylation of the Gly–Phe peptide bond, amidation of the carboxyl terminus, or replacement of the L-Phe with the D-isomer. Such analogues show even more enhanced analgesic activity than the D-Ala^2 analogues (189). Finally, angiotensin-converting enzyme (ACE) also shows enkephalinase activity and, like NEP, cleaves the Gly–Phe bond (190). However, ACE has a low ($\sim$1 nM) affinity for the enkephalins, and several specific ACE inhibitors do not significantly alter the overall enkephalin-degrading activity in brain tissue (191).

Intensive research efforts have focused on the discovery of potent and specific inhibitors of the enkephalin-degrading enzymes for novel analgesic agents. Because the enkephalinases are metallo (Zn) enzymes (192), inhibitor design was based on the synthesis of compounds with a strong metal coordinating group and which display energetically favorable interactions with one or more of the subsites surrounding the catalytic core (193). A potent and specific NEP inhibitor (K_i of 4.7 nM), thiorphan [76721-89-6] (194), produced analgesia on its own and potentiated analgesia elicited by enkephalin analogues (188). Subsequently, a number of modifications were made in order to increase the selectivity and bioavailability of thiorphan (188). Other classes of thiol-based inhibitors, such as the *N*-mercaptoacetyldipeptides, also show high potencies as NEP inhibitors. Another important class of enkephalinase inhibitors is the N-protected amino acid hydroxamates. These transition-metal chelators display nanomolar K_i values in inhibiting NEP (195), and modifications have led to extremely potent and specific NEP inhibitors (196). Finally, phosphorus-containing dipeptides, such as phosphoramidon [36357-77-4], are also potent inhibitors of NEP (197).

APN inhibitors include substituted aminoethanols (198) and phenyalanine-based compounds (199). Phe-containing dipeptides such as Tyr-Phe-NHOH are highly selective and potent inhibitors of DAP. Various hydroximate- and thiol-containing compounds have also been synthesized as mixed enkephalinase inhibitors. Compounds based on kelatorphan [92175-57-0], for example, potently inhibit NEP, APN, and DAP (195). Development of compounds in which several inhibitors are linked by disulfide bonds has led to systemically active mixed enkephalinase inhibitors that are very potent in antinociceptive tests (200). In addition to their promise as analgesic agents, mixed peptidase inhibitors with specificity for NEP and ACE have been found to possess antihypertensive activity (see Cardiovascular agents). A review of the design and potential clinical applications of mixed peptidase inhibitors is available (201).

Little is known about metabolic inactivation of β-endorphin and the dynorphins. NEP, and to a lesser extent APN, are only weakly active against β-endorphin (183). Enzymes are known which degrade β-endorphin *in vitro* under nonphysiological conditions (202) or which inactivate β-endorphin by N-acetylation (203). A lack of specific degradative enzymes for these peptides may account for their relatively long half-life *in vivo*, though this has not been definitively established.

6. Endogenous Opiate Alkaloids

Although the opioid peptides have long been identified as the primary endogenous opioid ligands in brain, several groups have identified the opiate alkaloid morphine and related compounds in the tissues of several species. A nonpeptide opioid has been isolated from toad skin in sufficient quantity for purification and has the same profile as morphine in high performance liquid chromatography (hplc), gas chromatography/mass spectrometry, radioimmunoassay, opiate receptor binding assay and bioassay (204) (see Analytical methods;

Chromatography; Immunoassay; Mass spectrometry). A nonpeptide opioid was also identified in bovine brain and adrenal gland, as well as rabbit and rat skin, that corresponded to morphine in hplc analysis. However, the concentration of the compound in these tissues was too low for further purification. Morphine and codeine have been identified in bovine hypothalamus and adrenal gland, as well as rat brain, and the presence of 6-acetylmorphine has been demonstrated in the bovine brain (205). This latter compound is a metabolite of heroin that had not previously been identified in plants or animals. The potential biological importance of 6-acetylmorphine is that it readily enters the central nervous system, where it is then converted to morphine. Thus, it has been speculated that this compound may be a peripherally synthesized hormone that targets the central nervous system. The biological activity of endogenous opiate alkaloids has not been determined, and it is not known how they may interact with endogenous opioid peptides. Although these compounds have been shown to be synthesized *in vivo*, their biosynthetic mechanism(s) and potential physiological significance have yet to be elucidated.

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