REVIEW

Evidence for the Existence of the β -Endorphin-Sensitive “ ϵ -Opioid Receptor” in the Brain: The Mechanisms of ϵ -Mediated Antinociception

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ABSTRACT—Recently, μ -, δ - and κ -opioid receptors have been cloned and relatively well-characterized. In addition to three major opioid receptor types, more extensive studies have suggested the possible existence of other opioid receptor types that can be classified as non- μ , non- δ and non- κ . Based upon anatomical and binding studies in the brain, the sensitive site for an endogenous opioid peptide, β -endorphin, has been postulated to account for the unique characteristics of the opioid receptor defined as a putative ϵ -opioid receptor. Many ϵ -opioid receptors are functionally coupled to G-proteins. The functional ϵ -opioid receptors in the brain are stimulated by bremazocine and etorphine as well as β -endorphin, but not by selective μ -, δ - or κ -opioid receptor agonists. ϵ -Opioid receptor agonists injected into the brain produce profound antinociception. The brain sites most sensitive to ϵ -agonist-induced antinociception are located in the caudal medial medulla such as the nucleus raphe obscurus, nucleus raphe pallidus and the adjacent midline reticular formation. The stimulation of ϵ -opioid receptors in the brain facilitates the descending enkephalinergic pathway, which probably originates from the brainstem terminating at the spinal cord. The endogenous opioid Met-enkephalin, released in the spinal cord by activation of supraspinal ϵ -opioid receptors, stimulates spinal δ_2 -opioid receptors for the production of antinociception. It is noteworthy that the ϵ -opioid receptor-mediated pain control system is different from that of other opioid systems. Although there appears to be no ϵ -selective ligand currently available, these findings provide strong evidence for the existence of the putative ϵ -opioid receptor and its unique function in the brain.

Keywords: ϵ -Opioid receptor, β -Endorphin, Descending enkephalinergic pathway, Antinociception

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Abbreviations used are (in alphabetical order): AS, antisense oligodeoxynucleotide; BNTX, 7-benzylidene naltrexone; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Phe-Thr-NH₂; CWS, cold-water swimming; DAMGO, [D-Ala², MePhe⁴, Gly⁵-ol]enkephalin; 5,7-DHT, 5,7-dihydroxydopamine; DPDPE, [D-Pen^{2,3}]enkephalin; DSP-4, N-(2-chloroethyl)-N-2-bromobenzylamine; EKC, ethylketocyclazocine; β -FNA, β -funaltrexamine; GTP γ S, guanylyl-5'-O-(γ -thio)-triphosphate; 5-HT, serotonin; i.c.v., intracerebroventricular; i.t., intrathecal; nor-BNI, nor-binaltorphimine; NTB, naltriben; NTI, naltrindole; POMC, proopiomelanocortin; PTX, pertussis toxin.

I. Introduction

Opioid analgesic drugs and endogenous opioid peptides exert a wide spectrum of physiological and behavioral effects on pain perception, mood, motor control and autonomic function. The myriad opioid-induced effects are mediated via a family of specific membrane-bound receptors located on nervous tissue, namely the opioid receptor. Early studies of the bindings of various ligands have suggested the existence of a multitude of distinct types of receptors that can interact with opioid drugs or endogenous peptides. More evidence for the existence of opioid receptor types has recently been confirmed by molecular cloning, which has already proved valuable in furthering our understanding of the actions of opioid receptors at the molecular level. The three major opioid receptor types have been cloned and sequenced. In late 1992, the δ -opioid receptor was the first such gene to be cloned by two independent groups, Evans et al. (1) and Kieffer et al. (2). Following the cloning of the δ -opioid receptor, μ - and κ -opioid receptors have been cloned in the past 4 years (3–12). A cDNA clone encoding a structurally related receptor with amino acid similarity to these three receptor genes as high as 65% has been reported by several groups (13–15). Although all three major types of opioid receptors are able to mediate analgesia/antinociception, their individual binding profiles and other pharmacological activities clearly distinguish one from another.

In addition to three major opioid receptor types, μ -, δ - and κ -opioid receptors, extensive pharmacology and bioassay studies have also indicated that there is another receptor type that has been classified as non- μ , non- δ and non- κ in nature. The sensitive site for an endogenous opioid peptide, β -endorphin, has been postulated to account for the unique characteristics of the opioid receptor defined as a putative ϵ -opioid receptor (16–21). Receptor binding studies employing [3 H] β -endorphin (22, 23), [3 H]diprenorphine (24, 25) and [3 H]ethylketocyclazocine (EKC) (26, 27) provide biochemical evidence supporting the presence of ϵ -opioid receptors in the brain. Thus, the subject of the present review is to focus on the evidence for the existence of the β -endorphin-sensitive ϵ -opioid receptor and its function in the brain. Especially, this review describes the neural mechanism of ϵ -opioid receptor-mediated antinociception.

II. Endogenous opioid peptides

II-1. Enkephalins and dynorphins

The relatively large number of opioid peptides isolated in recent years is a reflection of the complexity of the endogenous opioid system. The major opioid peptides are

cleavage products of three distinct proteins, which are the primary products of three genes. These precursor proteins are proenkephalin, prodynorphin and proopiomelanocortin (POMC).

Proenkephalin was originally discovered in bovine adrenal cortex and pig brain, where enkephalin biosynthesis was elucidated (28). It contains one copy of Leu-enkephalin, four copies of Met-enkephalin, and one copy each of a Met-enkephalin C-terminal-extended heptapeptide and octapeptides, all flanked by basic di-peptides, where processing generally takes place. Using immunohistochemical techniques, which have proven extremely useful in mapping the distribution of opioid peptides, enkephalins have been widely found in many neuronal systems from the telencephalon to the spinal cord (29). The enkephalins have been shown to form local circuits or to be transported via lengthy neuronal projections to areas distant from the cell bodies in which they were synthesized. The distributions of Leu-enkephalin and Met-enkephalin have so far been found to be identical. These two enkephalins have been defined as endogenous ligands for the δ -opioid receptors, although they also have appreciable affinity for μ -opioid receptors.

Prodynorphin has been isolated from mammalian tissues such as brain, anterior pituitary, adrenal gland, spinal cord and reproductive organs. All active opioid peptides processed from prodynorphin are C-terminal extensions of Leu-enkephalin. They are α - and β -neodynorphin, dynorphin A-[1–17], dynorphin A-[1–8], and dynorphin B-[1–29], also called leumorphin, which are processed at double basic amino acids that serve as processing signals (30). Prodynorphins have their highest concentrations in the hypothalamus and substantia nigra (29). Moderate concentrations of prodynorphin are found in the hippocampus and globus pallidus (29). These dynorphins, especially dynorphin A-[1–17], are proposed to be endogenous ligands for κ -opioid receptors (8).

II-2. β -Endorphin and newly discovered peptides, endomorphins

POMC was the first of three opioid peptide precursors to be sequenced (31). The major source of POMC is the pituitary gland; in its intermediate lobe, β -endorphin, the 31-amino acid C-terminal peptide of β -lipotropin, is the predominantly active opioid peptide product. The pharmacological properties of β -endorphin are more complex than other endogenous peptides (32, 33). Since β -endorphin binds to the μ - and δ -opioid receptors with comparable affinity (22, 34, 35), the argument that β -endorphin acts as the endogenous ligand for the μ - and δ -opioid receptors may hold in some brain areas for producing some pharmacological effects. However, the

contention that β -endorphin-induced responses are produced solely by stimulation of μ - and/or δ -opioid receptors in all of the brain and spinal regions has been questioned. It should be noted that a poor correlation exists between the anatomical distribution of β -endorphin-containing nerve fibers and terminals and the distribution of μ - or δ -opioid binding sites (see Table 1).

High concentrations of β -endorphin are found in the arcuate nucleus of the medial basal hypothalamus and the nucleus of the solitary tract, which lies within the medulla oblongata (36). In contrast, relatively few μ -opioid receptors are seen in the hypothalamus (29). The μ -opioid sites are most dense in the frontal cortex, nucleus accumbens, caudate putamen and hippocampus (29), whereas little or no β -endorphin-containing neurons can be observed in these areas (36). Immunoreactive β -endorphin fibers appear very dense in the periaqueductal gray (36), an area that plays an important role in the induction of opioid antinociception, while relatively lower levels of μ -opioid receptor binding and its mRNA expression are seen in this area (29, 37). Although the cloned μ -opioid receptor-like immunoreactivity is shown with high concentrations in the primary afferent fibers within the superficial layers (laminae I and II) of the dorsal horn of the rat (38), very low concentrations of immunoreactive β -endorphin are detected in this area (39). In the case of δ -opioid receptors, distribution is more restricted, and they appear the densest in the cortex, olfactory-related neural areas, neocortex, caudate-putamen, nucleus accumbens, hippocampus, amygdala and dorsal horn of the spinal cord (29). Little or no δ -binding can be detected in the thalamus, hypothalamus, periaqueductal gray and brainstem. The findings of different brain distributions between the μ - or δ -opioid receptors and the β -endorphin-containing nerve

fibers and terminals argue strongly against the contention that β -endorphin is the endogenous ligand for μ - and δ -opioid receptors.

In 1997, Zadina and colleagues (40) reported the isolation of two new μ -selective peptide ligands from the mammalian brain. These peptides, which are called endomorphin-1 (Tyr-Pro-Trp-Phe-NH₂) and -2 (Tyr-Pro-Phe-Phe-NH₂), differ structurally from previously described endogenous opioid peptides (such as endorphins, enkephalins and dynorphins, which all have the amino-terminal amino-acid sequence Tyr-Gly-Gly-Phe) in their N-terminal (Tyr-Pro) sequence, C-terminal amidation and tetrapeptide length. Based on the receptor binding assay, endomorphins have the highest specificity and affinity for the μ -opioid receptor of any endogenous substance so far described in the mammalian nervous system. More interestingly, the studies of the distribution of endomorphin-1 by radioimmunoassay indicate that endomorphin-1-like immunoreactivity is shown with high concentrations in the thalamus, hypothalamus, cortex and striatum. Thus, endomorphin-1 like immunoreactivity is present in several brain areas also known to contain high concentrations of μ -opioid receptors and their mRNA expression (29, 37). Although the cloning of the gene for the precursor pro-endomorphin should be studied, it is most likely that endomorphins are natural selective ligands for the μ -opioid receptor (33, 40). Table 2 classifies the new hypothesized endogenous opioid precursors and selected major opioid peptide products.

III. β -Endorphin-specific ϵ -binding site in the brain

III-1. Designation of the ϵ -site in the brain

Studies in peripheral bioassays and brain tissue in vivo

Table 1. Different distribution of μ/δ -opioid receptors and β -endorphin in some rat brain and spinal cord regions

Brain region	μ -Receptors		δ -Receptors		β -Endorphin
	Binding	mRNA expression	Binding	mRNA expression	(POMC or/and β -endorphin containing neurons)
Brain					
Cortex	+++	++	++	+++	—
Hippocampus	++++	++	++	++	—
Olfactory tubercle	+	+	+++	++++	—
Nucleus accumbens	++++	+++	++++	+++	+
Caudate-putamen	++++	+++	++++	++++	—
Hypothalamus	—	++	—	++	++++
Periaqueductal gray	++	++	—	++	++++
Spinal cord					
Lamina I and II (Dorsal horn)	++++	—	++++	—	—

++++ = very dense; +++ = dense; ++ = moderate; + = low; — = lower or undetectable. Derived from Refs. 29, 36–39.

Table 2. Endogenous opioid precursors and selected major opioid peptide products

Precursor	Selected major peptide (preferred opioid receptors)	Amino acid sequence
Proenkephalin	Leu-enkephalin (δ , μ)	Tyr-Gly-Gly-Phe-Leu
	Met-enkephalin (δ , μ)	Tyr-Gly-Gly-Phe-Met
Prodynorphin	Dynorphin A-[1–17] (κ)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln
Proopiomelanocortin	β -Endorphin* (μ , δ , ϵ)	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu
Proendomorphin (?)**	Endomorphin-1 (μ)	Tyr-Pro-Trp-Phe
	Endomorphin-2 (μ)	Tyr-Pro-Phe-Phe

*Human β -endorphin. **Not determined yet, hypothetical name.

have provided much information for determining the subtypes of opioid receptors. Differences in the relative potencies of enkephalins in guinea pig ileum and mouse vas deferens bioassays lead to the distinction between μ - and δ -opioid receptors (41). Similarly, selective receptors for β -endorphin, designated the ϵ -opioid receptor, have been proposed based upon studies on the rat vas deferens (21, 42, 43). A number of binding studies using [3 H] β -endorphin that support the existence of specific β -endorphin binding sites within the central nervous system (22, 44–46) have been reported. It has been known for some time that 6,7-benzomorphans, such as EKC, label two populations of non- μ -, non- δ -opioid binding sites in the brain; they are κ - and ϵ -sites (47–50). The κ_1 -selective benzeneacetamide U69,593 has been shown to selectively label one of these two 6,7-benzomorphans sites that corresponds to the κ -opioid receptor site, but the other benzomorphans site lacks a selective ligand (51). The nature and designation of the U69,593-insensitive benzomorphans site has been debated, including the suggestion that it might be a κ -opioid receptor subtype because of high affinity interactions with certain κ -opioid ligands. However dynorphin and other prodynorphin derived peptides presumed to be the endogenous ligands for the κ -opioid receptor have a very low affinity for this site, which has a high affinity for β -endorphin (27). Compounds such as enkephalins and other synthetic peptides, [D-Ala²,MePhe⁴,Gly⁵-ol]enkephalin (DAMGO) and [D-Pen^{2,5}]enkephalin (DPDPE) that are selective for μ - and δ -opioid receptors; the κ_1 -selective ligand U50,488; and the σ -receptor ligand (+)SKF-10,047 have low affinities for this β -endorphin-sensitive site. In addition, non-receptor selective opioid drugs, such as etorphine and other benzomorphans, have high affinities for this site (26). Nock and colleagues (26, 27, 52) have thus proposed the designation “ ϵ ” for this site in the brain as per the nomenclature employed for 1) a non- μ , non- δ [3 H] β -endorphin binding site in the brain (23, 53, 54), 2) a β -endorphin-sensitive, benzomorphans binding site in the

brain (24, 25) and 3) a non- μ , non- δ , non- κ receptor, which has been hypothesized to mediate a variety of peripheral and central physiological effects of benzomorphans and β -endorphin (21, 42, 55–70). The ϵ -binding site has been identified in guinea pig, rat, chicken, pig and cow brains. Its pharmacological profile appears to be identical across those species (27). Table 3 lists the affinity of several opioid receptor agonists for the ϵ -site.

III-2. Search for the ϵ -opioid receptor gene family

Following the cloning of μ -, δ - and κ -opioid receptors, some groups have been conducting a search for additional members of the opioid receptor gene family. More recently, O'Dowd and colleagues (71) have successfully cloned and characterized two novel and related human gene (named GPR7 and GPR8) encoding receptors with structural features common to both opioid and somatostatin receptors. The genomic clone of GPR7 and GPR8 contains an intronless open reading frame of 984 and 999 nucleotides, encoding 328- and 333-amino-acid proteins, respectively; and the intronless coding sequences of these genes shares a 70% identity with each other. The available pharmacologic study for the receptor encoded by GPR7 indicates high-affinity binding to several opioid drugs such as a benzomorphans ligand, bremazocine, and β -endorphin, but low-affinity binding to μ - (DAMGO), δ - (DPDPE) and κ - (U50,488) opioid receptor agonists. These findings strongly suggest the existence of the β -endorphin-specific ϵ -site as a putative opioid receptor. Subsequently, more studies are needed to conclusively verify that the receptor encoded by GPR7 is indeed the ϵ -opioid receptor.

IV. ϵ -Opioid receptor-mediated supraspinal antinociception

IV-1. Different mechanisms mediating antinociception induced by β -endorphin and morphine

The pharmacological identification of types of opioid

Table 3. Affinity of opioid compounds for the putative ϵ -receptor in rat brain

Opioid drug	Preferred affinity for three major opioid receptor types* ($K_i \leq 15$ nM)	Affinity for ϵ -site** (Very Low; $K_i \geq 100$ nM Low; $K_i = 30-100$ nM Moderate; $K_i = 10-30$ nM High; $K_i \leq 10$ nM)
μ -preferred compounds		
DAMGO	selective- μ	Very Low
CTOP	selective- μ	Very Low***
β -Endorphin	μ and δ	High
δ -preferred compounds		
DPDPE	selective- δ	Very Low
Met-enkephalin	δ and μ	Low
Leu-enkephalin	δ and μ	Low
Naltrindole (NTI)	selective- δ	Very Low
κ -preferred compounds		
U50,488H	selective- κ	Very Low
U69,593	selective- κ	Very Low
Dynorphin A	κ	Low
Nor-binaltorphimine (nor-BNI)	κ and μ	Very Low
Ethylketocyclazocine (EKC)	κ and μ	High
Non-selective compounds		
Bremazocine	μ , δ and κ	High
Etorphine	μ , δ and κ	High
β -Endorphin-[1-27]	not determined	Moderate

*Derived from Ref. 35. **Derived from Ref. 26. ***Derived from Ref. 71.

receptors progressed rapidly due, in part, to the increasing availability of highly selective opioid agonists and antagonists. Such compounds have been critical in allowing investigators to be reasonably confident that particular opioid receptor types are involved in opioid-induced effects such as antinociception. β -Endorphin has been reported in both in vitro and in vivo studies to be a non-selective opioid receptor agonist (22, 23, 26, 72, 73). It is expected that β -endorphin may stimulate nonselectively any of these opioid receptors to produce a variety of opioid pharmacological responses. However, using these selective μ -, δ - and κ -opioid receptor antagonists for antinociceptive studies, we have clearly demonstrated that β -endorphin when given supraspinally produces its antinociception by specifically stimulating ϵ -opioid receptors, but not μ -, δ - and κ -opioid receptors (67, 69, 74-76).

β -Endorphin exhibits profound antinociceptive activity when injected into the brain in different species of animals and humans (77-81). Structure-activity studies carried out with fragments and synthetic analogs of β -endorphin have shown that the presence of enkephalin in its N-terminal Met-enkephalin segment of the molecule plus a complete polypeptide chain is essential for the production of full antinociceptive activity (82). Successive removal of an amino acid from its C-terminus one at a

time reduces the antinociceptive potency in a stepwise manner (82). However, these shorter peptides still retain receptor binding potencies (83, 84). A β -endorphin analog, β -endorphin-[1-27], when given directly into the brain at lower doses has been shown to inhibit specifically the antinociceptive effect induced by β -endorphin, given supraspinally (84), indicating that β -endorphin-[1-27] is useful as a selective ϵ -opioid receptor antagonist in the brain.

The antinociception induced by β -endorphin given intracerebroventricularly (i.c.v.) in mice has been found to be blocked by i.c.v. administered β -endorphin-[1-27], but not by μ -opioid receptor antagonists, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Phe-Thr-NH₂ (CTOP) and β -funaltrexamine (β -FNA), a δ -opioid receptor blocker, ICI 174,864, or a κ -opioid receptor blocker, nor-binaltorphimine (nor-BNI) (67, 74, 75). Unlike β -endorphin that produces antinociception mainly by the stimulation of " ϵ "-opioid receptors in the brain, the antinociception induced by morphine given i.c.v. in mice is found to be blocked by i.c.v. administration of both CTOP or β -FNA but not by β -endorphin-[1-27], ICI 174,864 or nor-BNI, indicating that the antinociception induced by morphine is mediated mainly by the stimulation of μ -, but not δ -, κ - or ϵ -opioid receptors. The results provide evidence for different

mechanisms of antinociceptive actions for β -endorphin and morphine in the brain.

IV-2. Mapping of the brain sites sensitive to ϵ -mediated antinociception

Using the microinjection technique, mapping studies of

the brain sites sensitive to opioids have demonstrated that the measures associated with the antinociceptive actions of intracerebrally administered opioids vary in different brain regions. The pharmacology of the effect of opioids given into these separate regions has indicated that the individual effects are mediated by a local action on recep-

Fig. 1. Brainstem sites sensitive to microinjection of β -endorphin ($2 \mu\text{g}$) for inhibition of the tail-flick response in pentobarbital-anesthetized rats. DPGi, dorsal paragigantocellular nucleus; Gi, gigantocellular reticular nucleus; GiA, gigantocellular nucleus-alpha; IO, inferior olive; LC, locus coeruleus; LPGi, lateral paragigantocellular nucleus; LRT, lateral reticular nucleus; PGi, paragigantocellular nucleus; Py, pyramidal tract; RMg, raphe magnus nucleus; ROb, raphe obscurus nucleus; Sol, nucleus solitary tract; Sp5, spinal trigeminal tract; Sp5l, spinal trigeminal nucleus, oral; Sp5o, spinal trigeminal nucleus, interpolar.

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tors in those brain regions having opioid characteristics. We have clearly demonstrated that the rat brain sites sensitive to β -endorphin and morphine for antinociception are different, although there is some overlap.

Evaluation of anatomical substrates mediating supraspinally-administered opioid-induced antinociception has first centered upon brainstem structures. In this area, the sites most sensitive to β -endorphin for the inhibition of the tail-flick response are located in areas in the caudal medial medulla such as the raphe obscurus and raphe pallidus and the adjacent midline reticular formation

(see Fig. 1, Refs. 68 and 69). Sites in the rostral medial medulla are less sensitive to β -endorphin for antinociception. The β -endorphin-sensitive sites in the caudal midline medulla appear to be restricted to within 1 mm from the midline; the regions 1 mm or more lateral to the midline are not sensitive to β -endorphin. In contrast, the sites sensitive to morphine for antinociception are located in areas of the rostral ventromedial medulla such as the raphe magnus nucleus, gigantocellular nucleus and gigantocellular reticular nucleus α . The raphe obscurus, which is sensitive to β -endorphin, is not sensitive to morphine

Fig. 2. Brainstem sites sensitive to microinjection of morphine (4 μ g) for inhibition of the tail-flick response in pentobarbital-anesthetized rats. For abbreviations, see figure 1 legend. (Reprinted with permission from Ref. 69)

for antinociception (see Fig. 2, Ref. 69). The locus coeruleus is sensitive to β -endorphin and morphine for antinociception.

In the midbrain areas, microinjection of either β -endorphin or morphine into the central gray produces tail-flick inhibition (85). In the forebrain regions, the sites most sensitive to β -endorphin for the inhibition of the tail-flick response are located in the ventromedial regions of the forebrain such as medial posterior nucleus accumbens, medial preoptic area and arcuate hypothalamic nucleus. These antinociceptive effects are blocked by the administration of β -endorphin-[1–27], but not the μ -opioid receptor antagonist CTOP (86). Other areas such as the anterior nucleus accumbens, dorsomedial hypothalamic nucleus, posterior hypothalamus, lateral hypothalamus, caudate nuclei, thalami and cerebral cortex are not sensitive to β -endorphin (86). The sites sensitive to morphine for the tail-flick inhibition are located in regions of the medial preoptic nucleus and arcuate hypothalamic nucleus. These effects are blocked by CTOP, but not β -endorphin-[1–27]. The posterior nucleus accumbens, which is sensitive to β -endorphin, is not sensitive to morphine for antinociception (86). The differential profiles of sites sensitive to β -endorphin and morphine for antinociception give strong evidence to support the hypothesis that antinociception mediated by activation of μ - and ϵ -opioid receptors are mediated by different neural mechanisms.

IV-3. Other ϵ -agonists

Other ϵ -opioid receptor agonists that produce profound supraspinal antinociception have also been reported. Although benzomorphan analogs have historically been classified as κ -opioid receptor agonists, many benzomorphans have high affinities for the ϵ -site (see Table 3, Refs. 26 and 27). Bremazocine is a 6,7-benzomorphan that functions as a potent analgesic when peripherally and centrally administered (87–89). We have demonstrated that the tail-flick response induced by bremazocine given i.c.v. is blocked by the i.c.v. co-administration of β -endorphin-[1–27] and nor-BNI (90). These findings indicate that in addition to the κ -opioid receptor activity, bremazocine possesses ϵ -opioid receptor activity to produce supraspinal antinociception.

Etorphine, an oripavine derivative, produces potent pharmacological effects by interacting with a variety of opioid receptors, including μ -, δ - and κ -receptors (91, 92). In receptor assays, etorphine has been shown to bind with similar affinity to μ -, δ - and κ -sites (47, 93, 94). Nock et al. (26) have also described that etorphine, like β -endorphin and benzomorphans, has a relatively high affinity for the [3 H]EKC sites that are proposed to be β -endorphin-specific ϵ -opioid receptors. In our laboratory, an anti-

nociceptive response induced by etorphine given i.c.v. is shown to be blocked by the i.c.v. administration of selective μ -opioid receptor antagonists, CTOP and β -FNA, but not by the δ -opioid receptor antagonist ICI 174,864 and the κ -opioid receptor antagonist nor-BNI (95). The antinociception induced by i.c.v. etorphine is also antagonized by the i.c.v. pretreatment of β -endorphin-[1–27] in both non-treated- and β -FNA-treated mice (95). These results indicate that the antinociception induced by etorphine given i.c.v. is mediated by the stimulation of both μ - and ϵ -opioid receptors.

V. ϵ -Opioid receptor and G-protein

V-1. β -Endorphin-stimulated [35 S]GTP γ S binding

Guanine nucleotide binding protein (G-protein)-coupled receptors comprise the majority of receptor types in the central nervous system since at least 80% of all known intracellular signaling factors produce biological responses by binding to and activating receptors that are G-protein coupled. With the cloning of the three major types of opioid receptors, μ -, δ - and κ -opioid receptors, it is apparent from their deduced structures that opioid receptors belong to the superfamily of seven-transmembrane-domain membrane receptors that transduce their signal via heterotrimeric G-proteins (96, 97).

Agonist-stimulated [35 S]guanylyl-5'-O-(γ -thio)-triphosphate (GTP γ S) binding to G-proteins has been recently developed as a sensitive tool for investigating the interaction of receptors with G-proteins (98, 99). This assay is based upon the fact that the inactive state of the G-protein α ($G\alpha$) subunit has a relatively high affinity for GDP over GTP, while activation of receptors by the agonist shifts the $G\alpha$ subunit into a higher affinity state for GTP versus GDP. This is followed by dissociation of $G\alpha$ -GTP from the $\beta\gamma$ subunits. Both $G\alpha$ -GTP and $\beta\gamma$ have the potential to stimulate downstream events. The activity of the subunits is terminated by the intrinsic GTPase activity of $G\alpha$, which hydrolyzes the terminal phosphate bond of bound GTP to regenerate $G\alpha$ -GDP, which reassociates with $\beta\gamma$ subunits. Addition of the poorly hydrolyzable GTP analog [35 S]GTP γ S leads to the formation of $G\alpha$ -GTP γ S. However, this results in permanent activation of $G\alpha$ because GTP γ S is only slowly susceptible to the intrinsic GTPase activity of $G\alpha$; thus $G\alpha$ -GTP γ S survives as a long-lived functional species. In other words, if the receptor is coupled to G-proteins, the receptor agonist increases the amount of [35 S]GTP γ S bindings. Thus, the opioid agonist-mediated stimulation of the [35 S]GTP γ S binding assay using tissue membranes provides a functional measurement of efficacy and intrinsic activity of the opioid agonist. In our laboratory, the ability of opioid agonists, including β -endorphin, to

activate G-proteins in mouse brainstem and spinal cord membranes has been recently demonstrated by studying [35 S]GTP γ S binding (100–103).

In the absence of GDP, the opioid agonist does not stimulate [35 S]GTP γ S binding to the brain membrane. Since the absolute level of [35 S]GTP γ S binding decreases dramatically with increasing unlabelled GDP, the optimal GDP concentration is chosen to demonstrate both the basal level of bound [35 S]GTP γ S and increased binding stimulated by opioid agonists. In our systems, the optimal GDP concentration is 3–30 μ M. Like GDP, NaCl reduces basal [35 S]GTP γ S binding more than opioid-stimulated binding, resulting in a measurable difference. [35 S]GTP γ S binding stimulated by opioids also requires Mg $^{2+}$ ions. In the presence of 30 μ M GDP, 100 mM NaCl and 5 mM MgCl $_2$, the μ -receptor agonist DAMGO increases the [35 S]GTP γ S binding in a concentration-dependent manner and reaches approximately 100% maximal increase (200% of control) in mouse brainstem membranes, which includes several key sites in the production of opioid agonist-induced antinociception. Assays are routinely performed at 25°C with 30–80 μ g/ml of membrane protein for 120 min of incubation to produce approximately 100% increase (stimulation) of 50 pM [35 S]GTP γ S binding over controls in the presence of a maximal concentration of DAMGO. The increase of [35 S]GTP γ S binding by DAMGO is inhibited by co-incubation with a selective μ -opioid receptor antagonist β -FNA in a concentration-dependent manner. Ten micromolar β -FNA, which alone has no effect on the basal

[35 S]GTP γ S binding level, completely inhibits the effect by 10 μ M DAMGO so that the level of [35 S]GTP γ S binding is comparable to the basal level. Comparable levels of [35 S]GTP γ S binding, stimulated by β -endorphin and selective δ - and κ -opioid receptor agonists, are also examined in mouse brainstem membranes. The maximal increase of [35 S]GTP γ S binding by the δ -receptor agonist DPDPE is approximately 30%. The κ -receptor agonist U50,488H is much less efficacious and produces only 10–15% of the maximal stimulation. Like DAMGO, β -endorphin stimulates [35 S]GTP γ S binding in a concentration-dependent manner. Interestingly, β -endorphin at 10 μ M produces maximal stimulation of approximately 200%; β -endorphin stimulates twice the level of [35 S]GTP γ S binding compared to 10 μ M DAMGO. The orders of efficacies and potencies of the various agonists are β -endorphin $\gg$ DAMGO $\gg$ DPDPE $>$ U50,488H (see Fig. 3). To determine the specificity of this effect of β -endorphin, brainstem membranes are incubated with β -endorphin in the presence and absence of selective μ -, δ - and κ -opioid receptor antagonists, β -FNA, naltrindole (NTI) and nor-BNI, respectively. Concentrations of antagonists are chosen so that $>95\%$ of the appropriate agonist-stimulated [35 S]GTP γ S binding is inhibited by the antagonist. The increase of [35 S]GTP γ S binding stimulated by β -endorphin is only partially attenuated by incubation with β -FNA (see Table 4). The antagonistic effect by β -FNA is saturable (up to 10 μ M). After co-incubation with 10 μ M β -FNA, 100% stimulation of [35 S]GTP γ S binding by 10 μ M β -endorphin still remains. Co-incu-

Fig. 3. Comparison of the stimulation of [35 S]GTP γ S (50 pM) binding to brainstem membranes of mice by DAMGO (μ), DPDPE (δ), U50,488H (κ) and β -endorphin (ϵ and μ/δ). Assays were performed in the presence of 30 μ M GDP, 100 mM NaCl and 5 mM MgCl $_2$ for 120 min at 25°C.

Table 4. Effects of various opioid receptor antagonists on β -endorphin-stimulated [35 S]GTP γ S binding in the mouse brainstem

Co-incubation with opioid antagonists	% Stimulation of 10 μ M β -endorphin-stimulated [35 S]GTP γ S binding
None	202.1 $\pm$ 12.0
3 μ M β -FNA (μ)	108.7 $\pm$ 17.6**
10 μ M β -FNA (μ)	95.62 $\pm$ 16.9**
10 nM NTI (δ)	202.2 $\pm$ 26.8
30 nM NTI (δ)	194.7 $\pm$ 24.8
30 nM nor-BNI (κ)	210.3 $\pm$ 2.4
100 nM nor-BNI (κ)	190.5 $\pm$ 13.0
10 μ M β -FNA + 30 nM NTI + 100 nM nor-BNI	83.3 $\pm$ 13.3**
10 μ M β -FNA + 30 nM NTI + 100 nM nor-BNI + 10 nM β -endorphin-[1–27] (ϵ)	49.7 $\pm$ 0.2** ^{##}

Highest concentrations of antagonists are chosen so that >95% of the selective agonist-stimulated [35 S]GTP γ S binding is inhibited by the antagonist. β -Endorphin-[1–27] at higher concentrations itself stimulates [35 S]GTP γ S binding. ** P < 0.01 vs none, ^{##} P < 0.01 vs 10 μ M β -FNA + 30 nM NTI + 100 nM nor-BNI.

bation with either NTI or nor-BNI has no effect on β -endorphin-stimulated [35 S]GTP γ S binding in both control and β -FNA-treated membranes. On the other hand, co-incubation with an ϵ -receptor antagonist β -endorphin-[1–27] in β -FNA-, NTI- and nor-BNI-treated membranes significantly attenuates further the 10 μ M β -endorphin-stimulated [35 S]GTP γ S binding compared to 10 μ M β -endorphin in β -FNA-, NTI- and nor-BNI-treated membranes (see Table 4). Our recent data clearly indicate that β -endorphin increases [35 S]GTP γ S bindings by stimulation in part by μ -opioid receptors and in part by ϵ -opioid receptors in mouse brainstem membranes (see Table 4). Our results provide additional evidence for the existence of β -endorphin-sensitive ϵ -opioid receptors in the mouse brainstem.

V-2. PTX-resistant mechanisms of the ϵ -mediated antinociception

It has been proposed that the ϵ -binding site in the brain exists in two interchangeable affinity states that are likely to be site coupled to and uncoupled from G-proteins (27). β -Endorphin readily discriminates the two affinity states having about a 100 times higher affinity for the putative G-protein coupled site. Interestingly, we have demonstrated in antinociceptive assays that pretreatment with pertussis toxin (PTX), a protein isolated from the culture medium of *Bordetella pertussis*, differentially modulates morphine- and β -endorphin-induced antinociception in mice (104–106). Pretreatment of mice or rats i.c.v. with PTX has been demonstrated to attenuate antinociception and other effects induced by i.c.v. administered morphine and other μ -opioid receptor agonists (104–112). The possible mechanisms at the molecular level for this effect

could involve ADP-ribosylation of the α -subunit of the Gi/Go proteins catalyzed by the toxin (113). The results of these in vivo studies thus indicate that pharmacological effects induced by μ -opioid receptor agonists are mediated by PTX-sensitive Gi/Go proteins. In our experiments, an i.c.v. pretreatment with PTX has been shown to cause a time- and dose-dependent attenuation of the tail-flick and hot-plate inhibition induced by i.c.v. challenged morphine-induced antinociception (106). The tail-flick and hot-plate inhibition induced by selective μ -, δ - and κ -opioid receptor agonists, DAMGO, [D-Ala²]-deltorphin II and U50,488H, respectively, given i.c.v. is also attenuated by the i.c.v. pretreatment with PTX (106). However, the same pretreatment with PTX has no effect on the antinociception induced by i.c.v. administered β -endorphin (104–106, 114). These findings indicate that, unlike μ -, δ - and κ -opioid receptors that are coupled to PTX-sensitive Gi/Go proteins, ϵ -opioid receptor functions may be PTX-resistant. Since many ϵ -opioid receptors in the brain are coupled to G-proteins, it will be worth investigating whether PTX-insensitive G-proteins, such as Gq and Gx/z, are involved in the ϵ -opioid receptor-mediated antinociception at the supraspinal site.

More recently, we have demonstrated that activation of protein kinase C by phorbol 12,13-dibutyrate leads to the desensitization of μ - but not ϵ -opioid receptor-mediated antinociception (115). Although current information about the mechanism of ϵ -receptor-modulated signal transduction is somewhat limited, these findings provide evidence for differential intracellular modulation on antinociceptive action of ϵ -opioid receptor agonists and three major types of opioid receptor agonists.

VI. Existence of the supraspinal ϵ -opioid receptor-mediated descending enkephalinergic pain control system

VI-1. Activation of noradrenergic and serotonergic systems by morphine

Many studies indicate that opiates produce analgesia/antinociception in part indirectly by the activation of intrinsic pain inhibitory systems, which originate in the brainstem, via a descending pathway in the spinal cord dorsolateral funiculus, which project to and inhibit the spinal and trigeminal pain transmission neurons (116–118). It is widely known that the spinopetal noradrenergic and serotonergic systems appear to be involved in the antinociception induced by i.c.v. administered morphine (119–121). The antinociception induced by i.c.v. administered morphine can be attenuated by the depletion of norepinephrine by an intrathecal (i.t.) or s.c. pretreatment with *N*-(2-chloroethyl)-*N*-2-bromobenzylamine (DSP-4) or 6-hydroxydopamine or by the depletion of serotonin (5-HT) with i.t. or s.c. administered 5,7-dihydroxytryptamine (5,7-DHT) (121, 122). The inhibition of the tail-flick response induced by morphine given i.c.v. is blocked by an i.t. injection of yohimbine or methysergide but not by naloxone (74, 76). These findings indicate that the antinociception induced by morphine is mediated by the activation of spinothalamic tract noradrenergic and serotonergic systems and subsequent stimulation of α_2 -adrenoceptors and 5-HT receptors but not opioid receptors in the spinal cord.

VI-2. Specific ϵ -mediated Met-enkephalin release in the spinal cord

Ultrastructural studies have demonstrated that enkephalin-immunoreactive terminals make synaptic contacts on the somata and dendrites of identified spinothalamic tract neurons in laminae I and II (123, 124). A major proportion of the enkephalin-containing terminals in the dorsal horn of the spinal cord probably originates from enkephalin-containing neurons located in the dorsal horn (125–128). However, another potential source (of the enkephalin-containing axon terminals in the dorsal horn) is a group of enkephalin-containing neurons located in the medulla. These enkephalin neurons are located in the midline raphe nuclei of the brainstem such as the raphe obscurus and raphe pallidus, nucleus raphe magnus, nucleus gigantocellularis pars α and nucleus paragigantocellularis lateralis (125–136). Using the technique of superfusion of the spinal subarachnoid space with artificial cerebrospinal fluid described by Yaksh and Elde (137), our laboratory has provided substantial evidence indicating that β -endorphin given intraventricularly activates ϵ -opioid receptors and releases endogenous Met-enkephalin from the spinal cord (138, 139). The release of

Met-enkephalin from the spinal cord by β -endorphin applied supraspinally has been demonstrated in mice, rats, guinea pigs and cats (140). High-performance liquid chromatography after Sephadex G-50 gel chromatography analysis indicates that the Met-enkephalin released by β -endorphin has a retention time identical to that of authentic Met-enkephalin. The δ -opioid receptor agonist [D-Ala²-D-Leu⁵]enkephalin (DADLE), the κ -opioid receptor agonist U50,488H or the μ -opioid receptor agonist morphine injected intraventricularly even at high doses fails to cause any release of Met-enkephalin from the spinal cord (141), indicating that the release of Met-enkephalin from the spinal cord appears to be specific to β -endorphin applied supraspinally.

It is well-known that the N-terminus of β -endorphin has the same amino acid sequence as Met-enkephalin. Therefore, we have performed further studies to exclude the possibility that the increase of Met-enkephalin immunoreactivity in the spinal perfusate after supraspinal application of β -endorphin in vivo may result from the diffusion of β -endorphin from the injected sites into the spinal perfusates and the degradation of β -endorphin in the spinal perfusates. β -Endorphin microinjected into the brainstem regions does not show any trace of immunoreactive β -endorphin diffused into the spinal perfusates, but does show the increased release of Met-enkephalin in the spinal perfusates (68, 69, 86). β -Endorphin analogs, β -endorphin-[1–30], β -endorphin-[1–29] and β -endorphin-[1–28], which have Met-enkephalin in their N-terminus but have a stepwise reduction in antinociceptive potencies, have a stepwise reduction in potency to elicit the release of Met-enkephalin from the spinal cord after intraventricular injection (141). Des-[Met⁵]-camel β -endorphin, which does not have a complete sequence of Met-enkephalin in its N-terminus but still retains 20% of β -endorphin antinociceptive potency (142) and therefore could not generate Met-enkephalin as a product of metabolism, causes the spinal release of Met-enkephalin (141). The evidence described above indicates strongly that the increase of Met-enkephalin in the spinal cord is the result of the neuronal release of Met-enkephalin but is not due to the degradation of β -endorphin itself.

The release of Met-enkephalin by intraventricular injection of β -endorphin is blocked by intraventricularly administered β -endorphin-[1–27], which itself does not cause any release of Met-enkephalin (143), but not by systemic injection of naloxone (144). These findings indicate that the Met-enkephalin released by β -endorphin is mediated by the stimulation of ϵ -opioid receptors but not other opioid receptors. Only Met-enkephalin and to a much lesser extent, Leu-enkephalin, is released by β -endorphin. Other neuropeptides such as dynorphin

A-[1-17], substance P or neurotensin are not released by β -endorphin applied supraspinally (138).

The brain sites sensitive to β -endorphin for the release of Met-enkephalin from the spinal cord have also been determined using the microinjection technique in anesthetized rats. The brainstem sites sensitive to β -endorphin for the release of immunoreactive Met-enkephalin are located in areas in the midline nuclei of the medulla such as the raphe magnus nucleus, raphe obscurus nucleus, raphe pallidus nucleus and adjacent midline reticular formation (see Fig. 4, Ref. 69). The regions 1 mm and more lateral to the midline are not sensitive to β -endorphin. Diffusion studies of microinjection of

^{125}I - β -endorphin shows that more than 90% of recovered radiolabeled β -endorphin is found within a radius of 0.4 mm around the injected site observed at 15 or 30 min after injection (68), ruling out the possibility that β -endorphin produces the effect at other sites by diffusion. The raphe obscurus nucleus is the brainstem site most sensitive to β -endorphin for Met-enkephalin release (Fig. 4) and also the site most sensitive to β -endorphin for inhibition of the tail-flick response (see the above description and Fig. 1, Ref. 68). Thus the correlation of the sites sensitive to β -endorphin for the production of antinociception and the release of Met-enkephalin suggests the hypothesis that activation of ϵ -opioid receptors by

Fig. 4. Brainstem sites sensitive to microinjection of β -endorphin ($2\ \mu\text{g}$) on the release of Met-enkephalin from the spinal cord in urethane-anesthetized rats. For abbreviations, see figure 1 legend.

(Reprinted with permission from Ref. 69)

β -endorphin in the brainstem regions produces antinociception by increasing the release of endogenous Met-enkephalin in the spinal cord.

It is interesting to note that the brainstem regions in which low doses of β -endorphin elicit an antinociceptive response and release endogenous Met-enkephalin in the spinal cord correspond to the anatomic regions in which endogenous endorphineric and enkephalineric systems are distributed. The midline raphe nuclei of the brainstem contain high concentrations of β -endorphin and receive dense β -endorphin nerve fiber innervation. Neurons in the raphe obscurus nucleus, raphe pallidus nucleus and other midline raphe nuclei contain Met-enkephalin and fibers of Met-enkephalin neurons project to the spinal cord and trigeminal nuclei (131, 132, 145–147). These anatomical relationships provide neuronal substrates for the pharmacological actions of β -endorphin. However, whether Met-enkephalin is released from Met-enkephalin neurons in the raphe-spinal and raphe-trigeminal projections or from the interneurons in the spinal cord or trigeminal nuclei has not yet been clearly determined.

Unlike β -endorphin given supraspinally, no sites sensitive to morphine are found for Met-enkephalin release (69, 138). Additionally, the raphe obscurus nucleus is not sensitive to morphine for antinociception (68, 69). The presence of different sites in the brainstem sensitive to β -endorphin and morphine for antinociception and the lack of effect of morphine to induce the release of Met-enkephalin suggest different neuronal pathways of ϵ - and μ -systems for producing antinociception.

VII. Modulation of spinal δ_2 -opioid receptors by activation of supraspinal ϵ -opioid receptors

VII-1. Involvement of spinal δ_2 -opioid receptors in supraspinally ϵ -opioid receptor-mediated antinociception

Accumulating evidence indicates that the antinociception induced by β -endorphin applied supraspinally is mediated mainly by the release of endogenous Met-enkephalin in the spinal cord. This contention is based on our early findings that β -endorphin injected supraspinally releases Met-enkephalin in the spinal cord (see description in the previous section), and the blockade of opioid receptors in the spinal cord by a high dose of naloxone given i.t. antagonizes the antinociception induced by β -endorphin given supraspinally in both mice and rats (68, 74, 85, 148). Recently our studies using an antibody directed against Met-enkephalin give additional support of this hypothesis. An i.t. pretreatment with antibody to Met-enkephalin, which selectively binds the released endogenous Met-enkephalin, dose-dependently attenuates the tail-flick inhibition induced by i.c.v. administered β -endorphin. Antibody to dynorphin, β -endorphin or Leu-

enkephalin given i.t. does not affect β -endorphin-induced antinociception (149). Additionally, the same i.t. pretreatment with antibody to Met-enkephalin dose-dependently attenuates the antinociception induced by i.c.v. administered bremazocine, but not by morphine and U50,488H (149). Moreover, the tail-flick inhibition induced by i.c.v. administered β -endorphin is not affected by the depletion of norepinephrine by the i.t. pretreatment with DSP-4 or 6-hydroxydopamine or by depletion of 5-HT with i.t. administered 5,7-DHT (121). The findings indicate that activation of ϵ -opioid receptors in the supraspinal site selectively releases endogenous Met-enkephalin, but not dynorphins, Leu-enkephalin, β -endorphin, 5-HT and norepinephrine, in the spinal cord for the production of antinociception.

Met-enkephalin has been suggested to be the neurotransmitter for δ -opioid receptors (see the above description). Peptidase inhibitors, which prevent degradation of Met-enkephalin, enhances i.t. Met-enkephalin-induced antinociception in mice (149, 150), and its effect is selectively blocked by i.t. pretreatment with a selective δ_2 -opioid receptor antagonist, naltriben (NTB) (150). In the spinal cord, the cloned δ_2 -opioid receptor-like immunoreactivity is present in fibers in the spinal gray, with highest densities in the superficial dorsal horn, autonomic spinal regions, around the central canal as well as in the ventral horn motor nuclei (151). It is well-characterized that naloxone is a non-selective opioid receptor blocker. The essential question to be addressed, therefore, is whether specific δ -opioid receptor antagonists, instead of naloxone, injected i.t. block the antinociception induced by the ϵ -opioid receptor agonist applied supraspinally. An i.t. pretreatment with δ -opioid receptor antagonists, ICI 174,864 and NTI, in the mouse antagonizes the i.c.v. administered β -endorphin-, but not morphine-, induced inhibition of the tail-flick response, whereas selective μ -opioid receptor antagonists, β -FNA and CTOP, and a selective κ -opioid receptor antagonist, nor-BNI, have no effect on the antinociception induced by β -endorphin (95, 152). Furthermore, the antinociception induced by other ϵ -opioid receptor agonists, bremazocine and etorphine, given i.c.v. is also attenuated by i.t. administered ICI 174,864 and NTI in mice (90, 95). Furthermore, δ_2 -, but not δ_1 -opioid receptors, in the spinal cord are involved in supraspinal ϵ -mediated antinociception because NTB and NTI, but not the selective δ_1 -opioid receptor antagonist 7-benzylidene naltrexone (BNTX), given i.t. dose-dependently attenuates i.c.v. β -endorphin-induced inhibition of the tail-flick response (153).

High affinity δ_2 -selective binding sites in the mouse spinal cord have been demonstrated to be functionally coupled to G-proteins. Co-incubation of GTP γ S with the

mouse spinal cord membrane leads to a decrease in the specific bindings of [^3H][D-Ser²,Leu⁵]enkephalin-Thr⁶ (DSLET), a selective δ_2 -opioid receptor ligand (154). The high affinity GTPase activity and [^{35}S]GTP γ S binding in the mouse spinal cord are increased in a concentration-dependent manner by a selective δ_2 -opioid receptor agonist, [D-Ala²]deltorphin II, indicating that spinal δ_2 -opioid receptors are coupled to G-proteins (102, 155). In antinociceptive assays, i.t. pretreatment with PTX for 96 hr dose-dependently attenuates the antinociception induced by i.t. administered [D-Ala²]deltorphin II (105). It is noteworthy that i.t., but not i.c.v., pretreatment with PTX blocks markedly the tail-flick inhibition induced by β -endorphin given i.c.v. in mice (105). Taken together, it is most likely that antinociception induced by β -endorphin given supraspinally is mediated by the PTX-resistant ϵ -opioid receptors at the supraspinal sites and subsequent activation of the δ_2 -opioid receptors in the mouse spinal cord that is linked to PTX-sensitive Gi/Go-proteins.

An antisense oligodeoxynucleotide is a short piece of synthetic DNA with a nucleotide sequence that is the reverse of and complementary to a part of an mRNA. It therefore binds (hybridizes) to the mRNA, under suitable conditions, according to the rules of the mRNA, and the encoded protein cannot be synthesized (156). Antisense oligodeoxynucleotides have proven to be useful pharmacological tools for studying neurotransmitter receptor activities both in vitro and in vivo (157). In our laboratory, an antisense oligodeoxynucleotide (5'-AGG GCA CCA GCT CCA TGG CG-3') to δ_2 -opioid receptor mRNA (δ_2 -AS) has been recently utilized to block expression of mouse δ_2 -opioid receptor in the spinal cord (158–160). We have demonstrated that repeated i.t. administration of δ_2 -AS selectively attenuates the i.c.v. administered β -endorphin-induced antinociception without any effect on the supraspinal μ - and κ -mediated antinociception (161). The findings confirm our series of pharmacological studies at the molecular level indicating an involvement of spinal δ_2 -opioid receptors in antinociception induced by supraspinally-administered β -endorphin.

VII-2. Enhancement of the spinal δ_2 -opioid receptor turnover by activation of supraspinal ϵ -opioid receptors

Studies with δ_2 -AS to inhibit the synthesis of δ_2 -opioid receptor protein has revealed that δ_2 -opioid receptors have turnover. The antinociception induced by i.t. treatment with [D-Ala²]deltorphin II, a selective δ_2 -opioid receptor agonist, is not significantly attenuated 1 day after i.t. treatment with δ_2 -AS, but declined progressively after 2 and 3 days of treatment (159). These data indicate that initially there are enough δ_2 -opioid receptors remaining in the cell membrane to maintain its normal antinociception, and then the capacity of δ_2 -opioid receptor

activity is reduced after 2 or 3 days. The time for the decline of [D-Ala²]deltorphin II-induced antinociception to 50% is roughly estimated to be 2.3 days. This value may reflect the $t_{1/2}$ of the hypothesized turnover for δ_2 -opioid receptor function in the mouse spinal cord.

The attenuation of i.t. administered [D-Ala²]deltorphin II-induced antinociception after i.t. δ_2 -AS treatment is completely prevented by i.t. pretreatment with a δ_2 -opioid receptor antagonist, NTB, but is markedly enhanced by i.t. pretreatment with peptidase inhibitors (162). Furthermore, i.t. co-treatment of antiserum to Met-enkephalin but not antiserum to Leu-enkephalin, β -endorphin or dynorphin A-[1–17], is found to block the attenuation of the [D-Ala²]deltorphin II-induced antinociception in the presence of δ_2 -AS. These findings provide further evidence that a turnover of δ_2 -opioid receptors in the mouse spinal cord results from, at least in part, constant stimulation of δ_2 -opioid receptors by spontaneously released endogenous Met-enkephalin. This contention is supported by the findings that attenuation of the antinociception induced by i.t. administered [D-Ala²]deltorphin II in the presence of δ_2 -AS is enhanced by i.t. pretreatment for 1 day with [D-Ala²]deltorphin II, but not DAMGO or U50,488H given together with δ_2 -AS.

In as much as β -endorphin given supraspinally releases Met-enkephalin and subsequently stimulates δ_2 -opioid receptors in the spinal cord, β -endorphin given i.c.v. can be expected to facilitate the turnover of the spinal δ_2 -opioid receptors. Under the condition that i.t. pretreatment with δ_2 -AS alone given 24 hr earlier does not have any effect on i.t. administered [D-Ala²]deltorphin II-induced antinociception, a concomitant i.c.v. pretreatment of β -endorphin together with i.t. administered δ_2 -AS for 1 day dose-dependently attenuates i.t. challenged [D-Ala²]deltorphin II-induced antinociception (163). This effect by β -endorphin is blocked by a concomitant i.c.v. pretreatment with β -endorphin-[1–27]. An i.c.v. pretreatment of DAMGO, [D-Ala²]deltorphin II or U50,488H with i.t. δ_2 -AS for 1 day does not affect i.t.-administered [D-Ala²]deltorphin II-induced antinociception (163). These findings indicate that the activation of the ϵ -opioid receptor-mediated descending enkephalinergic pathway by β -endorphin at the supraspinal site enhances the turnover of δ_2 -opioid receptors in the spinal cord.

VIII. Involvement of the ϵ -opioid system in antinociception induced by the cold water swimming

It has been well-documented that animals exposed to acute and intense stress display a significant decrease in their responsiveness to nociceptive stimuli (164). In our laboratory, a brief exposure (3 min) of mice to cold-water swimming (CWS, 4°C) has been used for stress-induced

We have found that the CWS-induced tail-flick inhibition is blocked dose-dependently by an i.c.v. 1-h pretreatment with antiserum against β -endorphin, which selectively binds and inactivates the released β -endorphin. In contrast, i.c.v. pretreatment with antiserum against Met-enkephalin, Leu-enkephalin or dynorphin A-[1-13] does not have any effect on the CWS-induced tail-flick inhibition (165). Using specific receptor antagonists, the

The rationale of the next studies was that if inhibition of the tail-flick response induced by CWS is mediated by the release of Met-enkephalin in the spinal cord, then i.t. injection of antiserum against Met-enkephalin should attenuate inhibition of the tail-flick response induced by CWS. We have found that the CWS-induced antinociception is blocked by i.t. pretreatment with antiserum to Met-enkephalin, but not by antiserum to Leu-enkephalin, β -endorphin or dynorphin A-[1-17] (150). Additionally, i.t. pretreatment with NTB, but not with CTOP, BNTX or nor-BNI attenuates the inhibition of the tail-flick response by CWS (150, 166). Moreover, i.t. pretreatment with δ_2 -AS once a day for 1 to 3 days causes a time-dependent attenuation of the CWS-induced antinociception (166). These results indicate that anti-

Fig. 5. Schematic representation of neuronal connections of ϵ -mediated descending pain inhibitory pathways. The stimulation of ϵ -opioid receptors, which is mainly located in the caudal medial medulla, by ϵ -opioid receptor agonists given supraspinally facilitates the descending enkephalinergic pathway and induces the release of Met-enkephalin in the spinal cord. The released endogenous Met-enkephalin stimulates δ_2 -opioid receptors in the spinal cord for the production of antinociception. (—), inhibitory effect induced by activation of δ_2 -opioid receptors; (+), excitatory effect by the release of nociceptive neurotransmitter; (?), neuronal connections not established.

nociception induced by CWS is mediated by the release of β -endorphin that acts on ϵ -opioid receptors in the brain and by the subsequent release of Met-enkephalin and stimulation of δ_2 -opioid receptors in the spinal cord for the production of antinociception. Thus, the ϵ -opioid system including descending enkephalinergic pathways may play an important role in environmentally-induced antinociception/analgesia.

IX. Conclusion

Over the past 20 years, a considerable amount of progress has been made towards understanding the multiplicity of the opioid receptors at the pharmacological, biochemical and physiological levels. Three major types of opioid receptors have recently been cloned and well-characterized at the molecular levels; however, the accumulating pharmacological evidence also suggests the possible existence of other types of opioid receptors. This review has focused on the presence of the ϵ -opioid receptor in the brain and its physiological function for the production of antinociception.

A series of anatomical, binding and pharmacological studies clearly indicate that the ϵ -opioid receptor can be classified as a putative opioid receptor. Regarding naturally-occurring opioid peptides, β -endorphin has a higher affinity for the ϵ -binding site than proenkephalin- and prodynorphin-derived peptides. Highly selective synthesized- μ -, δ - and κ -opioid receptor agonists have no affinity for this site. In contrast, drugs that have benzomorphan or oripavine structure as well as β -endorphin appear to have affinity to ϵ -opioid receptors.

A major finding on this receptor function for the last 15 years in our laboratory is that ϵ -opioid systems are associated with the descending enkephalinergic pathway (see Fig. 5). Using the superfusion technique of spinal subarachnoid space, we are able to identify that stimulation of ϵ -opioid receptors at the supraspinal site (mainly located in the caudal medial medulla areas) induces the release of Met-enkephalin in the spinal cord. The released Met-enkephalin in the spinal cord by ϵ -opioid receptor agonists stimulates the δ_2 -opioid receptors in the spinal cord. The descending enkephalinergic system is a major part of the ϵ -mediated pain control system including environmentally-induced antinociception. It is noteworthy that the ϵ -descending inhibitory system for the production of antinociception is different from that of μ -systems, which are associated with noradrenergic and serotonergic systems.

It is highly probable that an era of non- μ -, non- δ - and non- κ -opioid research lays before us. It should be noted that there is no selective ϵ -opioid receptor ligand so far. Further investigations to develop selective ϵ -opioid recep-

tor agonists and antagonists will be undoubtedly essential for a much greater understanding of ϵ -opioidergic functions. Furthermore, the development of these ligands may pave the way for the molecular analysis of the specific ϵ -opioid receptor.

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