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Review

Endomorphins: Promising Endogenous Opioid Peptides for the Development of Novel Analgesics

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Key Words
Endomorphins • Distribution •Ultrastructure • Neural circuitry • Coexistence • Antinociception • Modification • Clinical implications

Abstract
Endomorphin-1 (EM1) and endomorphin-2 (EM2) are two endogenous ligands that belong to the opioid peptide family and have the highest affinity and selectivity for the μ-opioid receptor (MOR). The neuroanatomical distribution, ultrastructural features and neural circuitry of EM-containing neuronal structures have been morphologically demonstrated. In addition, the modulation effects of the EMs in different areas reflect their potential endogenous roles in many major physiological processes, including their remarkable roles in the transmission and modulation of noxious information. The distinguished antinociceptive property of the EMs in acute and chronic pain, including neuropathic pain, cancer pain and inflammatory pain, has been revealed and investigated for therapeutic purposes. However, EMs exert adverse effects in the gastrointestinal, urinary, cardiovascular, and respiratory systems, which impede the development of EMs as new analgesics. Numerous studies have synthesized and investigated EM analogues and demonstrated that these EM derivatives had improved pharmacological properties, supporting their therapeutic perspectives. In the present review, the results of previous studies, particularly morphological and pharmacological studies, were summarized. Finally, EM modifications and their potential clinical implications were described. Applying this knowledge about EMs may provide information for further investigations in clinical application.

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Introduction

As the two endogenous ligands with the highest affinity and a remarkable selectivity for the μ-opioid receptor (MOR) of all known mammalian opioids in the opioid peptide family,

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endomorphin-1 (EM1) and endomorphin-2 (EM2) were first discovered and isolated from the bovine brain by Zadina et al. in 1997 [1-4]. EM1 (Tyr-Pro-Trp-Phe-NH2) differs from EM2 (Tyr-Pro-Phe-Phe-NH2) by one amino acid [1]. Since the endomorphins (EMs) were isolated in 1997, they have been shown to exist in the rodent, primate and human central nervous system (CNS) and peripheral nervous system (PNS) [5-7].

Previous studies strongly suggest that EM1 was mainly distributed throughout the brain and the upper brainstem, whereas EM2 was principally detected in the spinal cord and lower brainstem [3, 7-9]. Furthermore, EM2 shows immunoreactivities in the PNS and peripheral tissues, such as primary afferent fibres and the nervous cells from which they originated in the somatic and visceral sensory ganglia [10], gastrointestinal tract [11, 12] and immune system [13, 14]. EMs were prominently present in many, but not all, regions in which MORs have been reported to be concentrated [15, 16].

The neuroanatomical distribution of EMs and MORs provides a basis for the existence of the EMs-related neural circuitry [17-24]. The EMs were also reported to be co-localized with other neurotransmitters in different areas of the living body in animals [10-12, 25-27]. The intricate landscape of the EMs in the nervous system reflects their potential endogenous roles in many major physiological processes; of these processes, the EMs may play a role in the perception of pain and have analgesic effects due to their binding to the MOR, one of the dominant antinociceptive targets among the opioid receptors [28-30].

There are three classic opioid receptors, i.e., mu (μ)-opioid receptor (MOR), delta (δ)-opioid receptor (DOR) and kappa (κ)-opioid receptor (KOR), in the CNS and PNS. Each opioid receptor has natural specific ligands with a high affinity [31, 32]. Opiates act on these receptors and have been widely studied and used in clinical practice due to their potent analgesic effects. However, the traditional opiates induce serious adverse effects; for instance, morphine, which is an exogenous ligand of the MOR, easily induces tolerance, physical dependence and adverse effects in the gastrointestinal, urinary, cardiovascular, respiratory, and motor systems [33-35].

The discovery of the endogenous opioid EMs raises the possibility that EMs might be a good alternative to the exogenous opioid morphine. The results of *in vivo* and *in vitro* studies have revealed that EMs exerted a more potent analgesia in acute and neuropathic pain than other opiates, such as morphine [36-38]. Compared with other opiates, EMs had remarkably fewer side effects [39, 40]. However, EMs are associated with undesirable effects in certain animal experiments, such as tolerance and addiction, which restricted their development as therapeutic analgesics [41-43], and their low membrane permeability and susceptibility to enzymatic degradation also prohibit their direct clinical application [44, 45]. Therefore, many attempts have been made to remodel the EMs to identify possible solutions, indicating their potential value in clinical application [46-52].

In the present review, previous studies exploring the EMs are summarized with a focus on the following aspects: (1) the morphological features of EM-immunoreactive (-IR) structures and EMergic connections that are closely related to the transmission of noxious information and modulation of functions; (2) the involvement of EMs in the transmission and modulation of noxious information; (3) the neural mechanisms underlying the side effects of opioid substances as revealed by investigations of the EMs; and (4) examination and prospects of EMs and their modifications in clinical implications.

Morphological features of EMs

Localization of EMs

EMs in the CNS. EM1 and EM2, which were first discovered and isolated from bovine extracts, are two endogenous opioid peptides that not only exist in the bovine brain but are also distributed throughout the human, rodent and primate CNS and PNS as revealed by immunoassay and immunohistochemistry [1, 5-12].

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Fig. 1 illustrates areas of CNS distribution where both EM1 and EM2 have been identified. EM1- and EM2-containing neuronal cell bodies were identified primarily in both the hypothalamus and nucleus tractus solitarii (NTS) using light and electron microscopy or immunohistochemistry [7, 53, 54]. Both EM1- and EM2-IR terminals are abundant in areas related to the processing of nociceptive information, such as the bed nucleus of the stria terminals (BNST), periaqueductal grey (PAG), locus coeruleus (LC), parabrachial nucleus (PBN) and NTS [7-9, 53, 55].

However, there are also important differences in the neuroanatomical distribution of the two peptides. Compared with EM2, EM1 was more prevalent throughout the brain and the upper brainstem [7, 9]. In addition to those regions in which EM1 was as abundant as EM2, EM1-containing fibres were also rich in the amygdala, nucleus accumbens (Nac), cerebral cortex, diagonal band, thalamus and hypothalamus [3, 7, 9]. Compared to EM1, EM2 was mainly observed in the superficial laminae of the spinal cord and the spinal trigeminal nucleus, which was supported by immunohistochemistry findings and immuno-electron microscopic examinations [2, 4, 7, 55, 56], with a modest density in the substantia nigra, nucleus raphe magnus, ventral tegmental area (VTA), pontine nuclei, Nac and amygdala [7, 9, 53, 55]. EM2 was sparse in the cerebral cortex, whereas EM1 was abundant [7, 55].

In addition, EM-IR fibres were prominently present in many, but not all, regions in which MORs are reported to be concentrated. Notably, there were negligible EM1- and EM2-immunoreactivities in the striatum, which is a region known to express high levels of MORs [3, 7-9, 15, 53]. Thus, although the EMs are specific MOR ligands, they are likely not exclusive ligands of the MOR in the CNS.

Regarding the differences in the distribution of the EMs, two distinct EM precursors or two different processing pathways involving the same precursor have been proposed [57], but further studies are required. In addition, the morphological features of the EMs are related to their functions. Therefore, it is necessary to determine whether the different expression patterns of the EMs reflect their distinct functions, whether EM1 and EM2 function at overlapping sites and whether one peptide can compensate for the down-regulated expression of the other peptide.

EMs in the PNS and peripheral tissues. Since the 1970s, a substantial number of published reports have shed light on the EMs in the PNS and peripheral tissues [6, 10-13, 58]. The avidin-biotin-peroxidase method was performed in the brainstem, spinal cord and sensory ganglia from rats and identified a dense aggregation of EM2-IR fibres in the dorsal

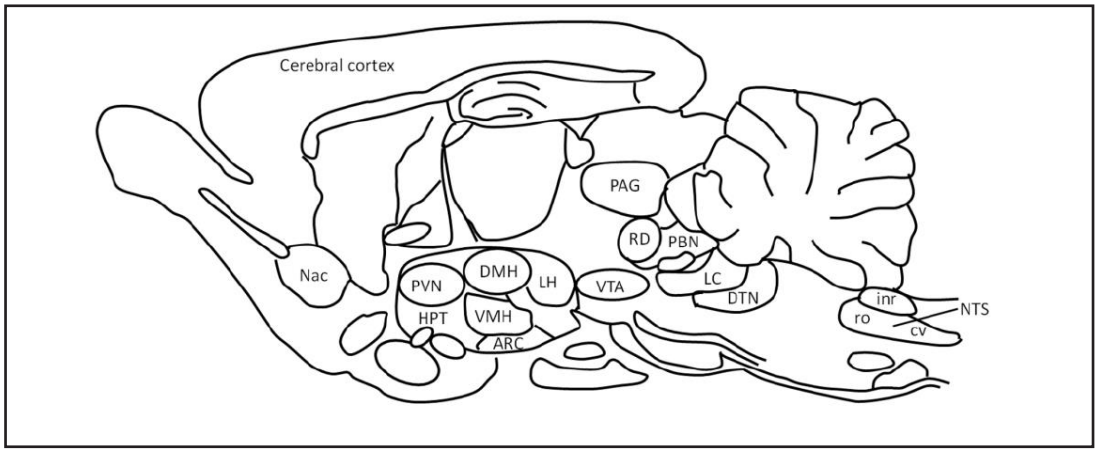

Fig. 1. Major sites in the central nervous system implicated in endomorphin expression. DMH, dorsomedial nucleus; DTN, dorsal nucleus; HPT, hypothalamus; LC, locus coeruleus; LH, lateral nucleus; Nac, nucleus accumbens; NTS, nucleus of the solitary tract (cv, caudal ventrolateral; im, intermediate, ro, rostral); PAG, periaqueductal gray; PBN, parabrachial nucleus; PVN, paraventricular nucleus; RD, caudal dorsomedial part of NTS; VMH, ventromedial nucleus.

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root, and EM2-IR neuronal perikarya were found in the dorsal root ganglion (DRG) and trigeminal ganglion (TG) [8]. Therefore, it was hypothesized that EM2 was synthesized in primary sensory neurons in the ganglia and then transported to the superficial dorsal horn. Subsequently, mechanical (deafferentation by dorsal rhizotomy) and chemical (exposure to the primary afferent neurotoxin capsaicin) methods of disrupting spinal primary sensory afferents were used to demonstrate that EM2-IR fibres in the spinal dorsal horn (SDH) primarily originated from neurons in the DRG [6, 10]. In addition, by transecting the trigeminal nerve sensory root, a significantly decreased expression of EM2 was observed in the ipsilateral medulla dorsal horn (MDH, which is also called the caudal subnucleus of the spinal trigeminal nucleus), indicating that the EM2-IR fibres in the MDH mainly originated from the trigeminal ganglion [23].

Moreover, a body of evidence has demonstrated the presence of EM2-immunoreactivities in the visceral ganglia (e.g., nodose ganglia, NG) and vagal afferents [58, 59]. Myenteric and submucosal plexus neurons in the rat colon were also identified to express a mass aggregation of EM2, but not EM1, and the EM2 in the rat colon was co-localized with a variety of neurochemicals [11, 12].

In addition to the nervous system, EM2 has been identified in the immune system. A report using radioimmunoassay (RIA) methods combined with reversed-phase high-performance liquid chromatography (HPLC) detected EM1- and EM2-immunoreactivities in spleen and thymus tissues from rats, and EM2-immunoreactivities were detected in the spleen in patient with Hodgkin's lymphoma [13]. This is the first report of EMs-immunoreactivities in tissues from the rat and human immune systems. Subsequently, another report demonstrated the presence of EMs in normal human blood lymphocytes [14]. Further studies identified the distribution of EM1 and EM2 in cells of the immune system, demonstrating that EM1- and EM2-IR staining was predominantly present in macrophages and B cells with minimal EM-IR staining in T cells [60].

Compared to the extensive morphological studies of EMs in the CNS, reports on EMs in the PNS and peripheral tissues were scarce, and the distribution of EMs outside the CNS must be further investigated. Since the genes of the two tetrapeptides have not been cloned, examining the distribution and expression levels of EMs proteins relies on immunohistochemistry methods, exerting high demands on the specificity of antibodies. Therefore, controlled experiments that include both a negative and a positive control are necessary.

Ultrastructural localization of EM2 in axon terminals. Using electron microscopy, it was observed that immunoreactive products in the EM2-IR axon terminals in the superficial laminae (lamina I and lamina II) of the spinal dorsal horn (SDH) were specifically located on the large dense-coated granular synaptic vesicles (LDGVs) (Fig. 2) [27, 56]. This result is consistent with the observation that EM2 is a neuropeptide that is located in the LDGV. These immunostained LDGVs were not the only synaptic vesicles in the EM2-IR axon terminals, and small clear vesicles were also observed, suggesting the potential coexistence of EM2 with other neurotransmitters and/or neuromodulators. In addition, a large number of EM2-IR axon terminals were in contact with both EM2-immunopositive and EM2-immunonegative processes in the SDH, which suggests EM2-containing neurons could modulate other EM2-containing neurons as well as those containing other neurotransmitters [56]. Subsequently, the EM2-IR axon terminals were demonstrated to mostly form asymmetrical synapses with dendrites immunostained for the MOR [2]. These results strongly suggest that EM2, which is a MOR agonist, in the SDH regulates the transmission of pain information via presynaptic or postsynaptic mechanisms.

EM-related neural circuits

It has been discussed in this review that the EMs are distributed throughout the CNS in regions in which MORs have been observed, but only the hypothalamus and NTS were detected to have a high density of EM1-containing cell bodies, and EM2 perikarya were mainly

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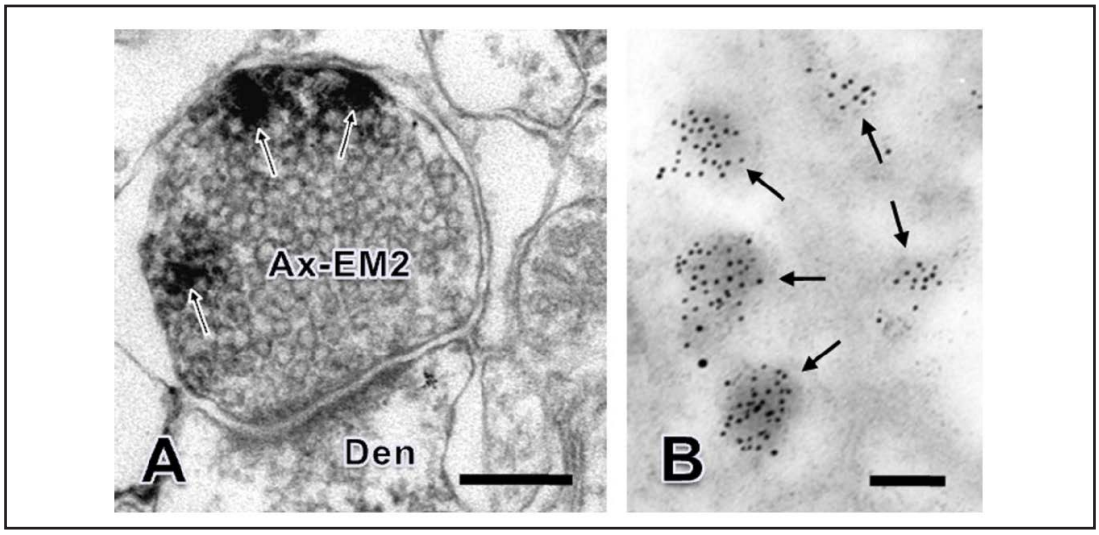

Fig. 2. Ultrastructural localization of EM2 in the axon terminals. A: Arrows indicate EM2-containg large dense-coated granular synaptic vesicles (LDGVs) in an axon terminal (Ax-EM2), visualized with pre-embedding staining method, which makes a synaptic connection with an unlabeled dendritic process (Den). B: Arrows indicate EM2-containg LDGVs in an axon terminal, exhibited with post-embedding staining method. Scale bars are 0.25 μm (A) and 0.15 μm (B), respectively.

found in the hypothalamus, NTS, DRG and trigeminal ganglia. Importantly, the hypothalamus is known as the centre responsible for controlling many homeostatic processes, and the NTS is the main terminal target of primary sensory information regarding visceral and taste inputs in the brainstem [61]. Therefore, it is hypothesized that endomorphinergic (EMergic) pathways exist in which EM1- (Fig. 3) or EM2-containing (Fig. 4) perikarya send axons to adjacent or distant areas that participate in relevant physiological processes. Examinations of EMergic pathways are useful for revealing the mechanisms of EMs as ligands of the MOR in many physiological functions [24].

By injecting the retrograde tracer fluorogold (FG) into the anterior or posterior VTA, some EM1- and EM2-containing neurons in the hypothalamus were revealed to project to the VTA [24]. The VTA is one of the most sensitive brain regions mediating the rewarding and locomotor effects of opioids [63]. Therefore, the EM-containing neurons in the hypothalamus that project to the VTA may modulate the reward and locomotor circuitry.

The hypothalamus has been reported to contain several subsets of neuropeptide-IR neuronal populations that project directly to the PBN [64]. Immunofluorescence histochemistry combined with the fluorescent retrograde tract-tracing method demonstrated that the EM-containing neurons in the hypothalamus and NTS reciprocally send axons to each other and the PBN [17, 20, 62]. In rats injected with FG into the PBN, most EMs/FG double-labelled neurons were distributed in the dorsomedial hypothalamic nucleus, centromedial hypothalamic region, and arcuate nucleus; a few of these neurons were also observed in the periventricular hypothalamic nucleus and posterior hypothalamic nucleus [17]. In 2009, a retrograde tract-tracing study showed that EM1/tetramethyl rhodamine dextran-amine (TMR) (injected into the PBN) and EM2/TMR double-labelled neurons were mainly observed in the medial, commissural, and dorsolateral subnuclei of the NTS [62]. The results of the anterograde tract-tracing study indicated that EM1- and EM2-IR fibres and axonal terminals were mainly found in the lateral PBN [62]. Since PBN has been implicated in a wide range of gustatory, autonomic, and nociceptive functions, it is reasonable to infer that EMs released from hypothalamus-PBN projecting neurons and NTS-PBN projecting neurons may modulate related functions via MOR-expressing PBN neurons [65-67]. The reciprocal projections between the hypothalamus and NTS might be involved in endocrine, nociceptive and autonomic functions [61].

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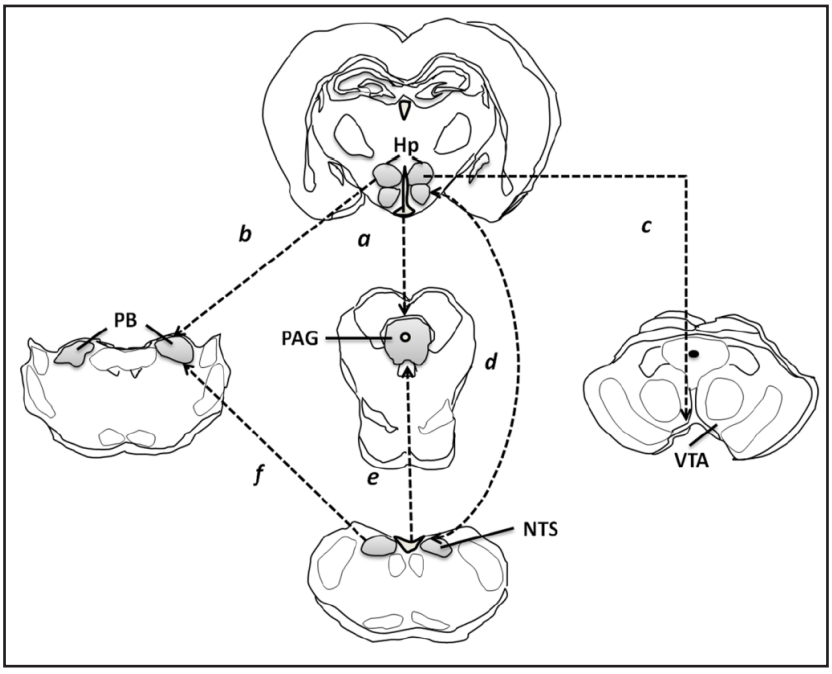

Fig. 3. Schematic summary of the projections of EM1-containing neurons in the central nervous system. EM1-ir neurons in the hypothalamus (Hp) principally project to PAG (a), PB (b), VTA (c) and NTS (d), respectively, while EM1-ir neurons in the NTS mainly send projection fibers to both PAG (e) and PB (f) [17, 18, 20, 22, 24, 62].

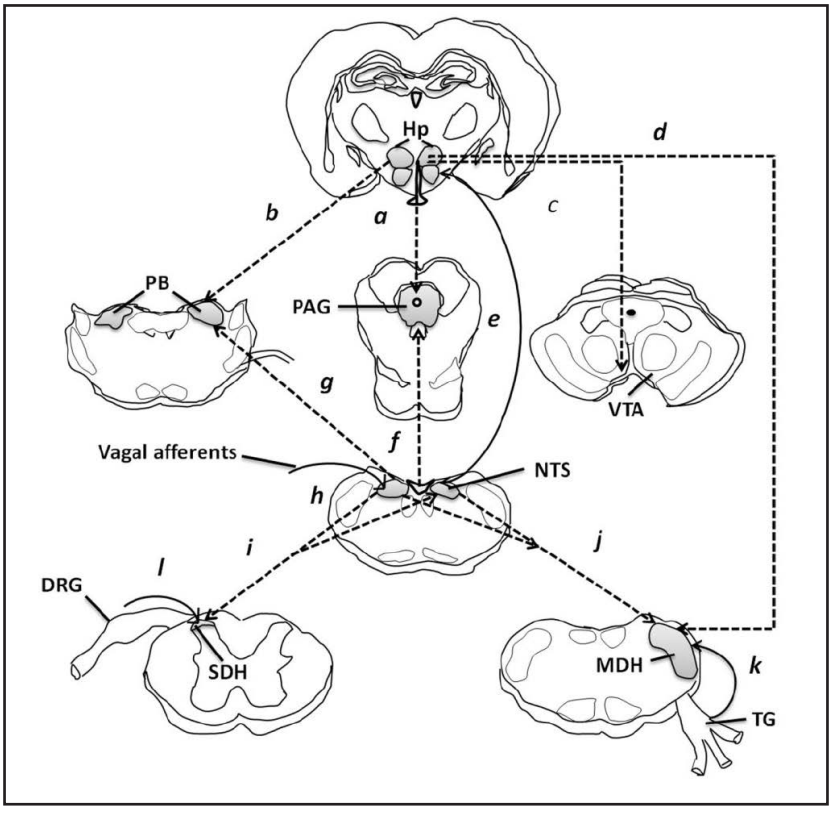

Fig. 4. Schematic summary of the projections of EM2-containing neurons in the central nervous system. The main targets of the EM2-ir neurons in the hypothalamus (Hp) are PAG (a), PB (b), VTA (c), MDH (d) and NTS (e), respectively. Whereas the major terminating area of the EM2-ir neurons in the NTS are PAG (f), PB (g), SDH (i) and MDH (j), respectively. EM2-ir vagal afferents terminate in the NTS (h). EM2-ir neurons in the TG and the DRG are the main sources of EM2-ir fibers and in the MDH (k) and SDH (l), respectively [17, 18, 20-24, 58, 59, 62].

The NTS is the main target in which primary visceral afferents terminate, and visceral ganglia and vagal afferents contain EM2 [58, 59, 61]. Therefore, EM2 in the NTS might be partially derived from primary visceral afferents, and this neural circuitry might be related to the role of EM2 in the modulation of visceral information [58].

The PAG is an important structure in pain modulation [68]. Reports regarding the origins of the EM-IR fibres in the PAG are contradictory. Initially, evidence indicated that EM1- and

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EM2-IR fibres exclusively originated from the hypothalamus [18]. Subsequently, the NTS was identified as one of the sources of the EM-IR fibres in the PAG [22]. The various injection sites and applications of tracers with different sensitivities can explain the contradictory results. Therefore, it is concluded that the origins of the EMergic fibres in the PAG are from both the hypothalamus and NTS [18, 22].

Previously in this review, we discussed that that EM2-IR fibres and terminals are densely aggregated in the superficial laminae of the SDH and MDH, and the EM2-immunoreactivity in these regions mainly originated from the DRG and trigeminal ganglia, respectively [6, 10]. However, an in-depth study exploring the origins of EM2-IR in the SDH showed that some of the EM2-IR originated from bilateral descending fibres from the NTS [21]. EM2-IR in the MDH was shown to originate not only from ipsilateral primary trigeminal afferents but also from bilateral fibres from the hypothalamus and NTS [23]. EM2 in the SDH and MDH regulates the transmission of nociceptive information [69-71] and plays potent analgesic roles when intrathecally injected [1, 36, 38, 72]. Therefore, the hypothalamus and NTS may also regulate pain transmission by projecting EM2-IR fibres to both the SDH and MDH.

In the sacral parasympathetic nucleus (SPN), EM2-containing axonal terminals formed symmetric synapses with MOR-expressing parasympathetic preganglionic neurons (PPNs), suggesting the existence of symmetric synapses. EM2 and MOR have been shown to be involved in homeostatic control and the transmission of micturition information [19].

In addition, synaptic connections were observed between the EM2-IR fibres and the motoneurons in lamina VIII of the spinal ventral horn [73]. To further investigate the effects of EM2 on these motor neurons, a whole-cell patch-clamp technique was used in this report [73]. It was found that EM2 could decrease both the frequency and amplitude of the spontaneous excitatory postsynaptic currents (sEPSC) in the motoneurons in lamina VIII. It could be concluded that EM2 might exert inhibitory effects on the motoneurons in lamina VIII through their synaptic connections. Motor impairment is one of the side effects of morphine, which is an exogenous agonist of MORs. This report might disclose a possible mechanism underlying this locomotion disorder [73].

Inhibitory interneurons in lamina II of the SDH and MDH play a critical role in the regulation of the transmission of nociceptive information by releasing γ–amino butyric acid (GABA) [74, 75]. Previous studies have shown that EM2 is co-localized with substance P (SP) in neurons in the DRG and terminals in the superficial laminae (lamina I and lamina II) in the SDH [10, 27]. Therefore, the connections between the EM2- or SP-IR terminals and GABAergic interneurons in the superficial laminae of the SDH and MDH were examined. A triple-immunofluorescent labelling method combined with immuno-electron microscopy revealed that EM2 and SP double-labelled fibres/terminals formed asymmetric synapses with GABA-IR dendrites in lamina II of the lumbar SDH [71]. This report also found that during the transmission of noxious information induced by formalin plantar injection, GABAergic neurons that were immunopositive for the FOS protein contacted EM2- or SP-IR terminals. In addition, the EM2-IR terminals formed synapses with GABA/MOR co-expressing dendritic processes and neuronal cell bodies in lamina II of the spinal trigeminal caudal subnucleus (also called MDH) [70]. These results suggest that the interactions between EM2- or SP-IR terminals and GABAergic interneurons might be involved in the transmission and modulation of pain.

Co-localization of EMs with other neurotransmitters and/or neuromodulators

The coexistence of EM2 with SP and MOR has been found in many fibres in the superficial laminae of the spinal cord and the spinal trigeminal nucleus [10, 27, 71]. Calcitonin gene-related peptide (CGRP) was also reported to be co-localized with EM2 in the primary afferent fibres in the SDH [6]. SP and CGRP are two primary peptide neurotransmitters involved in the transmission of painful stimuli [76, 77]. This evidence indicated that EM2 in primary afferent fibres may modulate pain perception by regulating pre- and postsynaptic MORs and interacts with these peptides to modulate pain. EM2 was also co-localized with SP, CGRP and

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MOR in the DRG [27, 78]. In the CNS, EM1 and EM2 were both shown to be co-localized with SP and CGRP in the NTS and SP, CGRP and MOR in the PBN [25].

In addition, immunofluorescent histochemical staining showed that notable EM2-IR nodose ganglion (NG) neurons expressed SP, CGRP, nitric oxide synthase (NOS) and vasoactive intestinal peptide (VIP). Most EM2-IR NG neurons also contained MORs. This phenomenon was observed in the vagal trunk [26]. In recent reviews, we discussed the distribution of EM2 in myenteric and submucosal plexus neurons in the rat colon [11, 12]. Previous studies have demonstrated that the activation of the MOR in the enteric nervous system (ENS) was responsible for the decreased gastrointestinal (GI) propulsive activity [79, 80]. The distribution of EM2-IR and MORs in the ENS indicated that EM2 played a role in the GI propulsive activity [11, 12, 16]. Although the immunofluorescence histochemical studies identified EM2-IR in the ENS, the cellular mechanisms by which MOR modulates intestinal motility have not been elucidated to date.

EM2 was widely expressed in interneurons and motor neurons in the myenteric plexus and co-localized with other neurochemicals. Double-staining of EM2 with choline acetyl transferase (ChAT), SP, VIP or NOS revealed a possible mechanism of the inhibitory effects of MOR in the gastrointestinal tract, i.e., enhancing spontaneous contraction and tension [11, 12].

Involvement of EMs in the transmission and modulation of noxious information

Pain is an unpleasant feeling that is caused by noxious stimuli, and in certain occasions, pain torments patients, particularly chronic pain, such as neuropathic pain, inflammatory pain and cancer pain, which have made the suffering unendurable. Pain signals originate from peripheral nociceptors and are transmitted to the superficial dorsal horn of the spinal cord through thinly myelinated (Aδ) and unmyelinated (C) primary afferent fibres whose sensory neuronal cell bodies exist in the DRG [81-83]. Then, ascending fibre tracts in the spinal cord transmit the noxious information to the cerebral cortex through the thalamus [84], PAG [85, 86], lateral PBN [87], etc., causing pain sensations. Both the spinal cord and descending fibres in the brain play an important role in the modulation of pain.

At the spinal cord level, inhibitory interneurons in the superficial laminae of the SDH modulate the transmission of noxious information from primary afferents to spinal projection neurons [75, 88]. In the brain, the PAG is the most important structure for modulating pain, and the rostral ventromedial medulla (RVM), lateral reticular nucleus (LRN) and LC are also important modulatory structures that send descending serotoninergic (5-HTergic), GABAergic or noradrenergic (NAergic) fibres [89-92]. To study the mechanisms of pain and identify potentially potent analgesic drugs, animal models with various types of pain have been established. Opioid peptides have been used for years in experiments and clinical practices and have been demonstrated to be potent analgesics that are currently widely used to ease patients' pain clinically. However, their side effects of addiction and tolerance are difficult to resolve [35, 93].

The two opioid tetrapeptides, i.e., EM1 and EM2, have been demonstrated to have naloxone-sensitive antinociceptive action and are more potent MOR agonists than (D-Ala2, N-MePhe4, Gly-ol)-enkephalin (DAMGO), and EM1 is as potent as morphine in producing naloxone-reversible analgesia in animal models [1]. Previous reports have confirmed that the EMs produced spinal and supraspinal analgesia with slightly fewer side effects in acute and neuropathic pain [1, 36-38] (Table 1). Moreover, the rewarding effects of the EMs can be separated from their analgesic effects [40].

Investigations of the mechanism underlying the analgesic effect of EMs contribute to the understanding of the pain control system in the body in-depth and expedite the clinic usage of EMs. As previously described, the central endogenous pain control system consists of the following two aspects: 5-HTergic fibres from the PAG, raphe nuclei and partial reticular nuclei [100, 101] and NAergic fibres from the LC [89, 102]. In addition, EM1 was shown to be more

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Table 1. The role of the EMs in the transmission and modulation of noxious information

Type of pain	Species	Pain regulating level	Site of administration	Key findings	References
Inflammatory pain	Rat	In the spinal cord	i.t.	The binding of EM2 to MORs suppresses the release of SP	[94]
		In peripheral nerve terminals and immunocytes		A peripheral regulating mechanism	[95]
Menopause-related pain	Rat	In the spinal cord	i.t.	Intrathecal EM2 can inhibit thermal hyperalgesia and inflammatory pain	[96]
Cancer pain	Rat	In the spinal cord	i.t.	Down-regulated spinal EM2 plays a critical role in bone cancer pain	[97]
Diabetic neuropathic pain (DNP)	Rat	In the spinal cord	i.t.	The decreased inhibition of SP signalling may account for DNP resulting from the decreased expression of presynaptic MOR	[94, 98]
				Microglial i.c.v. injection of EMs could improve DNP	
		In the brain	i.c.v.		[99]

potent than EM2 in inhibiting the synaptic transmission in the substantia gelatinosa (SG), and the EMs suppress both excitatory and inhibitory synaptic transmission by activating presynaptic MORs in the SG in the spinal cord [29]. Immunohistochemistry studies have shown the coexistence of EM2 and SP and the existence of synapses between EM-IR axons and GABA-IR neural cell bodies and dendrites [70, 71]. Therefore, the mechanism underlying the analgesic effects of the EMs in the spinal level can be explained by the following three aspects: 1). Inhibiting the release of SP from primary afferent terminals via presynaptic MORs [3, 27, 78]; 2). binding to postsynaptic MORs in GABAergic inhibitory interneurons to exert inhibitory effect on excitatory interneurons [30, 70, 71, 103] and 3) increasing the release of NA and 5-HT in the SDH from the descending terminals of the central endogenous pain control system [30, 104].

EM1 and EM2 have been shown to be involved in various rodent models of pain. A study using the complete Freund's adjuvant (CFA)-induced inflammatory pain model elucidated the mechanisms underlying the antinociception effect of EM2 in inflammatory pain in the spinal based on the following set of observations [94]: ① EM2 could significantly alleviate both mechanical and thermal hyperalgesia in the CFA rats; ② EM2 could serve as an functional antagonist of neurokinin-1 receptors (NK1Rs), which are specific receptors for substance P (SP); ③ a coexistence of MOR/SP was found in DRG neurons; and ④ EM2 induced NK1R internalization in CFA-induced inflammatory pain. It was concluded that the analgesic influences of EM2 might be through binding to MORs to suppress the release of SP. In addition, the upregulation of MORs in peripheral nerve terminals and the increased expression of EM1 and EM2 in immunocytes indicate the presence of a peripheral regulating mechanism via these opioid peptides in inflammatory pain [95].

Menopause-related pain tremendously impacts postmenopausal women, but the underlying mechanism remains unclear, and treatment is not effective. A study using an ovariectomized rat model (OVX) demonstrated the relationship between OVX-induced pain and a decrease in spinal EM2. Additionally, an intrathecal administration of EM2 can dose-dependently inhibit thermal hyperalgesia and inflammatory pain but the ED50 for thermal hyperalgesia is smaller than that for inflammatory pain responses [96].

The ipsilateral EM2-immunoreactivities in the spinal cord were markedly reduced in an established rat model of unilateral tibia cancer. In addition, the administration of EM2

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by an intrathecal injection significantly alleviated the cancer-related mechanical allodynia, providing support for the critical role of the down-regulated spinal EM2 in bone cancer pain [97]. Therefore, a new intervention that enhances the function of EM2 in the spinal cord may attenuate bone cancer pain. It has been demonstrated that adenosine triphosphate (ATP)-gated cation channel P2X receptors are critical facilitators of cancer-related pain, and developing blockers against P2X receptors was a hotspot for treating cancer-related pain [105]. Opioids have been shown to be involved in the modulation of P2X receptors in sensory neurons [106]. Hence, the hypothesis that EMs function as analgesics in cancer-related pain by controlling P2X receptors needs to be further investigated.

Painful diabetic neuropathy (PDN) is one of the most common complications tormenting patients during the early stage of diabetes mellitus (DM). Impairment in the endogenous opioid analgesia system mediated by EM2 via MOR might be involved during the early stage of the PDN [98]. Intracerebroventricular injection of EMs could improve PDN [99]. This down-regulation of endogenous antinociception may be due to the decreased inhibition of SP signalling resulting from the decreased presynaptic EM2 and MOR expression [94, 98].

Altogether, both EM1 and EM2 exhibit potent antinociception effects and are promising analgesics.

Neural mechanisms of opioid-induced side effects as revealed by studies of EMs

In addition to their role in the transmission and modulation of nociception, both EM1 and EM2 could cause many opioid-induced side effects (Table 2). EM1 and EM2 have been demonstrated to have significant vasodilator activity and decrease systemic arterial pressure in various species [107-109]. Decreases in heart rate, cardiac output, and total peripheral resistance were also observed, and the observed hypotension was believed to be associated with these changes [107]. Vasodilator responses induced by EMs were mediated by the activation of a naloxone-sensitive opioid receptor and the stimulation of nitric oxide release from the endothelium [28, 110]. These results suggest that EM2 and MORs might play a role in myocardial contractility and heart rate.

Respiratory depression is one of the severe side effects caused by opioid analgesics, and these two peptides also have this side effect, but the effect is more mild [39]. However, the underlying mechanisms of EM-induced respiratory depression are unclear. Understanding the mechanism of EM2-induced respiratory depression requires a further comprehension of opioid analgesics. It is believed that a decreased sensitivity in the brainstem respiratory centres to carbon dioxide is the main cause of opioid-induced respiratory depression. Investigations have shown that the dosage of EMs required to attenuate the hypercapnic ventilatory response (HCVR) was much larger than that of morphine [112]. Therefore, the distribution and pharmacological effect of EM2 in the ventral respiratory group (VGR), particularly the pre-Bötzinger complex (pre-BötC), which is the centre generating respiratory rhythm, were investigated. It was demonstrated that EM2-IR axonal terminals modulated NK1R-expressing neurons in the pre-BötC, and EM2 plays a role in respiratory depression through MORs in the pre-BötC [113]. In addition, MOR-IR neurons and terminals were also found in regions other than the pre-BötC in the VGR, and a microinjection of EM1 into these regions influenced the phrenic nerve frequency and amplitude, thus regulating respiration [111].

Investigations of EMs have revealed the potential mechanism of the urinary retention effect induced by opioid analgesics. Morphological evidence has shown that EM2-IR fibres were distributed in the sacral parasympathetic nucleus (SPN), and parasympathetic preganglionic neurons (PPNs) that express MORs in the SPN innervate the urinary bladder. EM2-containing axon terminals formed symmetric synapses with MOR-expressing PPNs in the SPN [19]. EM2 activated pre- and post-synaptic MORs, thereby inhibiting excitatory

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Table 2. Neural mechanisms of opioid-induced side effects as revealed by studies of EMs

Adverse effects	Species	Methods	Key findings	References
Cardiovascular responses	Rat, rabbit, cat	i.v.	Decreased heart rate, cardiac output, total peripheral resistance and arterial pressure by EMs were mediated by the activation of a naloxone-sensitive opioid receptor and the stimulation of nitric oxide release from the endothelium	[107-109]
Respiratory depression	Rat	i.v. Microinjection into different subdivisions of the VRG Double-labelling immunofluorescence	EMs attenuate the hypercapnic ventilatory response (HCVR) EM2-IR axonal terminals modulated NK1R-expressing neurons via MORs in the pre-Bötzinger complex (pre-BötC)	[39, 111-113]
Urinary retention	Rat	Double-labelling immunofluorescence	EM2 activated pre- and post-synaptic MORs, thereby inhibiting the release of excitatory neurotransmitters from presynaptic terminals and decreasing the excitability of PPNs due to the hyperpolarization of their membrane potentials	[19, 114]
Gastrointestinal reaction	Rat	Double-labelling immunofluorescence	EM2 was widely expressed in interneurons and motor neurons in the myenteric plexus and submucosal plexus of the rat colon and activated presynaptic receptors, thereby enhancing spontaneous contraction and tension	[11, 12]

neurotransmitter release from the presynaptic terminals and decreasing the excitability of PPNs due to the hyperpolarization of their membrane potentials. These inhibitory effects of EM2 on PPNs in the spinal cord may explain the mechanism of action of morphine treatment and morphine-induced bladder dysfunction in the clinic [19, 114].

Gastrointestinal reactions to opioids are also common in the clinic and hamper the development of opioid-based treatments. Opioids, such as morphine, inhibit intestinal smooth muscles via widely expressed MORs in the gastrointestinal tract, resulting in constipation, and the colon is the main target of opioid bowel dysfunction [115, 116]. EM2 was shown to be widely expressed in interneurons and motor neurons in the myenteric plexus and submucosal plexus of the rat colon and activate presynaptic receptors, thereby enhancing spontaneous contraction and tension [11, 12]. However, in diabetic mice, the inhibitory effect of EM2, but not of EM1, in the gastrointestinal tract was attenuated, suggesting the EM1 and EM2 have different functional responses in the gastrointestinal tract, and changes occur in the MOR subtypes in diabetic processes [117]. Fortunately, EMs with modifications in terms of reducing opioid bowel dysfunction have achieved promising performance [46, 118].

Potential Clinical implication of the EMs

Opioids, such as morphine, have been used as potent clinical analgesics for years. However, the accompanied adverse effects of these opioid alkaloids, such as drug dependence, tolerance, respiratory depression and gastrointestinal effects, have always triggered the demand for new alternatives [33-35]. The discovery of the EMs can expedite the development of novel analgesics. Compared with other opioid alkaloids, the potent antinociception effect of the EMs in acute [36, 37] and neuropathic pain [38], the slightly fewer side effects [39] and particularly the separation of the rewarding effect from the analgesic effect [40] open the

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Table 3. Clinical implications

Modifications	Species	Methods	Improved properties	Decreased properties	References
EM-1-$NHNH_2$	Guinea pig, mice	i.c.v.	Remarkably improved antinociception	Moderate colonic contractile and expulsive effects	[49, 119]
EM-2-$NHNH_2$			Significantly attenuated colonic propulsion	Slightly lower antinociception	
Tyr-(β-Pro/D-Ala)-Trp-Phe-$(Gly)_2$-$(Arg)_2$-OH	Guinea pig, mice	i.c.v. s.c.	Improved antinociceptive activity and had fewer gastrointestinal side effects	Less potent ED_{50} values	[51]
Tyr-(β-Pro/D-Ala)-Trp-Phe-$(Gly)_2$-$(Arg)_5$-OH					
(thienyl)-alpha-methylene-beta-amino acids (Map) modified EM1	Mice	i.v.	Improved blood brain barrier (BBB) permeability		[47]
EN-9 (Tyr-Pro-Phe-Phe-Gln-Pro-Gln-Arg-Phe-NH2)	Mice	i.c.v. i.v. s.c.	Potent, non-tolerance forming antinociception		[46]
Lipid- and sugar-modified EMs	Rat	i.v.	Improved penetration of the BBB and cell membrane		[50]
Tyr-c[D-Lys-Trp-Phe-Glu]-NH2 Tyr-c[D-Glu-Phe-Phe-Lys]-NH2; Tyr-c-[D-Lys-Trp-Phe-Asp]-NH2; Tyr-c[D-Lys-Trp-Phe-Glu]-Gly-NH2	Rat, mice	i.c.v. s.c. oral administration	Remarkably improved properties in respiratory depression, motor impairment, tolerance, immune reactivity, and reward/abuse liability		[120]
Engineered EM2 gene	Rat	Lumbar subarachnoid catheterization Subcutaneous inoculation	Reduced withdrawal syndrome attenuated chronic pain		[120, 121]

possibility of the pharmaceutical use of EMs in clinical trials. Unfortunately, the therapeutic uses of EMs as analgesics are currently not valuable because of the limitation of their low membrane permeability [44], susceptibility to enzymatic degradation [45] and few adverse effects [41-43]. Therefore, the discovery of novel peptides based on the structure of the EMs with a high efficiency and extremely attenuated undesired effects has become a hotspot in the field of medicine [46-51]. Substantial attempts have been made to study their structure-activity relationships, and modifications, such as methylation, glycosylation and lipidation, etc., directed at these sites have achieved promising results [46-52]. Here, we selectively report some of the important modifications of EMs (Table 3).

Reverse-phased chromatography purification of brain tissue combined with Edman degradation sequencing found that both EM1 and EM2 were endogenous tetrapeptides, and their structures were Tyr-Pro-Trp-Phe-NH2 and Tyr-Pro-Phe-Phe-NH2, respectively [1]. A series of EM2 analogues were developed by substituting the C-terminal amide group. The conversion of the C-terminal amide to hydrazide in EMs produced differential pharmacological and behavioural effects both in vitro and *in vivo* with little influence on their affinity [49, 119]. EM1-NHNH2 exhibited a higher antinociceptive effect but more moderate colonic contractile and expulsive effects than EM1, while EM2-NHNH2 displayed the contrary effects [49]. EM1 analogues with a C-terminal oligoarginine-conjugation displayed systemic antinociceptive activity and fewer gastrointestinal side effects without markedly influencing the MOR affinity and *in vitro* bioactivity [51]. (Thienyl)-alpha-methylene-beta-amino acids (Map) modified EM1 analogues synthesized by the same group showed improved blood

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brain barrier (BBB) permeability [47]. These authors also synthesized a chimeric peptide containing the functional domains of the endogenous opioid EM2 and neuropeptide FF, which was called EN-9. Pharmacological studies have shown that EN-9 produced a potent, non-tolerance forming antinociception effects with fewer side effects [46].

Glycosylation and lipidation modifications also achieved important improvements to the therapeutic possibility of EMs. The conjugation with carbohydrates confers an increased biodistribution to the peptide, which improved penetration through the BBB and cell membranes, and lipidation can also improve peptide stability and BBB transport [50, 122]. Researchers have designed a tripartite synthetic gene to directly produce, cleave and amidate EM2, making the local biosynthesis of EMs possible for the treatment of chronic pain [120].

Importantly, a report by Zadina et al. suggested that the EM analogues could provide a gold standard pain relief mediated by selective MOR activation but with remarkably safer side effect profiles compared to those of opioids, such as morphine [121]. These authors synthesized four types of cyclized, dextro-amino acid-containing EM analogues and compared these analogues to morphine; these analogues showed remarkably improved properties in respiratory depression, motor impairment, tolerance, immune reactivity, and reward/abuse liability in rats without impairing the affinity. The authors also showed favourable solubility and stable plasma half-life *in vitro*. Regarding the antinociceptive effects, the authors revealed potent, long-lasting and μ-selective analgesia after intravenous (*i.v.*), subcutaneous (*s.c.*), and oral administration. Of the four analogues, Analogue 4 displayed superior effects over the others in respiratory depression, motor impairment and abuse liability with the most potent antinociceptive effects. These findings are extremely encouraging regarding the prospect of novel clinical analgesics.

In addition, the high ratio of DOR: MOR in cell membranes and the activated DOR indicate a greater possibility of developing opioid tolerance [123, 124]. Therefore, it is hypothesized that MOR agonists with mixed MOR-agonist/DOR-antagonist activity might decrease dependence and tolerance. The development of EMs analogic analgesics with a high efficiency and few side effects in the future is highly important.

Moreover, the reduced withdrawal syndrome in an opioid dependent rat model was observed after administrating the engineered EM2 gene into the CNS, opening another potential application of EMs in the management of withdrawal syndrome in opioid dependent subjects [52].

Conclusion and Outlook

Since their discovery as specific MOR ligands in the 1997, increasing studies have been performed to quantitatively investigate the potent analgesic effects of EMs. Furthermore, numerous studies have revealed that the intricate morphological features of the EMs were related to their endogenous roles in many physiological processes. These reports deepen our understanding of the mechanisms underlying the pain control system in the body and brain functions. It is hypothesized that the EMs might be employed as analgesics to replace traditional opiates, which are associated with serious adverse effects. However, while animal experiments show that EMs have many advantages in pain relief and other functions, their limitations continue to hamper their clinical application. Therefore, in recent years, the modifications of EMs have become research focus. Thus far, these EM derivatives have shown improved properties in animal experiments. The application of EM analogues in the clinic is very promising.

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YQ Li and H Li initiated the project and design the present project. ZH Gu, B Wang and ZZ Kou wrote the draft for the manuscript. Y Bai, T Chen and YL Dong contributed to preparation of reference, critical reading and revising the manuscript. YQ Li and H Li revised and edited the manuscript and approved the final vision of the manuscript.

Disclosure Statement

The authors have nothing to disclose.

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