FORUM MINIREVIEW

Recent Advances in the Search for the μ -Opioidergic System

Isolation and Distribution of Endomorphins in the Central Nervous System

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ABSTRACT—Endomorphin-1 (Tyr-Pro-Trp-Phe-NH₂, EM-1) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH₂, EM-2) have the highest affinity and selectivity for the μ -opioid receptor (MOP-R) of all known mammalian opioids. They were isolated from bovine and human brain, and are structurally distinct from the other endogenous opioids. Both EM-1 and EM-2 have potent antinociceptive activity in a variety of animal models of acute, neuropathic and allodynic pain. They regulate cellular signaling processes in a manner consistent with MOP-R-mediated effects. The EMs are implicated in the natural modulation of pain by extensive data localizing EM-like immunoreactivity (EM-LI) near MOP-Rs in several regions of the nervous system known to regulate pain. These include the primary afferents and their terminals in the spinal cord dorsal horn, where EM-2 is well-positioned to modulate pain in its earliest stages of perception. In a nerve-injury model of chronic pain, a loss of spinal EM2-LI occurs concomitant with the onset of chronic pain. The distribution of the EMs in other areas of the nervous system is consistent with a role in the modulation of diverse functions, including autonomic, neuroendocrine and reward functions as well as modulation of responses to pain and stress. Unlike several other μ opioids, the threshold dose of EM-1 for analgesia is well below that for respiratory depression. In addition, rewarding effects of EM-1 can be separated from analgesic effects. These results indicate a favorable therapeutic profile of EM-1 relative to other μ opioids. Thus, the pharmacology and distribution of EMs provide new avenues both for therapeutic development and for understanding the neurobiology of opioids.

Keywords: Endomorphin-1, Endomorphin-2, μ -Opioid receptor, Immunocytochemistry, Neuropathic pain

Discovery of the endomorphins (EMs)

The discovery of opioid receptors (1) led to a concept that stimulated an intense race to discover endogenous opioid peptides: It seemed unlikely that this new receptor would be present in the nervous system only to respond to a ligand (morphine) derived from a plant (poppy), to which an organism may or may not ever be exposed. A more likely possibility was that the nervous system would produce a natural agonist for that receptor. Within a short time, three families of opioid peptides were discovered containing a common N-terminus amino acid motif of YGGF. A mystery remained, however, after these discoveries. The μ -opioid receptor (MOP-R) is known to be essential for the analgesic and euphoric actions of morphine, as shown

by the elimination of these effects in MOP-R knockout mice (2). Again, it seemed unlikely that the only natural ligands for the μ receptor would be those that either preferentially bind to other types of opioid receptors (enkephalin to δ and dynorphin to κ receptors) or indiscriminately bind to MOP-Rs or δ receptors (β -endorphin) (3). The plant-derived alkaloid, morphine, by contrast, binds to the MOP-R with nearly two orders of magnitude greater affinity compared with its next-preferred site, the δ receptor (3, 4). Peptides with clear selectivity for the μ receptor were later isolated from milk (casomorphin (5)), blood (hemorphin (6)), pituitary (7) and brain (Tyr-MIF-1 (8), Tyr-W-MIF-1 (9)). Those containing a Tyr-Pro-aromatic (Phe or Trp) motif showed the highest affinity, but were still well below the affinity of the endogenous opioids with a YGGF sequence.

From this group of peptides, we used the μ -selective

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peptide with the highest affinity and selectivity (Tyr-W-MIF-1, YPWG-NH₂) (4) as a lead compound to screen peptides in which each of the 20 possible natural amino acids was substituted in the fourth position following the YPW motif. One of the 20 peptides (Phe⁴, YPWF-NH₂) showed affinity and selectivity dramatically greater than all others in MOP-R binding assays (10). This peptide showed very potent inhibition of guinea pig ileum contractions, the classical *in vitro* opiate agonist test. *In vivo*, the peptide was a very potent analgesic after intracerebroventricular and intrathecal administration (10). Specific μ antagonists reversed both the *in vitro* and *in vivo* effects of the YPWF-NH₂ peptide.

To determine if this all-natural amino acid peptide was synthesized in the nervous system, a specific antibody was generated against the tetrapeptide sequence and used in combination with HPLC to screen extracts of bovine brain for immunoreactivity. Multiple purification steps yielded an immunoreactive fraction from which Edman degradation sequencing revealed 2 peptide sequences: YPWF-NH₂ and YPFF-NH₂. Tests of the second sequence showed that its affinity and activity in bioassays was only slightly lower than the first. Because of their selectivity for the MOP-R, they were named endomorphin-1 (EM-1) and -2 (EM-2) (10). Both sequences were then isolated from human brain (11).

Following the discovery of the EMs, many laboratories confirmed their μ -selective analgesic effects in a variety of pain paradigms. Work from two outstanding laboratories in this area are included in other Minireviews in this forum (12, 13). Demonstrations that the peptides exhibited the cellular responses expected of a selective μ agonist were also the focus of many studies. Early examples included the inhibition of calcium currents (14) and stimulation of GTP γ S binding (15). Differential effects of EMs and β -endorphin in GTP γ S binding are described in an accompanying Minireview (16).

Distribution of the EMs in the spinal cord and medulla

To characterize a peptide as an agonist for the MOP-R, an important criterion is that it should be present in proximity to that receptor. In addition, if the peptide is a natural modulator of pain processes, one would anticipate that it would be present in pain pathways with the MOP-R. Early studies in our laboratory established this to be the case. In the initial characterization of the peptides, radioimmunoassay (RIA) was used to detect EM-1 in brain areas important for μ -opioid actions (10). In that same year, we published the first report that EM-2 was present in the superficial layers of the dorsal horn of the spinal cord and medulla (17). These regions, where primary afferents from the dorsal root ganglia (DRG) and trigeminal nerve terminate, are among the regions with the highest densities of

MOP-Rs in the nervous system. Similarly, the EM-2 like immunoreactivity (EM2-LI) in this region is among the highest in the nervous system. Thus, EM2-LI and MOP-Rs are both present at the earliest stages of pain transmission, at the synaptic region of primary afferents.

The source of the EM2-LI (i.e., the cell bodies), however, needed to be addressed. In the initial study (17), immunoreactive cell bodies were not detected within the dorsal horn and were observed in the DRG. However, the staining of the DRG cells was resistant to preabsorption by the cognate peptide, preventing conclusive identification as EM-2-containing cells. EM2-LI fibers in the DRG, however, were preabsorbed, consistent with the DRG being the source of the immunoreactivity in the dorsal horn. Compelling evidence to confirm this possibility was generated in two laboratories. Mechanical disruption of the primary afferents by rhizotomy (severing the primary afferent neuron proximal to the DRG) nearly eliminated the staining in the dorsal horn (18, 19). Similar effects were observed by chemical disruption of c- and a- δ primary afferents with the toxin capsaicin (18). Subsequent studies, in addition to replicating the DRG fiber staining, demonstrated EM2-LI in skin, consistent with its presence in the peripheral terminals of the primary afferents (20). Colocalization studies showed that EM2-LI was present in fibers containing immunoreactivity for MOP-Rs, substance P (SP) (18), and calcitonin gene-related peptide (CGRP) (19), which is derived from primary afferents (22). Taken together, these studies clearly support the idea that the primary afferents, with their cell bodies in the DRG, are the source of most, if not all, EM2-LI in the dorsal horn of the spinal cord.

These findings addressed several long-standing questions concerning the role of opioids in the primary afferents and dorsal horn. For example, the MOP-R was known to be present presynaptically (on primary afferents) and postsynaptically (on intrinsic neurons of the dorsal horn), because dorsal rhizotomy reduced, but did not eliminate MOP-R in the dorsal horn (23). The natural agonist for MOP-Rs on the primary afferents, however, was not clear. The hypothesis that enkephalin fibers should synapse on primary afferents (24), thus providing an endogenous ligand for the presynaptic receptors, was not supported in several studies showing that such connections were rare (25–28). Similarly, dynorphin contacts with primary afferents were found to be rare (29). An alternative raised by the results described above is that the MOP-R on the primary afferents could serve as an autoreceptor, with EM-2 as its natural agonist. Opioids are known to regulate the release of glutamate, SP and CGRP from primary afferents, and EM-2 would therefore be in position to modulate release of these excitatory transmitters. In addition, EM2-LI is present in dense-cored vesicles in dorsal horn axons (30)

and is released by stimulation of the dorsal roots (31). EM-2 is therefore well-positioned to provide fine control of pain perception at its earliest stages by modulating primary afferent firing as well as post-synaptic excitability through MOP-Rs on dorsal horn cells. As described below, changes in EM2-LI in these regions may contribute to the chronic pain associated with nerve injury.

Distribution of EMs in brain

The early studies described above clearly demonstrated that the novel μ agonist EM-2 was localized with or near MOP-Rs in circuits mediating pain transmission at its earliest stages. Subsequent studies provided detailed mapping of EM-2 (32, 33) and of both EM-1 and EM-2 (21) throughout the nervous system. Consistent with their potential role as endogenous μ agonists, immunoreactivity for both peptides was observed in numerous regions that contained high densities of MOP-Rs. Many of these regions are known to be involved in the processing of nociceptive information, by direct input from primary afferents, or as relay nuclei to other pain-processing circuits, or both. These areas include the caudal nucleus of the spinal trigeminal tract, the parabrachial nucleus, the nucleus tractus solitarius (NTS), the periaqueductal gray, nucleus ambiguus, locus coeruleus, midline thalamic nuclei and the amygdala. EMs could play a modulatory role at several stages of pain processing. Projection neurons from lamina I of the spinal cord, for example, terminate in the parabrachial nuclei, which in turn conveys nociceptive information to the central nucleus of the amygdala. This spino (trigemino)ponto-amygdaloid pathway could modulate emotional-affective, behavioral and autonomic reactions to noxious stimuli (34). EM-containing neuronal elements are present in most regions of this pathway.

Although both EM1- and EM2-LI are present in most areas where either was observed, several differences in the distributions and staining intensity were observed. EM2-LI predominates in the spinal cord and parts of the medulla, while EM-1 is more prevalent in brain. Brainstem nuclei where both peptides are relatively abundant provide striking examples of differential staining in subregions. In the NTS, for example, intense EM2-LI varicose fibers in the ventrolateral nucleus contrast with the EM1-LI present in punctate terminal field elements in the dorsomedial nucleus. In the parabrachial nucleus, EM1-LI fibers are primarily lateral to the superior cerebellar peduncle while EM2-LI is primarily ventral to it. The differences in EM-1 and EM-2 could reflect different precursors or differential processing of a single precursor and could have implications for differential functional roles of the two peptides. For the NTS and parabrachial nucleus, this may be particularly important for differential effects on autonomic function.

Several diencephalic and telencephalic structures are

enriched in EM-LI and indicate a role for the peptide in neuroendocrine, homeostatic, limbic and reward function. These include most areas of the hypothalamus, the diagonal band of Broca, septal nuclei, bed nucleus of the stria terminalis, amygdaloid complex and the nucleus accumbens.

Reminiscent of β -endorphin neurons, cell bodies immunoreactive for EM-1 and EM-2 appear to be confined to part of the hypothalamus, from which fibers emanate throughout the nervous system. After i.c.v. colchicine treatment, EM1- and EM2-immunoreactive cell bodies were visualized in regions separate from those of β -endorphin, primarily in the periventricular hypothalamus, upper arcuate nucleus, and between the ventromedial and dorsomedial hypothalamus (21, 33). Except for the NTS, where EM1-LI cell bodies could be observed without colchicine (21), and the DRG as discussed above, EM-LI cells have been observed only in the hypothalamus. It should be noted, however, that the restricted diffusion of colchicine after i.c.v. injection may have limited the detection of cell bodies to nuclei near the ventricles and future studies may reveal additional cells that synthesize EMs. Regardless, these early histochemical studies clearly implicate the EMs in the modulation of pain, autonomic function and stress responses, and raise the possibility that they serve numerous other functions in the nervous system.

Dissociation of the effects of EM-1 on analgesia from its effects on reward and respiratory depression

Because activation of the MOP-R is, in general, the most effective means of pain relief, and the EMs have been shown in numerous studies to be potent analgesics, there is considerable potential for development of therapeutics based on their structure. As with all new medications, EM-based compounds would have to meet requirements of fewer unwanted side-effects than are observed with currently used compounds in the class. Two issues of concern for μ opioids are their rewarding properties, which can contribute to abuse potential, and respiratory depression, which can be life-threatening.

μ Opioids are well known for their rewarding properties. The conditioned place preference (CPP) test is one of the most widely accepted methods for testing opioid-induced reward. In this paradigm, animals are given injections of test compounds in one compartment and the vehicle in an adjacent compartment. The two compartments are distinct on several perceptual dimensions so that the animal can easily distinguish the compartment where the drug was given from that in which vehicle was injected. After several days of injections, the animal is placed in a neutral area with access to both compartments. The relative time spent in each compartment provides an index of whether the animal finds the compound appetitive, aversive or neutral.

Using this paradigm, we showed that, in contrast to morphine, EM-1 could induce potent analgesia in rats at a dose that did not induce place preference (35). It was anticipated that EM would not be void of rewarding properties (an effect recently shown in mice (36)). However, this was the first demonstration that analgesia induced by central administration of a μ -selective agonist could be separated from reward.

μ Opioids are also known to induce respiratory depression. In a recent study, we tested the possibility that four μ agonists may differ in their induction of respiratory depression relative to their ability to induce analgesia (37). Despite the fact that DAMGO, EM-1, EM-2 and morphine have similar affinities for the MOP-R (10) and induced analgesia at similar doses, the doses at which cardiovascular and respiratory side-effects occurred greatly differed. The ventilatory depressant effects required significantly higher doses of EM-1 than EM-2, DAMGO or morphine. For EM-1, the dose inducing ventilatory depression was above that inducing analgesia, while for EM-2, DAMGO and morphine, ventilatory depression was seen at doses below the analgesic dose. In addition, the transient decrease in blood pressure seen after doses as low as 20 nmol/kg DAMGO or 300 nmol/kg EM-2 was not observed with EM-1 at doses up to 10,000 nmol/kg. Thus, the "therapeutic ratio" for analgesia relative to adverse cardio-respiratory effects was more favorable for EM-1. The results support the concept that the analgesic and respiratory effects of μ agonists can be dissociated and that EM-1 shows several characteristics that could provide the basis for novel, safer analgesics.

Changes in EM during chronic pain

Tissue and nerve injury can produce prolonged hypersensitivity to pain (hyperalgesia) as well as painful responses to normally innocuous stimuli (allodynia). These changes are accompanied by extensive neuroplasticity in primary afferent and dorsal horn neurons. A more complete understanding of these neuroplastic changes will be essential for improved therapy of the painful syndromes.

In recent studies, we tested whether spinal EM2-LI is altered in animal models of nerve injury and chronic pain (38). After partial ligation of the sciatic nerve, chronic pain is present ipsilateral to the injury and manifests as reduced latency to withdraw the paw from a heat stimulus. Using this model, we showed that EM2-LI was dramatically reduced in the dorsal horn ipsilateral to the injury. The time course of the reduction paralleled the development of chronic pain with decreases in EM2-LI apparent by day 2 that endured throughout the 2-week study. The decrease was specific for the medial portion of lumbar segments, where the sciatic nerve afferents terminate. A decrease in SP was also observed, but was less dramatic and delayed

relative to the decrease in EM2-LI. CGRP was not altered within the 2-week period. This contrast in two peptides (EM-2 and CGRP) that are both derived mainly or entirely from primary afferents indicates that they are differentially regulated and that the change in EM2-LI is not the result of non-specific decreases in peptide content. In addition, the loss of an excitatory neuropeptide (SP) with a delay relative to the onset of pain is consistent with an adaptation to the injury rather than a cause. By contrast, the more rapid decrease in EM2-LI concurrent with the onset of pain is consistent with the loss of an inhibitory influence on pain transmission. These results provide the first evidence that reduction of an endogenous opioid in primary afferents is associated with injury-induced chronic pain. Together with the demonstration (39, 40) that EMs are more effective than morphine at alleviating some forms of allodynic pain, these findings support the possibility of novel, EM-based therapies for neuropathic pain.

Concluding remarks

The EMs are high affinity endogenous opioids selective for the MOP-R. They are potent analgesics and their distribution in nociceptive pathways and in regions of high MOP-R density implicates them as particularly important for the modulation of pain. Changes in EM content during the development of chronic pain further support this concept. Additional functions are also implicated by the immunocytochemical data, including modulation of autonomic, homeostatic, neuroendocrine and reward functions. The analgesic effects of EM-1 relative to reward and cardiorespiratory function indicate a pharmacological profile favorable for development of new therapeutics.

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