

Endomorphin peptides: pharmacological and functional implications of these opioid peptides in the brain of mammals. Part one

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Actualización por temas

SUMMARY

The present paper describes several aspects of the biological activities, physiological and behavioral responses displayed by the most recent discovered opioid peptides: *endomorphins*. Endomorphins comprise two endogenous C-terminal amide tetrapeptides, named as endomorphin-1 (EM1; Tyr-Pro-Trp-Phe-NH₂) and endomorphin-2 (EM2; Tyr-Pro-Phe-Phe-NH₂), which were discovered a decade ago (1997) by Zadina's group. Initially, they reported the identification of two endogenous opioid peptides that displayed high binding affinities and selectivities for the μ -opioid receptor among other identified and cloned opioid receptors. These led authors to support the hypothesis that endomorphin peptides represent the endogenous ligand agonists for the μ -opioid receptor. Both peptides were identified and isolated from bovine and human brains. They consist of four amino acids that share a 75% structural homology among amino acids, and which display the structural α -amidated form of C-terminal -Phe- residue, as demonstrated for many other bioactive neuropeptides.

These peptides are structurally distinct from other endogenous opioid substances identified in the brain of mammals, although they share some similarities with other amide neuropeptides such as Tyr-W-MIF-1, found also in the mammalian brain. Here, we review the structure-relationship activity of both endomorphin molecules comparing their binding properties to different opioid receptors. Both EM1/EM2 peptides appear to be vulnerable to enzymatic degradation when exposed to the activities of different proteolytic enzymes, as occurs with many other neuroactive peptides found in the SNC of mammals. Immunohistochemical studies showed the wide and asymmetric distribution of both EM1-2 peptides in the brain, leading to the extensive pharmacological, cellular, and physiological studies that demonstrated the wide and varied bioactivities displayed by these peptides at both central and peripheral tissues. These studies led several authors to suggest the potential endogenous role of these peptides in major physiological processes (e.g. analgesia or antinociception). Based on the generation of specific (rabbit) polyclonal antibodies and the use of combined radioimmunoassay

(RIA) techniques and immunohistochemical procedures, it was shown the wide distribution of EM1-2-LI (endomorphin 1-2-like immunoreactivities) throughout the brain of different species (e.g. rat, primate, human), particularly co-localized in specific areas where μ -opioid receptor has been shown to be expressed. IHC mapping of endomorphin material in the CNS showed a parallelism with the neuroanatomical distribution of other endogenous opioid peptides (e.g. Met/Leu-enk, Dynorphin A, β -endorphin) previously reported.

These studies showed for instance that, whereas EM1-LI was shown to be widely and densely distributed throughout the brain, particularly in forebrain structures (e.g. nucleus accumbens [NAc]; cortex [Cx]; amygdala [AMG]; thalamus [Th], the hypothalamus [Hyp], the striatum [CPu]), including the upper brainstem (BS); and dorsal root ganglia (DRG); EM2-LI is highly expressed in spinal cord and lower brainstem. Interesting enough is the demonstration of the expression of EM1-2-LI outside the CNS (e.g. spleen, thymus and blood), and detected in immune cells (e.g. macrophages/monocytes, lymphocytes, and polymorphonuclear leucocytes) surrounding inflammatory foci. Pharmacological studies showed that these peptides displace with high potency several μ -opioid receptor ligands agonists in a concentration-dependent manner. Moreover, EM1-2 peptides have been shown to modulate the release of several conventional transmitters from neurons (e.g. DA, NA, 5-HT, ACh) besides on active neurohormones. Additionally, *in vitro* and *in vivo* studies showed that both EM1/EM2 peptides produce their pharmacological and biological effects by stimulating either μ_1 or μ_2 -opioid receptors, which mediate the distinct pharmacological activities detected for each peptide. Cellular studies showed that both EM1/EM2 peptides induce a potent granule/vesicle endocytosis and trafficking of μ -opioid receptor in cells transfected with the μ -opioid receptor cDNA; following some endocytosis responses and μ -opioid receptor trafficking mechanisms shown in enteric neurons; cells previously reported to express naturally μ -opioid binding sites on cells.

Endomorphins have been shown to induce potent antinociceptive responses after ICV or IT administration into mice; to modulate nociceptive transmission and pain sensation into the brain after

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Recibido: 3 de noviembre de 2009. Aceptado: 29 de enero de 2010.

stimulating peripheral nociceptors on primary neuronal afferents; and to generate cross-tolerance between endomorphin peptides and between EM1 and opiate compounds, such as morphine.

Key words: Endomorphins, peptides, nociception, analgesia, mu-opioid receptor, physiology, immunoreactivity.

RESUMEN

Este artículo resume varios aspectos de las múltiples actividades biológicas, celulares, efectos farmacológicos, respuestas fisiológicas y conductuales de dos nuevas sustancias péptidas de naturaleza opioide, descubiertas recientemente y denominadas *endomorfinas*. Las *endomorfinas* son dos péptidos opioides, clasificados como endomorfina-1 (EM1, Tyr-Pro-Trp-Phe-NH₂) y endomorfina-2 (EM2, Tyr-Pro-Phe-Phe-NH₂), cuyas secuencias péptidas fueron identificadas y aisladas del cerebro de bovino y humano por el grupo de Zadina en 1997. Estudios de unión radioligando-receptor demostraron que estos péptidos se unen con alta afinidad de unión al receptor opioide μ en relación con su capacidad de unión a otros subtipos de receptores opioides (κ [kappa], δ [delta]), previamente identificados en el SNC de mamíferos. Ambos péptidos están compuestos por cuatro aminoácidos y son estructuralmente distintos de las demás sustancias opioides endógenas conocidas.

Esta revisión detalla con precisión diversos aspectos de la farmacología y actividades celulares de estos opioides y sus implicaciones en la modulación de distintas circuitos o vías neurales y funcionamiento del SNC de los mamíferos, respectivamente. Los estudios relacionados con la función estructura-actividad de estos péptidos han mostrado que, al igual que la mayoría de los péptidos bioactivos endógenos de naturaleza opioide y no opioide, son vulnerables a la escisión peptídica por cortes enzimáticos mediante la exposición a distintas enzimas proteolíticas que pudiesen participar en la degradación endógena de las endomorfinas, y la obtención de diversos productos de degradación. Asimismo, este artículo menciona la amplia distribución neuroanatómica que poseen las endomorfinas en distintas regiones del cerebro, particularmente en aquellas que regulan el procesamiento y la transmisión de la información nociceptiva y que, por tanto, reflejan el papel potencial de estos péptidos en procesos fisiológicos de analgesia, entre muchos otros (memoria y otro aprendizaje). En este contexto, diferentes estudios basados en el empleo de ensayos inmunológicos (radioinmunoensayos [RIA] y técnicas de inmunohistoquímica [IHC]) que requieren el uso de anticuerpos específicos generados contra las secuencias consenso de las endomorfinas mostraron una amplia distribución de material inmunoreactivo a endomorfina (vg., EM1-LI, EM2-LI) en tejidos neurales de humano, bovino y roedores. Por ejemplo, la EM1-LI mostró una distribución relativamente abundante en una gran mayoría de las

regiones del SNC de mamíferos estudiados, particularmente en la región rostral y superior del tallo cerebral, así como en el núcleo *accumbens* (NAc), la corteza prefrontal y frontal (PFCx), la amígdala (AMG), el tálamo (TH), el hipotálamo (HPT), el estriado (CPu) y fibras nerviosas de la raíz del ganglio dorsal (DRG). En contraste, la expresión de EM2 mostró ser muy abundante en la región de la médula espinal y en la región caudal del tallo cerebral.

La distribución de material inmunoreactivo a EM1-2 en el SNC de mamíferos mostró similitudes en cuanto a la distribución neuroanatómica reportada para otros péptidos opioides endógenos, previamente identificados (vg., encefalinas, dinorfinas, endorfinas). Así mismo, estudios paralelos lograron identificar la presencia de EM1-2-LI en órganos periféricos (vg., bazo, timo, células inflamatorias del tipo de macrófagos-monocitos, linfocitos y leucocitos PMN) y en plasma. Más aún, diversos estudios farmacológicos han mostrado que las actividades biológicas y respuestas fisiológicas de las EM1-2 están mediadas a través de la estimulación de los subtipos de receptores opioides $\mu 1$ y $\mu 2$. Estudios de inmunohistoquímica (IHC) demostraron la colocalización del receptor opioide μ y las EM1-2 en diversas regiones del SNC de mamíferos. Esto ha permitido proponer que las EM1-2 representan una nueva familia de péptidos opioides con funciones neuromoduladoras relevantes en el SNC, las cuales intervienen en la regulación de los procesos biológicos de percepción del dolor; respuestas de estrés; funciones límbicas de placer y recompensa inducidas por incentivos naturales y/o sustancias psicotrópicas; funciones de estado de alerta y vigilia, funciones cognitivas (de aprendizaje y memoria) y actividades de regulación neuroendocrina.

Además, diversos estudios celulares han mostrado que ambos péptidos opioides son capaces de inducir la internalización aguda o endocitosis del receptor opioide μ en células somáticas transfectadas con el ADN (ADNc) que codifica este mismo receptor opioide. Al igual que otros péptidos opioides (vg., encefalinas), diversos estudios mostraron el catabolismo enzimático de estos péptidos amidados mediante la actividad de enzimas proteolíticas (vg., carboxipeptidasa Y, aminopeptidasa M), lo que ha permitido sugerir que estos péptidos opioides son degradados por rutas de degradación enzimática similares que rigen para múltiples péptidos bioactivos moduladores en el SNC de los mamíferos. Al igual que otros péptidos endógenos, ambas endomorfinas mostraron la capacidad de modular la liberación neuronal de neurotransmisores (DA, NA, 5-HT, ACh) y hormonas péptidas en áreas específicas del cerebro de los mamíferos. Asimismo, ambos péptidos mostraron una capacidad de generar efectos antinociceptivos potentes en forma dosis-dependiente posterior a su administración ICV o IT en animales experimentales, además de generar respuestas de tolerancia cruzada entre ambas endomorfinas y/o entre la EM1 y alcaloides opiáceos del tipo de la morfina.

Palabras clave: Endomorfina, péptidos, nocicepción, analgesia, receptor opioide mu, fisiología, inmuno reactividad.

INTRODUCTION

Extensive studies on opiate receptor pharmacology led to the initial identification of the three major families of endogenous opioid ligands referred to as endorphins,^{1,2} enkephalins,³ and dynorphins,^{4,5} and most recently the nociceptin/OFQ peptide.^{6,7} Cloning and molecular characterization of the genes that encode the different propeptide precursors —namely pro-opiomelanocortin,²

proenkephalin A,⁸⁻¹⁰ prodynorphin,¹¹ and pronociceptin/OFQ peptide—¹²⁻¹⁴ showed that these large opioid propeptide precursors contain different copies of active peptide sequences flanked by pairs of basic amino acids (e.g. Arg-Arg or Arg-Lys), which upon tissue specific endo and exopeptidases¹⁵⁻¹⁷ mature opioid peptides are released from LDCV vesicles into the extracellular space by stimuli-coupling dependent mechanisms.¹⁸⁻²¹ Activation of the G-protein coupled membrane opioid receptors²²⁻²⁶ by

endogenous opioid peptides results in the expression of a vast number of pharmacological, physiological, neuroendocrine, and behavioral responses in the CNS of mammals and humans.²⁷

I. STRUCTURE-RELATIONSHIP ACTIVITY OF ENDOMORPHINS

Naturally occurring opioid peptides identified and cloned from mammalian tissues —such as casomorphin heptapeptide (Tyr-Pro-Phe-Pro-Gly-Pro-Ile), obtained from tryptic digests of β -casein;²⁸ hemorphin-4 (Tyr-Pro-Trp-Thr), obtained from hemoglobin digests;²⁹ the amide tetrapeptides, Tyr-MIF-11 (Tyr-Pro-Leu-Gly-NH₂), and Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH₂), isolated from the bovine and human brains, respectively— display preferential binding affinities and selectivity for the μ -opioid receptor when compared to both δ - and κ -opioid receptors, respectively.^{30,31} Nonetheless the previous observation, these peptides displayed low-to-medium binding affinities (Ki= 10-80 nM) for the μ -opioid receptor.³²

A large number of synthetic analogs of Tyr-W-MIF-1 peptide (Tyr-Pro-Trp-Gly-NH₂), which contain all possible natural amino acid substitutions at position 4 with exception of the Gly residue, were used to screen out peptide compounds capable of displaying high and selective binding properties at the μ -opioid receptor (Ki \leq 1.0 nM). Zadina's group reported the identification of a potent peptide sequence (Tyr-Pro-Trp-Phe-NH₂), named as endomorphin 1 (EM1), which displayed the highest binding affinity (Ki=360 pM, 0.36 nM) and selectivity for the μ -opioid receptor (e.g. 4000-15,000-fold over δ - and κ -opioid receptors, respectively), when compared to other endogenous opioid ligands, including β -endorphin.^{33,34} This peptide sequence identified and isolated from cortical tissues of mammalian brains (e.g. bovine and human).^{33,35} This newly identified opioid

peptide was extremely potent in the guinea pig ileum assay, a classic test for μ -opioid receptor agonist activity³³ shown to display a potent and specific antinociceptive effect in vivo, as shown in the tail-flick test^{33,35} and thereby modulate both supraspinal and spinal antinociception) after ICV or IT injection of the peptide.³⁶⁻⁴⁰ A structurally close related peptide sequence, named as, endomorphin-2 (EM2) (Tyr-Pro-Phe-Phe-NH₂) was subsequently isolated from mammalian tissues³³ and shown to be almost as potent as endomorphin-1.³³

Thus, endomorphins molecules represent the first peptides isolated from brain shown to bind the μ -opioid receptor with high affinity and selectivity and categorized as the natural opioid peptide ligands for the μ -opioid receptor in mammals, including humans.³⁴ However, despite the whole range of activities reported for these peptides (see below), no existing evidences have been reported on the cloning of specific mRNA(s) encoding a precursor molecule(s) for these opioid peptides, as demonstrated for other major family of opioid peptides found in mammals (e.g. proenkephalin A, prodynorphin, POMC, proNOC/OFQ peptide).^{8,12,41}

Structurally, the peptide sequences encoded by each endomorphin molecules (EM1, EM2) have been shown to be completely different from the consensus motif encoded by several endogenous opioid peptides (e.g. endorphins, enkephalins, and dynorphins) as depicted in figure 1. As shown, classical opioid peptides share the Tyr-Gly-Gly-Phe motif at the N-terminus domain of each peptide, whereas EM1 and EM2 contain two pharmacophoric amino acid residues (Tyr [1] and Trp[3] in EM1; Phe[3] replacing Trp [3] in EM2) joined together via a Pro(2) amino acid residue, used as molecular spacer in both peptides. Both phenolic groups (Tyr, Phe) and/or aromatic rings (Trp, Phe) encoded by each amino acid residue represent structural and chemical requirements for opioid receptor recognition.³²

Using nuclear magnetic resonance approaches to reveal amino acid conformation of endomorphin molecules and

Leu-Enkephalin	(Homo sapiens)	YGGFL
Nociceptin/Orphanin FQ	(Homo sapiens)	FGGTGARKSARKLANQ
Dynorphin A	(Homo sapiens)	YGGFLRRIRPKLKWDNQ
Dynorphin B	(Homo sapiens)	YGGFLRRQFKVVTRSQEDPNAYSGELFDA
Met-Enkephalin	(Homo sapiens)	YGGFM
β -Endorphin	(Homo sapiens)	YGGFMTSEKSQTPLVTLFKNAIKNAYKKGE
Endomorphin-1	(Bos Taurus)	YPWF
Endomorphin-2	(Bos Taurus)	YPFF

Figure 1. Representative structural features of active opioid peptides in the CNS of mammals. The figure displays the amino acid sequences of different endogenous opioid peptides previously identified and cloned from the brain of mammals, including the most recent EM1/EM2 peptides, identified and isolated from the human and bovine brain. Structural homology shared by each opioid peptide listed is outlined by the shaded amino acids at the N-terminal domain of each peptide. Implications of the structural homology and identity among mammalian and non-mammalian species (not shown here) demonstrates conservation of shared amino acid sequences along evolution (text and figure were adapted from Darlison and Richter, 1999, and modified for the present review).

the structural domains required to recognize opioid receptors, these studies revealed that both Tyr(1) and Trp(3) side chains display opposite orientations with respect to Pro(2), which provides the necessary stereochemical properties of endomorphin-1 for binding the μ -opioid receptor.⁴² Moreover, using pseudo-proline containing analogs of EM2, it was shown that the Tyr-Pro amide bond forms a cis-conformation structure along the peptide backbone, allowing its molecular conformation to bind and recognize the μ -opioid receptor.⁴³

II. DISTRIBUTION OF ENDOMORPHINS IN CENTRAL AND PERIPHERAL TISSUES OF MAMMALS

Immunological assays (e.g. radioimmunoassays, immunocytochemical analyses) using different rabbit antisera raised against synthetic peptides of EM1 and EM2 showed that endomorphin-1-like immunoreactivities (EM1-2-LIs) are widely distributed throughout the neuroaxis of the rat, primate, and human brains,^{35,44-50} displaying a preferential localization in areas where MOR-LI (μ -opioid receptor-immunoreactivity) is highly expressed (see representative immunoreactive areas in figure 2).⁵¹ Although EM1-2-LIs were localized in cells and fibers in similar regions of the rat CNS (e.g. stria terminalis [ST]; periaqueductal gray [PAG]; locus coeruleus [LC]; parabrachial nucleus [PBN]; hypothalamus [HPT]; nucleus of the solitary tract [NTS]), major differences about the neural distribution of these peptides were detected.^{44,47,49} For instance, EM1-LI was shown to be widely and densely distributed in several forebrain structures (e.g. nucleus accumbens [NAC]; the cortex [Cx]; amygdala [AM]; thalamus [TH]; the hypothalamus [HTH]; striatum [ST]), together with the upper brainstem (e.g., nucleus of the solitary tract [NTS]) and the dorsal root ganglia,^{47,50} whereas EM2-LI was localized mostly in the spinal cord and lower brainstem,^{47,49} showing densely stained cell bodies and axon fibers within the nucleus of the solitary tract (NTS); substantia gelatinosa of the medulla and superficial layers of the spinal cord/dorsal horn, including the lateral hypothalamus.^{44,47,50} Although these studies showed that the anatomical distribution of EM1-2-like immunoreactive material along the rat CNS parallels the regional localization of several endogenous opioid peptides and their cognate opioid receptors,^{52,53} differences observed between the endomorphin material, enkephalin,^{54,55} and dynorphin⁵⁶ peptide systems showed that EM2-LI in the mammalian brain^{47,50} is highly analogous to the distribution of the μ -opioid receptor peptide ligand, β -endorphin (BE).⁵⁷

Moreover, it has been shown that endomorphins do not co-localize with brain areas expressing μ -opioid receptors (e.g. striatum) containing low-to negligible amounts of EM1-2-LI material.⁴⁹ This asymmetric localization between

endomorphins peptides and their corresponding binding sites in areas of the rat brain does not preclude the synaptic activation of membrane bound opioid receptors. However, μ -opioid receptors and EM1-2-LI fibers have been shown to co-localize in limbic structures of the rat brain (e.g. septal nuclei, the bed nucleus of the stria terminalis, NAC, amygdaloid complex, hypothalamic nuclei), whereas in other rat brain regions (e.g. amygdala, thalamus, hypothalamus, PAG), showing for instance the co-localization of low levels of EM1-2-LI in areas displaying high density of opioid-binding sites (figure 2).^{47,49,58}

Very few reports have documented the extraneuronal distribution of endomorphin material in peripheral tissues,³² in contrast with the vast reports describing the distribution and expression of μ -opioid receptors in extraneuronal tissues.²⁷ For instance, using RIA assays in combination with reversed phase high-performance liquid chromatography techniques (RP-HPLC),⁵⁹ it was found the expression of detectable amounts of EM1-LI and EM2-LI in human spleen, as compared to high levels of EM1-2-like immunoreactive material detected in non-neuronal tissues of the rat, such as spleen and thymus, including plasma. These results contrast the low amounts of EM1-2-LI detected in anterior and posterior rat pituitaries, showing that secretion of opioid material from the pituitary gland does not contribute at all to the significant high levels of EM1-2 detected in plasma.⁵⁹ Based on these results, authors

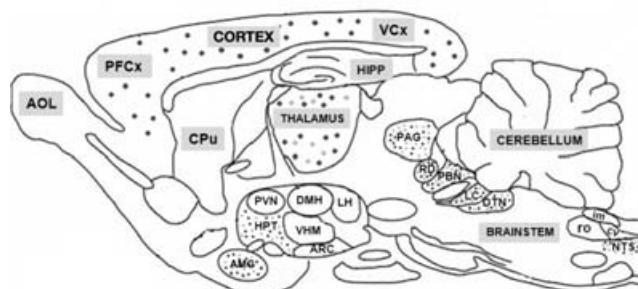


Figure 2. Schematic representation of the distribution of endomorphin immunoreactivity in functional structures of the rat brain implicated in nociceptive transmission and pain modulation. The figure depicts representative brain areas involved in modulation nociceptive transmission, pain sensation. Spotted areas denote the distribution of endomorphin-1 and endomorphin-2-like immunoreactivities (EM1-ir/EM2-ir) in functional areas of the rat brain. Immunoreactive brain areas shown here represent major structures in the CNS where EM1-2 peptides have been shown to display their antinociceptive opioid-dependent effects. Positive areas of the rat brain shown to contain EM1-ir and/or EM2-ir were found to express moderate to dense immunoreactive fibers and somata (not shown) among the distinct regions involved in regulating antinociceptive bioactivities, as depicted in the illustration. Abbreviations: AMG, amygdala; ARC, arcuate nucleus; DMH, dorsomedial, nucleus; DTN, dorsal nucleus; HIPP, hippocampus; HPT, hypothalamus; LC, locus coeruleus; LH, lateral nucleus; NTS, nucleus of the solitary tract (cv, caudal ventrolateral; im, intermediate; ro, rostral); PAG, periaqueductal gray; PBN, parabrachial nucleus; PFCx, prefrontal cortex; PVN, paraventricular nucleus; RD, caudal dorsomedial part of NTS; VCx, visual cortex; VHM, ventromedial nucleus (text and figure were obtained and adapted from Fichna et al., 2007, and modified for the present review).

proposed that EM1-2-like immunoreactive material secreted from the nerve fibers and/or presynaptic terminals localized in the spinal cord may explain the high content of endomorphin material observed in human and/or rat plasma samples.⁵⁹ Moreover, endomorphins were shown to be expressed by immune cells (e.g., macrophages/monocytes, lymphocytes, and polymorphonuclear leukocytes),^{60,61} including inflamed subcutaneous tissue.⁶² Endomorphins have been found to alter a variety of immune parameters and functions,⁶³⁻⁶⁶ as well as to inhibit spleen cell antibody formation when added to in vitro culture systems at femtomolar concentrations (10^{-13} M to 10^{-15} M) and reverted after application of specific EM1/EM2 polyclonal rabbit antisera.⁶⁷

III. BINDING ASSAYS AND CELLULAR ACTIONS OF ENDOMORPHINS

A large number of pharmacological and molecular studies have clearly demonstrated that the μ -opioid receptor mediates the spectrum of opiate-related activities of endomorphins in the CNS of mammals.³² The proposed hypothesis suggesting that endomorphin molecules are the endogenous ligands for the μ -opioid receptor, aroused from classic binding assays and functional studies, which showed that both endomorphin peptides displaced DAMGO (Tyr-DAla-Gly-MePhe-Gly-ol); naloxone, and other μ -opioid receptor-selective ligands, in a concentration-dependent manner⁶⁸ and stimulate the binding of [35 S]guanosine 5'-O-(3-thio)triphosphate ([35 S] γ GTP) in riched mu-opioid receptors-membrane preparations, isolated from thalamus⁶⁹⁻⁷¹ periacuectal gray area (PAG)⁷² and pons/medulla.⁷³ Moreover, autoradiographic studies using radiolabeled tracers of endomorphin molecules showed that these ligands matched the neuroanatomical distribution of DAMGO-binding sites (a conventional μ -opioid receptor selective ligand used as standard in binding studies) in the rodent's brain, postulating thus the role of endomorphins «as the preferential endogenous ligands of the μ -opioid receptor in the brain».³²

However, based on the lower efficacy of endomorphins in stimulating μ -opioid receptors in several bioassays (e.g. ([35 S] γ GTP binding) compared to DAMGO and morphine, both peptides have been regarded as partial agonists of μ -opioid receptors.^{71,74} The extent to which these relative efficacies of endomorphins apply in the expression of functional bioactivities is still unclear and further analysis is needed to solve this issue.

Moreover, pharmacological studies showed that both EM1 and EM2 produce their biological effects by functionally stimulating different μ -opioid receptors subtypes, μ 1 (MOR1)/ μ 2 (MOR2)-opioid receptors, which appear to be responsible for the distinct pharmacological activities

mediated by opiate alkaloids, including both EM1-2 peptides.^{38,40,75} For instance, the μ 1 receptor antagonist, naloxonazine, displayed a potent efficacy in blocking the antinociception response induced by EM2 compared to that exhibited by EM1. Conversely, the μ -opioid receptor antagonist, β -funaltrexamine, was capable of inhibiting the antinociceptive responses induced by both EM1-2 molecules.^{38,40,75}

Pharmacological and molecular studies, showed that spinal administration of specific antisense oligodeoxy-nucleotides (used to knockdown the expression of the μ -opioid receptor genes) differentially attenuated the antinociception induced by either EM1 and/or EM2 peptides.^{76,77} These studies proved that endomorphin molecules activate and modulate μ 2-receptor induced-responses (e.g. spinal analgesia, respiratory depression, and inhibition of gastrointestinal motility),⁷⁸ whereas EM2 appears to modulate μ 1-dependent responses, which include supraspinal analgesia, acetylcholine (ACh), and prolactin release from hypothalamus.⁷⁸ Thus, these data provided significant insights on the differential regulation of μ -opioid receptor subtypes and their interaction with endomorphin peptides.

Quite interesting to note is that in vivo studies using radioligand binding techniques, combined with autoradiographic procedures, demonstrated that endomorphin peptides labeled some binding sites shown for DAMGO in specific reports of the rat brain,^{36,70,71} support the endogenous role of EM1-2 as μ -opioid receptor peptide ligands (partial agonists of μ -opioid receptor) involved in the regulation of several μ 1/ μ 2 opiate-receptor related bioactivities.³²

Furthermore, a vast number of cellular and pharmacological studies showed that chronic exposure of μ -opioid receptor-agonists mediate long-lasting changes and cellular adaptations that underlie the development of opiate tolerance and physical dependence in animals and humans.⁷⁹⁻⁸¹ These observations led pharmacologists to limit a wide range of μ -opioid receptor-binding agents, clinically used to treat pain-related disorders.^{82,83} In vitro studies have shown that chronic administrations of EM1-2 peptides stimulated the development of tolerance in specific neuronal-tumor cell lines (e.g. SH-SY5Y neuroblastoma cells).^{84,85} For instance, chronic exposure of EM1/EM2 peptides on Chinese hamster ovary cells (functionally transfected with a μ -opioid receptor cDNA) induced a naloxone-dependent increase of forskolin-stimulated adenylyl cyclase activity above baseline responses, whereas in monkey kidney cells a specific EM1-2-dependent increase of type I and V-adenylyl cyclase isozymes was observed.⁸⁶

In extension to the aforementioned studies, intracellular signaling pathways and trafficking molecules have been shown to mediate vesicle-endocytosis in brain cells expressing μ -opioid receptors, exposed to both opiates and opioid

peptide ligand agonists.^{87,88} For instance, endomorphins have been shown to induce potent granule/vesicle endocytosis and trafficking of μ -opioid receptor in cells (transfected with the μ -opioid receptor cDNA), inducing similar sort of endocytosis and trafficking mechanisms for μ -opioid receptors in enteric neurons (shown to synthesize and express naturally opioid-receptor binding sites).⁸⁵ This peptide-inducing regulated endocytosis and trafficking of μ -opioid receptor in cells has been shown to mediate desensitization, resensitization, and down-regulation of distinct molecular mechanisms that mediate the cell responsiveness to ligand stimulation, involved in the development of opioid tolerance and drug addiction.⁸⁹

In vivo experiments showed that endomorphin peptides induced a naloxone-precipitated withdrawal response in rats, effects similar to those induced using same dose-unit of morphine.⁹⁰ Using a preestablished scoring system used to detect specific withdrawal signs in rats (e.g. chewing, sniffing, grooming, wet-dog shakes, stretching, yawning, rearing, jumping, teeth grinding, ptosis, diarrhea, penile erection), Chen et al. demonstrated that endomorphin pre-treatment for 5 days (20 μ g, ICV administration), followed by naloxone (4mg/kg, IP) administration, rats displayed physical signs of opioid dependence, exhibiting different potency for different withdrawal signs.⁹⁰

The documented μ -opioid receptor ligand agonists (involved in the development of tolerance and physical dependence in animals and humans) have been suggested to operate through the interaction of conventional transmitter systems (e.g. dopamine[DA],⁹¹⁻⁹³ norepinephrine [NE],⁹⁴⁻⁹⁷ cholinergic [ACh],^{98,99} benzodiazepine,¹⁰⁰ GABA¹⁰¹ and N-methyl-D-aspartate [NMDA],¹⁰² imidazoline¹⁰³ and glutamatergic neural pathways).¹⁰⁴ However, no data on the interactions between endomorphin peptides and aforementioned transmission systems have been reported.³²

Nitric oxide (NO) has been suggested to participate in the cellular mechanisms involved in peptide(s)-inducing physical dependence, based on observations that *NO synthase* inhibitors attenuated some signs of naloxone-precipitated withdrawal and cell-hyperactivity at the locus coeruleus (LC).¹⁰⁵ In addition, the intrathecal (IT) infusion of morphine was shown to increase the N-methyl-D-aspartate (NMDA) binding activity, besides of inducing an up-regulation of NO synthase expression in neurons as well.¹⁰⁶

IV. DEGRADATION OF ENDOMORPHINS: PEPTIDE CATABOLITES AND BIOACTIVITIES

Endomorphins have been shown to be vulnerable to enzymatic cleavage and several enzymes have been proposed as participants in endomorphin degradation.¹⁰⁷ Studies performed to analyze the enzymatic degradation of endomorphins upon exposure to the activity of

proteolytic enzymes (e.g. carboxypeptidase Y and A, proteinase A, and aminopeptidase M) showed a chromatographic profile of recovered endomorphin metabolites eluted from the HPLC column (figures 3A-B).¹⁰⁸ These studies led to the proposal that enzymatic cleavage mediated by aminopeptidase M (EC 3.4.11.2) and/or aminopeptidase P (EC 3.4.11.9) degrade N-terminal Tyr-Pro peptide bonds, thus releasing the N-terminal amino acid (Tyr) and generating a tripeptide fragment from endomorphin molecules.^{109,110} Moreover, it has been shown that peptide sequences containing N-terminal hydrophobic residues followed by a Proline (Pro) residue; the preceding two amino acids (e.g., H₂N-Tyr-Pro) in the peptide moiety may be released by aminopeptidase M, as an intact dipeptide (e.g. Tyr-Pro).¹¹¹ In addition, enzymes—such as carboxypeptidase Y (a serine peptidase, EC 3.4.16.5) and/or proteinase A (a non-pepsin-like acid endopeptidase [EC 3.4.23.6]), shown to display deamidase activities—have been postulated to catalyze the C-terminal amide group and catalyze the hydrolysis of the Xaa(3)-Phe(4) peptide bond (Xaa= natural amino acid), possibly by hydrolyzing EM1-2 peptide sequences into peptide acids, after releasing ammonia and cleaving off the C-terminal Phe(4) amino acid residue (figures 3A-B).^{108,112}

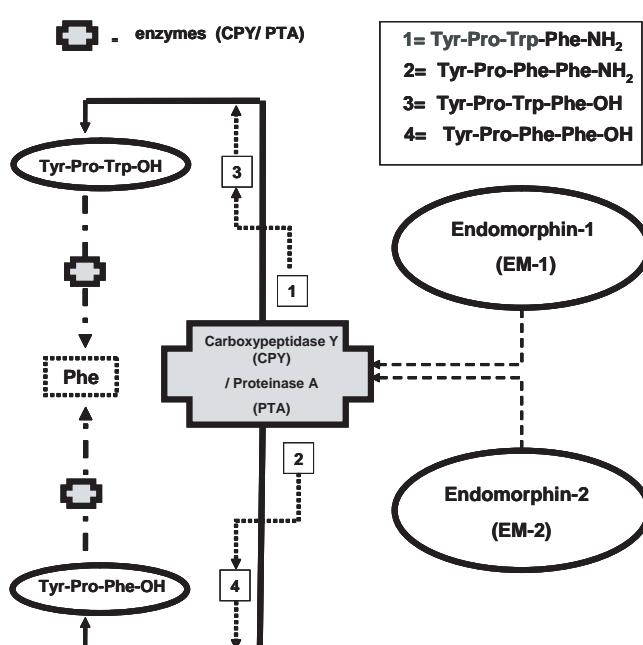
Other enzymes, shown to display peptidase activities on distinct polypeptides, have been included as potential degrading enzymes of endomorphins. For instance, the membrane-bound serine proteinase, *dipeptidyl-peptidase IV (DPP-IV)* (EC.3.4.14.5), has been shown to remove N-terminal dipeptide sequences from different polypeptides that contain the Pro residue at the penultimate position (H₂N-Tyr-Pro....Xn-COO).¹¹³ In vivo studies have shown that two groups of enzymes (e.g. proteinase A, aminopeptidase M/P), working together in related catalytic pathways, may catalyze the degradation of endomorphins from formed dipeptides into single amino acids. In this context, the serine proteinase/DPP-IV system could trigger the catalytic activity of EM1/EM2 by cleaving the Pro(2)-Trp(3) and Pro(2)-Phe(3) peptide bonds, respectively, followed by the aminopeptidase M/P activities, recruited to catalyze secondary peptide cleavages. That is, hydrolyzing released dipeptides (Tyr-Pro/Trp-Phe in EM1; Tyr-Pro/Phe-Phe in EM2) into single amino acids.¹¹⁴ Although the Tyr(1)-Pro(2) peptide bond might also be cleaved during the first step of enzyme processing pathway (dipeptide formation), some authors argued that EM1 appears to be more resistant to enzymatic degradation in vivo than EM2.¹¹⁵ Such proposals appear to be in good agreement with the observations showing that the spinal antinociceptive effects induced by EM1 are significantly longer than those induced by EM2.¹¹⁶ In a similar context, the analgesia induced by EM1 required a longer pre-treatment time than that required for EM2, before tolerance is detected in mice.¹¹⁷ In addition, the antidepressant-like activity induced by EM2 results in a relatively shorter

duration than that detected for EM1 in mice, after ICV administration of each peptide (figures 3A-B).³²

One crucial aspect to elucidate the byproducts formed during the degradation of endomorphins is that the catabolites produced during peptidase activity could display related bioactivities similar to the parent compound. Thus, competition between endomorphin byproducts and full peptide sequences for the m-opioid receptor binding sites would lead to the expression of cellular and related opioid-bioactivities.^{32,118} In such context, radioligand binding studies demonstrated that the primary degradation products of EM1 (Tyr-Pro-Trp-Phe-OH and/or Pro-Trp-Phe-OH) displayed a very low m-opioid receptor binding affinity, showing no substantial activation/stimulation on Go/Gi proteins and non-antinociceptive activity after ICV administration of cleaved catabolites of EM1 in mice (figures 3A-B).¹¹⁸ Parallel studies used to evaluate the

relationship between the binding activity of EM1/EM2 peptides and byproducts of peptide catabolism showed that peptidase inhibitors (e.g. diprotin A [Ile-Pro-Ile] a specific DPP-IV inhibitor) have no effect on the binding properties displayed by [³H]-EM2 on rat brain membrane preparations. However, when no inhibitor was added to the incubation mixture, 40% of the radioligand was degraded by endogenous membrane-bound proteinases.¹¹⁹ Moreover, ICV administration of a different DPP-IV inhibitor (e.g. Ala-pyrrolidonyl-2-nitrile) produced a significant increase in the antinociception activity induced by EM2, as shown for the altered analgesic related-parameters (e.g., magnitude, duration, and potency) detected in the rat tail-flick test.¹²⁰ Similar EM2- induced analgesic effects were observed with diprotin A (a DPP-IV inhibitor), which produced a five-fold increase in potency with respect to EM2 itself in the paw withdrawal test¹¹⁴ or with actinonin (a selective serine-

A



B

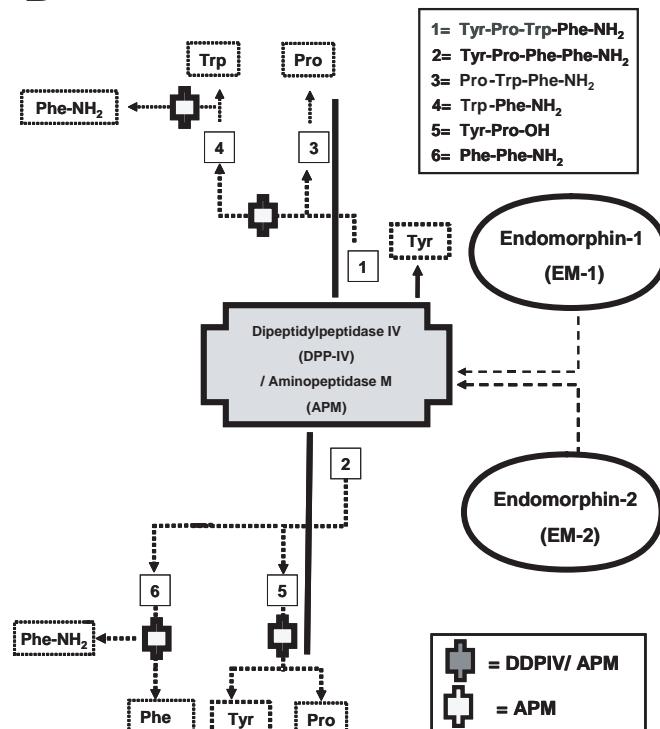


Figure 3. Schematic representation of the proposed enzymatic pathways involved in the catalysis of endomorphin peptides in the brain. Both panels illustrate enzymes and pathways implicated in the degradation of endomorphin peptides in both neural and non-neuronal tissues. Panel A depicts the enzymatic degradation of endomorphins at the C-terminal domain of both molecules. Endomorphins exposed to *Carboxypeptidase Y* (CPY, a serine peptidase, EC 3.4.16.5) and/or *Proteinase A* (PTA, a non-pepsin type acid endopeptidase, EC 3.4.23.6) have been proposed as the enzymatic route implicated in the initial catalysis of the C-terminal domain of both amide tetrapeptides. Thus, both CPY and PTA may hydrolyze the C-terminal amide group (Phe-CO-NH₂) into a carboxyl group (-Phe-OH) followed by enzymatic hydrolysis of the Xaa(3)-Phe(4) peptide bond in EM1 and EM2 molecules, releasing the Phe residue, as shown (see text for more details). Panel B depicts the proposed degradation of endomorphin peptides at the N-terminal domain of both molecules, where the aminopeptidase M (APM, EC 3.4.11.2) and/or aminopeptidase P (APM, EC 3.4.11.9) (not shown) enzymes could hydrolyze the N-terminal domain of both opioid amide tetrapeptides; releasing from either EM1 and/or EM2 peptides, the N-terminal amino acid (Tyr) together with the corresponding tripeptide sequence (EM1; Pro-Trp-Phe-NH₂; EM2; Pro-Phe-Phe-NH₂). Both APM and the membrane bound serine proteinase, dipeptidylpeptidase IV (DPP-IV, EC 3.4.14.5), have been proposed to participate in the initial release of the dipeptide (Tyr-Pro) from both EM1-2-molecules, where APM may hydrolyze the dipeptide bond enhancing the releasing of both N-terminal amino acids from the peptide structure (see text for more details) (text and figure were adapted from Fichna et al., 2007 and modified for the present review).

proteinase/peptidase inhibitor), which blocked the peptide catabolism of EM1, when incubated in a 6-day-old rat spinal cord homogenate, producing a potent antinociception effect compared to the peptide alone.¹²¹

V. ENDOMORPHINS REGULATING CONVENTIONAL NEUROTRANSMITTERS SYSTEMS AND RELEASE OF PEPTIDE HORMONES

Both exogenous and endogenous opioid receptor ligands display their biological activities by modifying the function and release of conventional transmitters in the CNS of mammals, including the evoked-release of various neurohormones from neuroendocrine tissues.³² Opioid ligands regulating neurotransmitter release have been extensively reviewed and reported elsewhere.^{32,122,123}

In this context, μ -opioid receptor agonists have been shown to regulate the neuronal release of *dopamine* (DA),¹²⁴⁻¹²⁷ *norepinephrine* (NA),^{125,128,129} *serotonin* (5-HT),^{122,123,125} and *acetylcholine* (ACh),^{130,131} in areas where μ -opioid receptors have been found to be co-localized with each neurotransmitter in specific areas of the mammalian brain.³²

a) Dopamine (DA) transmission system

Immunohistochemical studies (IHC) revealed that μ -opioid receptors are densely expressed on dopamine neurons in the NAC and striatum in the CNS of rodents, including primates and humans,¹³²⁻¹³⁴ and they play an important regulatory role on the DA transmission system in these brain structures.³² In the striatum, opioid ligands have been shown to act on presynaptic opioid-receptors, producing indirect effects on DA turnover. This effect on DA activity varies according to the sort of opioid receptor bound by ligands.¹³⁵⁻¹³⁷ For instance, striatal administration of ligand agonists acting on postsynaptic μ -opioid receptors induced an increased DA efflux from neuronal cells¹³⁸ without affecting the DA uptake or the levels of DA metabolites (DOPAC, HVA).¹³⁹ Interesting enough is that the modulatory activity of DA release from striatal neurons by μ -opioid receptor agonists, requires the integrity of both cholinergic and GABAergic neurons,¹⁴⁰ including the nigrostriatal neural pathway, where μ -opioid receptor agonists (e.g. morphiceptin) acting at a presynaptic level prevented the neuronal release of DA.^{139,141}

In the accumbal region (NAC), microdialysis studies showed that μ -opioid receptor ligands agonists infused into the NAC induced an increased accumbal DA release or DA efflux in rats¹⁴² as shown for EM1-2 peptides, which produced a dose-dependent increase in DA efflux from NAC in freely moving rats.¹⁴³ Whereas an accumbal infusion of EM1 showed a dose-dependent increase of DA efflux, which was abolished by intraccumbal perfusion of

CTOP or by systemic administration of naloxone and/or naloxonazine,¹⁴³ DA efflux induced by local infusion of EM2 was not abolished by selective μ -opioid receptor antagonists. This suggests that the EM2-induced effects may be mediated through different receptors other than the μ -opioid receptor.¹⁴³ These studies support previous data showing that EM2 induces a dose-dependent stimulation of DA release and levels of DA metabolites (e.g. DOPAC, HVA) from the accumbal shell region¹²⁶ as a result of the disinhibition of local GABA neurons.¹²⁶

b) Noradrenergic (NA) transmission system

Noradrenergic system arises from a clustered group of NA-containing cell bodies, that arises from the locus coeruleus (LC) localized at the rostral area of the pons,¹⁴⁴ and which projects axon fibers to forebrain areas and limbic structures. NA projecting fibers from LC represent the primary source of NA in cortical and hippocampal areas of the brain.¹⁴⁵⁻¹⁴⁷ The LC-NA neurons innervate almost all regions of the neuraxis, influencing several bioactivities and behaviors.¹⁴⁸⁻¹⁵⁰ LC-NA neurons also provide descending fibers to both medulla and spinal cord involved antinociceptive bioactivities.¹⁵¹⁻¹⁵³ Neuroanatomical studies using autoradiographic and electrophysiological procedures using intracellular recording techniques revealed the expression of a high-density of μ -opioid receptor at the LC,¹⁵⁴⁻¹⁵⁶ where endogenous opioid peptides (e.g. morphiceptin, hemorphin-4, Tyr-MIF-1, Tyr-W-MIF-1), including endomorphins and non-natural synthetic peptides (e.g., Tyr-D-Arg-Phe-Sar; Tyr-D-Arg-Phe-Lys-NH₂), shown to bind and activate μ -opioid receptors, were shown to modify the physiological activity of LC neurons.^{32,155}

All peptides tested displayed opiate-alkaloid activities on LC-NA cells,¹⁵⁵ producing a dose- and time- dependent electrophysiological responses (e.g. decreased neuronal excitability; inhibition of spontaneous cell-firing, opioid-induced hyperpolarization effect mediated through the opening of inward-rectification potassium channels, and other membrane bioactivities).¹⁵⁵ Among the tested peptides, EM1 and EM2 represented the most effective peptides displaying equipotent electrophysiological responses (e.g. hyperpolarizations) on LC-NA neurons at very low concentrations, when compared to β -endorphin, DAMGO, or morphine.^{32,155,157} These results led authors to assume that the presence of the aromatic group on the Phe residue at position 4, in addition of the structural C-terminal amidation found in both EM1-2 peptides, represent crucial structural features by which these peptides exert their electrophysiological responses on LC-NA neurons after binding their cognate μ -opioid receptors on these cells.^{32,155}

Ultrastructural studies showed that EM1-LI was mostly localized in axon terminals forming synaptic specializations between tyrosine hydroxylase (TH) and CRF-containing dendrites in peri-LC and Barrington's nucleus (localized at

the brainstem dorsal pontine tegmentum) in the rat brain.¹⁵⁷ Both areas were shown to express high-densities of μ -opioid receptors^{158,159} and were suggested to be targeted by EM1-2 peptides, producing a significant reduction of LC-NA neuronal excitability.¹⁵⁷ Thus, EM1 could modulate LC neurons directly by tonically inhibiting neurons within the Barrington's nucleus.¹⁵⁷ Furthermore, physiological studies showed the interaction between EM1 and NA at the level of the spinal cord, showing that the IT administration of EM1 in rats induced an evoked-release of NA from presynaptic axon terminals that arise from LC-NA descending neural pathways impinging into the dorsal horn of the rat's spinal cord.¹⁶⁰ Thus, EM1 produced a potent analgesia activity (measured in different behavioral models of analgesia) by activating μ 2-opioid receptors.^{32,160}

c) Serotonin (5-HT) transmission system

The serotonin (5-HT) transmission system has been shown to mediate a vast number of physiological and behavioral responses in mammals (e.g. nociceptive, appetitive, emotional, stress and anxiety, motor, cognitive, and autonomic functions).¹⁶¹⁻¹⁶⁵ Several bioactivities mediated by serotonergic tone in the brain have been found to be influenced by the endogenous opioid system (EOS).³² The serotonin (5-HT) transmission system is functionally dependent on the serotonergic tone of the CNS, which is regulated by the firing activity of cell bodies (somata) which are clustered in different nuclei along the midline of the brainstem; namely, the dorsal raphe nucleus (DRN) and the median raphe nucleus (MRN).¹⁶² Both nuclei have been shown to project axon fibers to almost all forebrain structures along the neuroaxis (e.g. mesolimbic areas, hippocampus, and cortex), where 5-HT axons projecting to terminal fields regulate the expression of different bioactivities and behavioral repertoires required for survival and adaptive functions in species.¹⁶² Thus, the 5-H/DRN located at the ventral aspect of the PAG has been shown to be crucial for the integration stress-responses,¹⁶⁶ and pain and stressful stimuli activating peptidergic neurons at the PAG have been shown to modulate the firing activity of 5-HT neurons (at the somatodendritic level) via activation of μ -opioid receptors expressed at synaptic endings in short axons, innervating the 5-HT/DRN.^{167,168} Finally, 5-HT/DRN neurons project axon fibers to several limbic structures (e.g. amygdale, PFCx, hippocampus, NAC) involved in handling emotional and stress-related responses.^{167,169,170} However, the cellular mechanisms by which opioid agonists affect the activity 5-HT neurons have not been completely elucidated.

For instance, previous electrophysiological studies showed, on the one hand, that μ -opioid receptor agonists induced significant inhibitory effects on cell-firing of DRN/5-HT neurons.¹⁷¹ On the other hand, in vivo microdialysis showed that opioid agonists induced an increased activity on 5-HT neurons.^{167,172} Several mechanisms have been

offered to explain such inconsistencies based on the structural organization of the DRN. This brainstem nucleus receives neural inputs from local GABAergic neurons and from descending glutamatergic axon fibers from the cortex, which control the cellular excitability of 5-HT neurons and the net equilibrium between both neural systems, will provide the result on the neuronal release or efflux of 5-HT into the external milieu.¹⁶² In this context, it has been shown that μ -receptor agonists move the net equilibrium of both neural systems into an increased release and extracellular levels of 5-HT at the DRN, possibly mediated through an opioid-enhanced activity of the glutamatergic (excitatory) input on 5-HT/DRN neurons and/or opioid-induced disinhibitory effect of GABAergic neurons.¹²²

Microdialysis studies revealed that endomorphins peptides influenced the activity of DRN/5-HT neurons by increasing the net efflux of 5-HT (increased extracellular levels of the aminergic transmitter) within the DRN. This effect was blocked by selective μ -opioid receptor antagonists such as β -funaltrexamine.¹²² In a similar context, endomorphins have shown to modulate 5-HT activity in other brain areas. For instance, EM1-2 peptides infused into the VTA induced a significant decreased of 5-HT activity in the rat PFCx (cortex) and NAC (ventral striatum), thus suggesting that μ -opioid receptors could potentially mediate a neuronal depletion of 5-HT in these brain areas.¹²⁵ However, no effect on the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA), was found in the NAC.¹²⁶ In forebrain areas, DAMGO and EM1 produced a strong, CTOP-reversible suppression of the 5-HT_{2A}-induced excitatory post-synaptic currents on layer V-pyramidal cells, localized at the medial prefrontal cortex (PFCx).¹⁷³ These studies showed that μ -opioid receptor agonists act presynaptically in cortical layer V pyramidal cells,¹⁷⁴ and the interaction of 5-HT_{2A} and μ -opioid receptor ligands appear to occur on projecting glutamatergic afferents sent from thalamic or amygdale nuclei (e.g. basolateral nucleus)¹⁷⁵⁻¹⁷⁷ that locally synapse layer V-pyramidal cells.¹⁷⁴

d) Acetylcholine (ACh) transmission system

With regard to the ACh-transmision system, several studies showed that EM1-2 peptides modulate cholinergic neurotransmission in peripheral tissues, such as the respiratory system and the gastrointestinal (GI) tract (see corresponding sections xiv and xv in the following chapter, Part II, in this journal) acting on prejunctional μ -opioid receptors that inhibit the release of ACh that mediate the contractile muscle-activity and peristalsis in the GI tract.³² In a similar context, recent experiments showed that EM1 regulates ACh-transmission system in the rat inner hair cells.¹⁷⁸ Using patch-clamp recording techniques, these authors showed that EM1 inhibited the nicotinic receptor-induced ACh-evoked currents in both frog saccular hair cells and rat inner hair cells, respectively.^{32,178}

e) Release of neuropeptides hormones

Electrophysiological studies *in vivo* showed that EOS regulates the electrical activity of the neuron cells that synthesize and secrete oxytocin (OT) (from supraoptic nuclei [SON] localized within the hypothalamus) and arginine vasopressin (AVP) (from the anterior paraventricular nucleus of the hypothalamus [PVN]).^{179,180} Previous studies showed that μ -opioid receptor agonists produced a potent inhibition on OT-secreting cells (SON), whereas neuronal-activity of AVP-secreting cells (PVN) is decreased by the same receptor agonists.¹⁸¹ In a similar context, endorphin peptides showed to display related activities on OT/SON and AVP/PVN cells, respectively, showing that ICV administration of EM1 into rats induced a more potent inhibition effect of OT cells than on AVP cells.¹⁸² These effects detected on SON versus PVN-cells were attributed to the number of cell-surface opioid receptors expressed on each neurosecretory cell.¹⁸² Recent studies showed that the oxytocin (OT) induces important antinociceptive effects in rodents and humans,¹⁸³⁻¹⁸⁸ as shown after ICV,¹⁸⁹ IP,¹⁸³ or IT/IC¹⁸⁴ administration of the peptide hormone, in addition of the injection of the peptide directly into the PAG and NRM (nucleus raphe magnus)^{190,191} (sections x-xiii in the following chapter, Part II, in this journal).

Recent studies shed important data concerning the interaction between μ -opioid receptors and OT-inducing antinociceptive responses in the rat CNS.^{192,193} These studies showed that μ -opioid receptors antagonists (e.g. β -FNA) inhibited OT-induced antinociceptive effects in response to the application of either thermal or mechanical stimuli in rats. So far, no experimental data have elucidated yet any role for the endogenous opioid system (e.g. β -endorphin), including both EM1-2 peptides, mediating the OT-induced antinociception responses in animals.³²

Most of the experimental data reporting on the interaction between the AVP/PVN system and μ -opioid receptor agonists have been shown in stress-related behaviors³² (section xiii, in the following paper Part II, in this journal). However, no experimental works have clearly elucidated the mechanisms by which EOS and endorphin peptide interact with the AVP/PVN peptide system. Nonetheless, recent documents showed that acute stress leads to an increase synaptic concentration of AVP in the rat amygdala or hypothalamus;^{194,195} where AVP released from PVN-neuronal cells was shown to activate its cognate membrane receptor (e.g. V1 receptor) and mediate crucial anxiogenic and depressive behavioral responses in rodents.¹⁹⁵

With regard to somatostatin, μ -opioid receptors ligand agonists,^{196,197} including endorphins,¹⁹⁸ have been reported to regulate gastric-somatostatin and neuroendocrine activity at the GI tract (section xv, in the following chapter Part II, in this journal). For instance, EM1 was shown to exert

an important inhibition on somatostatin-induced stimulation in the perfused rat stomach, which was partially blocked with CTOP.¹⁹⁸ These results posit that EM1 acting on μ -opioid receptors mediate in large part the opioid-inhibitory effect on somatostatin activity.¹⁹⁸ Mediated by the EM1 peptide, this inhibitory effect might be similar for other regulatory peptide-hormones in several systems.³² However, the interaction between peptide-hormone activity and EM1-2 peptides needs further investigation.

VI. ENDOMORPHINS MODULATING NOCICEPTIVE TRANSMISSION AND TOLERANCE

With regard to μ -opioid receptor-mediated pain modulation, *in vivo* studies showed that the intracerebroventricular (ICV) administration of endorphins produced a potent antinociceptive effect in wild type mice,^{33,34,68} showing no significant effect in μ -opioid receptor mutant knockout mice.^{34,199-201} In a similar context, intrathecal (IT) administration produced significant antinociception effects in adult rodents exposed to the tail-flick, paw-withdrawal, tail pressure, and/or flexor-reflex tests, respectively (figures 4A-B).^{31,33,36,116,117,202,203}

Based on the aforementioned description of the neuroanatomical distribution of endorphins and μ -opioid receptors in the rodent, primate and human CNS (see section ii); several authors have categorized the functional role of EM1-2 peptides as neurotransmitters or neuromodulators, regulating different biological and physiological processes, as previously described.³² Such modulatory-related activities mediated by endorphins include pain perception, stress-related responses, complex neural functions —such as reward, arousal, and vigilance—, as well as autonomic, cognitive, neuroendocrine, and limbic homeostasis.³²

Endorphin peptides have been implicated in the modulation of neural pathways of pain and/or nociceptive transmission in several areas of mammals brain,³² particularly, the spino(trigemino)-ponto-amygdaloid pathway, where nuclei and neuronal elements (e.g. caudal nucleus of spinal trigeminal tract, parabrachial nucleus, the NTS, PAG, nucleus ambiguus, LC, and midline thalamic nuclei) have been shown to express EM1-2-LI material (see reviews in^{204,205}) (figures 2 and 4A-B). Based on the anatomical distribution of EM2-LI^{34,62} and its co-localization with μ -opioid receptors —densely expressed in spinal cord neurons and along primary afferent fibers penetrating the superficial layers of the dorsal horn—,⁴⁶ EM2 was found to modulate nociceptive transmission pathway, modulating the release of excitatory transmitters (e.g. glutamate [Glu], substance P, γ -aminobutyric acid [GABA], glycine [Gly], including the calcitonin gene-related peptide [CGRP peptide]) and by stimulating presynaptic μ -opioid receptors

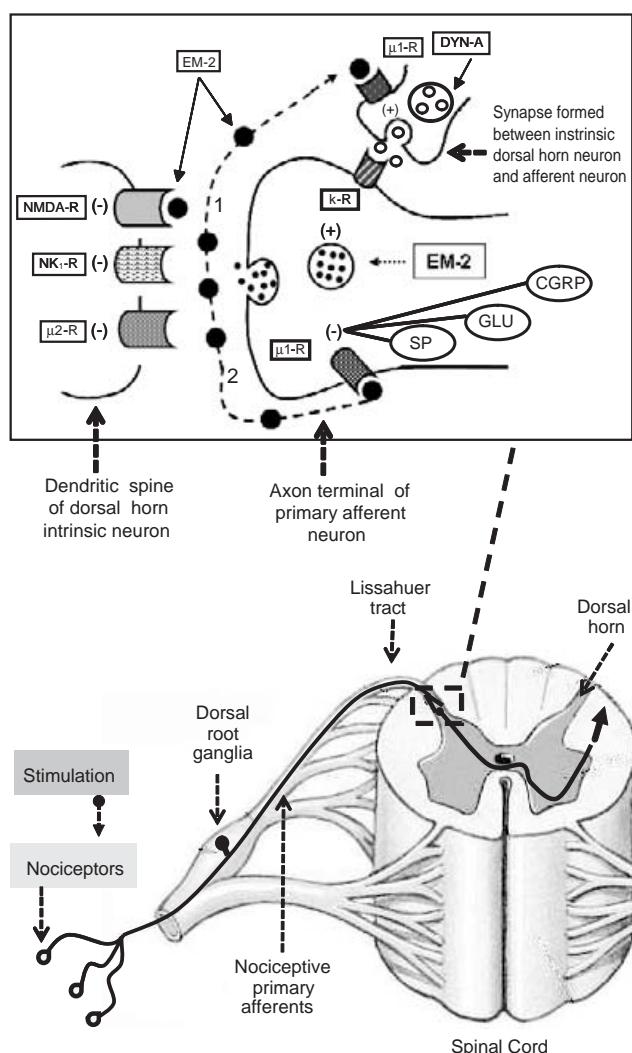
expressed in axon terminals along the Lissahuer tract that penetrates the superficial dorsal horn and established synaptic contacts with intrinsic and relaying neurons of the spinal cord (figure 4A).^{206,207}

Supporting evidences for the modulatory role of EM2 on primary nociceptive transmission pathways come from electrophysiological studies that showed the evoked-release

of EM2 from dense-cored vesicles from spinal neurons after electrical stimulation from the dorsal root ganglion fibers,^{208,209} in addition to the EM-2-induced hyperpolarizing responses on cell-membranes of intrinsic spinal neurons, and the decreased excitability of postsynaptic μ -opioid receptors in intrinsic relay neurons as well.²¹⁰

Furthermore, supraspinal (ICV) and spinal (IT) administration of EM1/EM2 showed that both amide tetrapeptides influence several neurotransmitter systems, in a similar fashion as demonstrated for morphine and DAMGO (preferential agonists of the μ -opioid receptor subtypes MOR1/MOR2) in the rat's CNS.^{33,34} In such a context, anatomical and pharmacological studies have

A



B

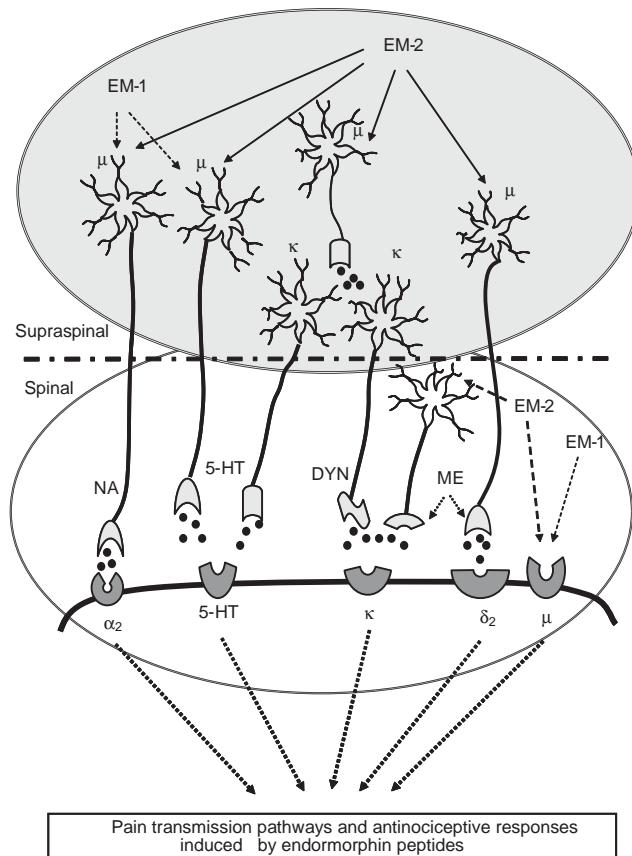


Figure 4. Schematic representation of the putative role of endomorphin-2 modulating nociceptive transmission and pain perception in the brain of mammals. Panel A illustrates a hypothetical situation whereby endogenous EM-2 peptide might be involved in the regulation of nociceptive transmission through the activity of intrinsic spinal neurons. Stimulation of peripheral *nociceptors* will relay nociceptive transmission enhancing the release of EM2 from vesicles localized in axon terminals of penetrating primary neuronal afferents to superficial dorsal horn (Lissahuer tract). (1) EM-2, exerting a regulatory function on postsynaptic cells, may decrease the activity of postsynaptic μ 2-opioid receptors. (2) EM-2 may exert an autoregulatory function via the activation of its presynaptic cognate μ 1-opioid receptor, expressed either on axon terminals of incoming neural afferents or on intrinsic dorsal horn neurons. Thus, presynaptic activation of μ 1-opioid receptors will lead to significant inhibition on the local release of excitatory peptide (Substance P [SP]; calcitonin gene-related peptide [CGRP]) and non-peptide transmitters Glu from primary neuronal afferents; and conversely, EM-2 may enhance the release of dynorphin A (DYN-A) from local spinal neurons (see inset, upper left) (see text for more details).

Panel B depicts different neural pathways involved in nociceptive transmission and modulation of pain during supraspinal and supraspinal administration of EM-1 and EM-2. Abbreviations: DYN, dynorphin A; ME, met-enkephalin; 5-HT, serotonin; NA, noradrenaline; α 2-R, alpha-2 noradrenergic receptor; δ 2-R, delta-2 enkephalin-opioid receptor subtype; μ -R, mu-opioid receptor subtype (see text for more details) (text and figures were adapted from Fichna et al., 2007, and modified for the present review).

extensively shown that neurotransmitters systems and chemical substrates that mediate mesencephalic analgesia induced by morphine (at the rostral ventromedial medulla) include the serotonin (5-HT),^{211,212} the inhibitory GABAergic system,^{213,214} the Glutamatergic and N-methyl-D-aspartate excitatory system,^{215,216} and the neurotensin peptide system as well (figures 4A-B).²¹⁷

Pharmacological studies showed that both NA and 5-HT (acting on spinal cord, α 2- and 5-HT receptors subtypes) mediate antinoceptive/analgesia responses induced by ICV administration of DAMGO,^{218,219} and conversely, the induced-depletion of both neurotransmitters (NA, 5-HT) after IT administration of 6-hydroxydopamine (6-OHDA) and/or 5,7-dihydroxytryptamine (5,7-DHT), respectively, or IT administration of yohimbine and methysergide (used to block spinal α 2-adrenoreceptors and serotonin receptors) respectively; attenuated the antinociception response induced after ICV administration of morphine.²²⁰⁻²²³ In a similar context, the antinoceptive responses induced after ICV administration of EM1/EM2 peptides were completely abolished after depleting (> 90%) the spinal NA system with IT administered 6-OH-DA (3-day pretreatment period with neurotoxic compound).¹²⁹ However, administration of 5,7-DHT (which depleted both 5-HT [$\geq 90\%$] and NA [$\geq 25\%$]) attenuated, but did not block the endomorphin-induced antinociception. These results clearly showed the relevance of the NA system over the 5-HT system in mediating the induced-antinociception by the supraspinal (ICV) administration of endomorphin molecules (figure 4B).^{32,129}

Complementary results showed that the antinoception-induced effect detected after the spinal administration of EM1/EM2 peptides into mice was abolished with μ -receptor antagonists (e.g. naloxone, β -funaltrexamine), but not by the neurotoxic agents, 6-OHDA or 5,7-DHT, respectively,^{129,202} suggesting that the spinal induced-antinociception by EM1/EM2 peptides is caused by the direct stimulation of μ -opioid receptors, without affecting the NA or 5-HT transmission.^{32,129,202} Moreover, parallel studies showed that the supraspinal antinociception induced by ICV administration of EM2, but not EM1, were shown to be mediated by the activity of dynorphin A₁₋₁₇ and Met-enkephalin peptides, acting on their cognate κ - and δ ₂-opioid receptor subtypes, respectively.⁴⁰ Thus, these data demonstrated functional differences in the spinal and supraspinal antinociceptive effects induced by EM1 and EM2 substances.⁴⁰

Several studies provided strong evidence that the peripheral administration of endomorphins produce similar analgesic effect shown after IT or ICV administration of same opioid tetrapeptides. Albeit that the EM1/EM2 peptide-induced analgesia was demonstrated to be mediated through the activation of μ - and δ -opioid receptors expressed on primary neuronal afferents penetrating the dorsal horn (see above and figures 4A-B),²²⁴

²²⁶ the precise mechanisms and neural pathways mediating antinociception from peripherally-administered opioids are still unclear. In this context, several studies showed that EM1 produced a dose-dependent and naloxone-dependent reversible analgesia after i.p. administration in rats, exhibiting a delayed and less pronounced analgesic response when compared to the faster and increased antinociceptive effect induced from ICV or IT administered opioid peptides.²²⁷ The reduced analgesic effect observed after peripheral administration of opioid peptides could be explained by the rapid enzymatic degradation of EM1/EM2 peptides in peripheral tissues (by unspecific membrane proteasas or esterases), besides the low permeation capability of these peptides to cross the brain-blood barrier (BBB), producing a small amount of active substances to elicit a centrally-mediated analgesia response.^{228,229} Whether peripheral administered-opioid peptides produced their antinociceptive/analgesia effect via this route or after permeating the BBB, is still a controversial issue, which requires further investigation (figures 4A-B).³²

With regard to opioid-peptides inducing tolerance, in vivo studies showed that pre-treatment with different doses of EM1 or EM2 peptides in rodents (mice, rats)^{37,84,117} or the initial administration of single high doses of EM1 or EM2 peptides²³⁰⁻²³³ produced acute tolerance to the antinociceptive responses, induced by EM1/EM2 peptides after IT or ICV administration. Although tolerance development by EM1/EM2 was much faster than that induced by morphine, tolerance induced by EM1 required a longer pretreatment time-period when compared to pretreatment with EM2, before the acute antinociceptive tolerance was observed. Such variations in the EM1/EM2 producing acute antinociceptive tolerance responses were proposed to be due to several pharmacological and cell-related mechanisms (e.g. different peptide half-lives, differences in μ -opioid receptor selectivity, divergent neuronal mechanisms), but not to the degree of how EM1-2 peptides stimulate membrane-bound μ -opioid receptors or induced μ -opioid receptor desensitization.²³² Moreover, cross-tolerance studies (evaluating the induced-antinociceptive effects of EM1/EM2 peptides and morphine in both tail-flick and paw pressure tests in rats) showed that animals displayed a tolerance effect to EM2, exhibiting a partial antinociceptive cross-tolerance to EM1. However, rats displaying tolerance to EM1 showed no cross-tolerance to EM2.²³¹

Similarly, these studies showed a cross-tolerance between morphine and EM1, but not for EM2, thus suggesting that morphine/EM1 mediate their analgesic/tolerance effects via a common target opioid receptor (e.g. the μ 2-opioid receptor subtype), whereas EM2 appears to mediate their opioid-related responses via a different membrane-bound opioid receptor (e.g. the μ 1-opioid receptor subtype) or via a spliced variant of the μ 2-opioid receptor and/or the same receptor displaying a different

physical state of the functionally expressed μ -opioid receptor subtype.²³¹

Moreover, cross-tolerance studies (evaluating the induced-antinociceptive effects of EM1-2 peptides and morphine in both tail-flick and paw pressure tests in rats) showed that animals made tolerant to EM2 exhibited a partial antinociceptive cross-tolerance to EM1, whereas rats tolerant to EM1 showed no cross-tolerance to EM2.²³¹ Similarly, these studies showed a cross-tolerance between morphine and EM1, but not for EM2, thus suggesting that morphine/EM1 mediate their analgesic/tolerance effects via a common target opioid receptor (e.g. the μ 2-opioid receptor subtype), whereas EM2 could mediate their opioid-related responses via a different membrane-bound opioid receptor (e.g., the μ 1-opioid receptor subtype) or via a spliced variant of the μ 2-opioid receptor and/or the same receptor displaying a different physical state of the functionally expressed μ -opioid receptor subtype.²³¹ Albeit the several studies used to characterize the development of tolerance to the antinociceptive effects mediated by EM1-2 peptides in animal models of pain, tolerance responses to other endomorphin-inducing activities have not been elucidated and/or compared to morphine, its parent compound. For instance, tolerance does not develop for all of the alkaloid-mediating or inducing behavioral or functional responses (e.g. tolerance does not develop during morphine-inducing locomotor activity responses) (see corresponding sections vii-ix in the following chapter, part II, in this journal).²³⁴

ACKNOWLEDGEMENTS

This review is dedicated to the memory of profesor Ramon de la Fuente Muñiz, whose vision and knowledge in distinct areas on the neuroscience research led to the support and development of several projects in our lab. Moreover, we gratefully thank the following institutions for the funding of the present manuscript: National Institute of Psychiatry (Instituto Nacional de Psiquiatria) Mexico-Project INPRF-NC092318.1; INP-2040, ICYTDF-2007; CONACYT-SALUD-2003-COI-14; CONACyT/SEP-COI-47804; CONACyT-FOSSIS/SALUD-2007-COI-69373; Megaproyecto UNAM MP6-16; Fundación Gonzalo Rio Arronte A.C. CONADIC and BIRMEC are also acknowledged for supporting in the preparation of this manuscript.

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