Endogenous Opioid Peptides

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There exist three main types of endogenous opioid peptides, enkephalins, dynorphins and β-endorphin, all of which are derived from their precursors. These endogenous opioid peptides act through opioid receptors, including mu opioid receptor (MOR), delta opioid receptor (DOR) and kappa opioid receptor (KOR), and play important roles not only in analgesia, but also many other biological processes such as reward, stress response, feeding and emotion.

β-endorphin dynorphin A [Met]5Enkephalin-Arg6-Phe7 endormorphins mu opioid receptor MOR OPRM1 alternative splicing G protein β-arrestin biased signaling

1. Introduction

Discovery of the three main types of endogenous opioid peptides, enkephalins, dynorphins and β-endorphin in the 1970s [1][2][3][4] with help by early established opioid receptor binding assays [5][6][7] revolutionized the opioid field and further advanced our understanding of opioid receptor subtypes. Decades of research have revealed that all these endogenous opioid peptides play important roles in many biological systems by acting through opioid receptors. Molecular cloning of the delta opioid receptor (DOR-1) in 1992 [8][9] quickly led to isolate the mu opioid receptor (MOR) [10][11][12][13] and kappa opioid receptor (KOR-1) [14][15][16]. These discoveries not only validated the pharmacologically defined opioid receptor subtypes, but also provided essential tools to investigate the mechanisms and functions of the endogenous opioid peptides. A single-copy gene was identified for each of these receptors. The MOR gene (OPRM1) undergoes extensive alternative pre-mRNA splicing, producing multiple splice variants or receptor isoforms (see reviews: [17][18][19]. Although several splice variants were identified in OPRD1 [20][21] and OPRK1 genes [21][22][23], the extent of the OPRM1 alternative splicing is far larger and more complex than the OPRD1 and OPRK1. Conservation of the OPRM1 alternative splicing from rodent to human also suggests the evolutionary importance of the OPRM1 alternative splicing and resulting splice variants.

2. The Opioid Receptors and Endogenous Opioid Peptides

The opiates derived from opium have been used for thousands of years. However, the concept of opiate receptors was only proposed several decades ago based on the strict structural requirements needed for opiate activity [24][25][26][27][28][29]. Subsequently, Martin proposed the existence of opioid subtypes in his proposal of receptor dualism

[30] and then suggested M and N receptors, which later were referred to mu (morphine) and kappa (ketocyclazocine) receptors, respectively [31]. Soon afterwards, the delta-opioid receptor was proposed as the recognition sites for the enkephalins [32][33][34]. In 1973, three laboratories experimentally demonstrated opioid binding sites in the central nervous system for the first time using various ^{3}H-labeled ligands, including ^{3}H-naloxone [5], ^{3}H-dihydromorphine [6] and ^{3}H-etorphine [7]. The high stereospecificity and selectivity of the binding for opiates were consistent with the basis for a receptor [35]. Biochemical and pharmacological studies further confirmed the protein nature of the binding sites by their sensitivity to proteases, including trypsin and chymotrypsin [7][36][37], as well as the reagents targeting sulfhydryl groups [36][37], and their insensitivity to DNase, RNase, neuraminidase and phospholipase C [7][36][37].

The identification of opioid receptor binding sites in the brain quickly let to the quest of their endogenous ligands. The endogenous opioid-like substances in the brain were first disclosed by several labs at a meeting of the Neuroscience Research Program in Boston in 1974 sponsored by the Massachusetts Institute of Technology [38]. Subsequently, Kosterlitz and Hughes were the first to report the sequences of two pentapeptide enkephalins [32]. This was quickly followed by the isolation of two other endogenous opioid peptides, dynorphin and β-endorphin [1][2][3][4][39]. Similar to most neuropeptides, all these peptides are produced through post-translational modifications of their precursors, proenkephalin, prodynorphin and proopiomelanocortin (POMC), by several processing enzymes and peptidases (Figure 1) [40]. Both proenkephalin and prodynorphin generate several opioid peptides, while POMC yields only β-endorphin in addition to some non-opioid peptides such as adrenocorticotropin and α-melanocyte-stimulating hormone.

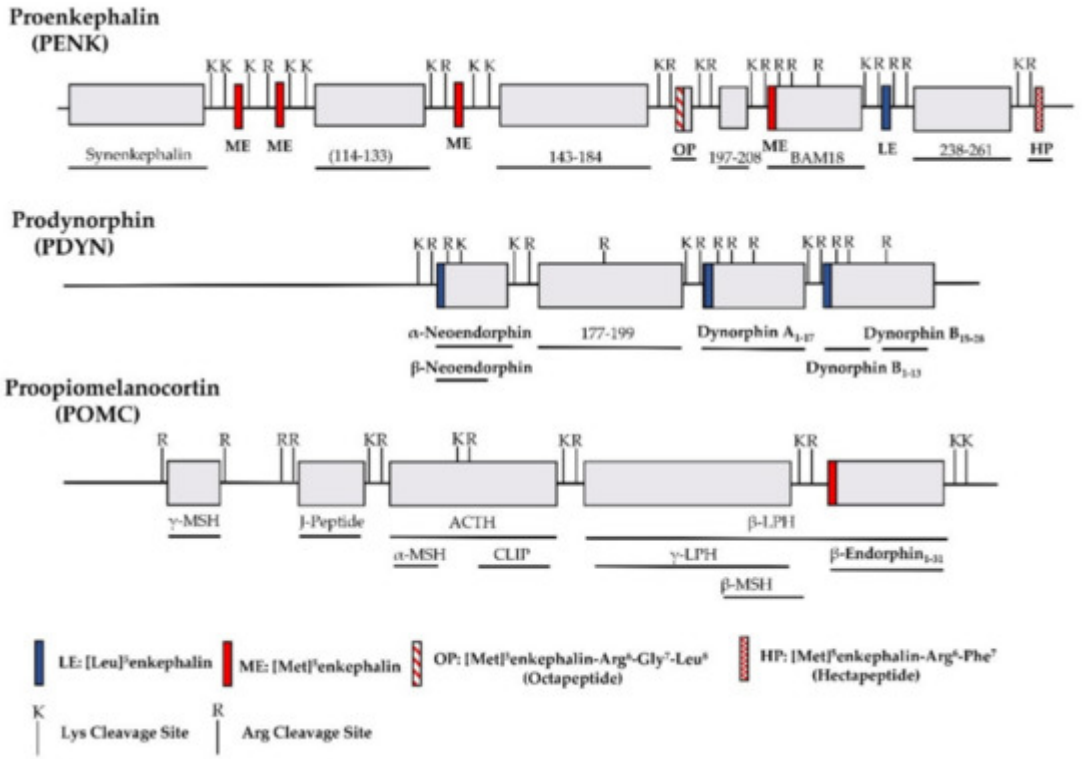

Figure 1. Schematic of the major endogenous peptides processed from human proenkephalin (PENK), prodynorphin (PDYN) and proopiomelanocortin (POMC). BAM: bovine adrenal medulla peptide; MSH: melanocyte

stimulating hormone; ACTH: adrenocorticotropic hormone; CLIP: corticotropin-like intermediate lobe peptide; LPH: lipotropin.

All the endogenous opioid peptides contain the enkephalin sequence, Tyr-Gly-Gly-Phe-Leu or Tyr-Gly-Gly-Phe-Met, at the *N*-terminus with different *C*-terminal sequences (Table 1). The enkephalins are the endogenous ligands for the delta-opioid receptor (DOR-1). Although dynorphins are considered endogenous agonists for the kappa1-opioid receptor (KOR-1), they bind to the mu-opioid receptor (MOR-1) and DOR-1 with high affinities as well [41][42]. Additionally, β-endorphin is thought to be an endogenous agonist of MOR-1, but has high affinity for DOR-1 [42].

Table 1. Amino acid sequences of selected human endogenous opioid peptides.

Precursor	Opioid Peptide	Copies of Peptide	Structure	Other Peptides
Proenkephalin (PENK)	[Leu]^{5}enkephalin	1	Tyr-Gly-Gly-Phe-Leu	Synenkephalin
	[Met]^{5}enkephalin	4	Tyr-Gly-Gly-Phe-Met	
	[Met]^{5}enkephalin-Arg^6-Gly^7-Leu^8(Octapeptide)	1	Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu	
	[Met]^{5}enkephalin-Arg^6-Phe^7 (Heptapeptide)	1	Tyr-Gly-Gly-Phe-Met-Arg-Ph	
Prodynorphin (PDYN)	Dynorphin A_{1-17}	1	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln	α-neoendorphin, β-neoendorphin, Big dynorphin, Leumorphin
	Dynorphin B_{1-13}	1	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr	
Pro-opiomelanocortin (POMC)	β_h-Endorphin$_{1-31}$	1	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu	γ-MSH, ACTH, α-MSH, CLIP, β-LPH, γ-LPH, β-MSH
Unknown	Endomorphin-1		Tyr-Pro-Trp-Phe-NH_2	
	Endomorphin-2		Tyr-Pro-Phe-Phe-NH_2	

Another group of endogenous opioid peptides are endomorphins, including endomorphin-1 (Tyr-Pro-Trp-Phe-NH2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH2) [43]. Both endomorphins lack the common enkephalin motif (Try-Gly-Gly-Phe) shared by other opioid peptides. However, they are the ligands highly selective for the mu-opioid receptor

(MOR-1). The distribution and function of endomorphins have been extensively studied [44]. However, the precursors for these endomorphins or their genes remain to be identified.

Enkephalins are widely distributed in the central nervous system, such as the striatum, hypothalamus, thalamus, hippocampus, pons, medulla and spinal cord. Dynorphins have similar distributions as enkephalins with a few exceptions. POMC is mainly synthesized in the pituitary gland. POMC mRNA is highly expressed in the hypothalamus and detected in the caudal nucleus tractus solitarius and the commissural nucleus, as well as in peripheral tissues such as testis, gut, kidney, adrenal and skin. Extensive studies showed that all these endogenous opioid peptides play important roles in a variety of biological functions. In addition to analgesia, they can modulate reward, addiction, stress response, emotion and feeding (see reviews: [42][45][46][47][48][49][50][51]). Several transgenic mouse models targeting either the precursors or encoded peptides were generated to study in vivo function of these endogenous opioid peptides [52][53][54][55][56].

3. Alternative Splicing of Mu-Opioid Receptor Gene, OPRM1

The mu-opioid receptor has a special place within the opioid receptor family because it mediates the actions of most of the clinically used opioids such as morphine and fentanyl, as well as drugs of abuse such as heroin. The existence of multiple mu-opioid receptors has been long suggested by clinical observations that patients often show different sensitivities towards various mu opioids not only in analgesia, but also in their side-effects including tolerance, dependence, itch, constipation and addiction. Furthermore, incomplete cross tolerance in patients has led to the clinical practice of opioid rotation in which patients who develop tolerance to one mu opioid must use much higher doses of the opioid for pain relief can take back analgesic control by switching to another mu opioid with lower doses. Similar observations were seen in animal models [57][58][59][60][61]. It is difficult to interpret these observations using a single mu receptor mechanism. Early pharmacological studies defined mu_1 and mu_2 receptors using in vivo behavioral assays and in vitro opioid receptor binding assays with newly synthesized antagonists including naloxazone and naloxonazine [62][63][64][65][66][67] and also morphine-6β-glucuronide (M6G) receptor [68][69][70][71]. However, genomic characterization of the MOR gene using the MOR cDNA clones and the human genome sequencing project revealed only a single copy of the MOR gene, OPRM1, raising questions about how a single copy of OPRM1 gene reconciles multiple mu-opioid receptors suggested by clinical observations and the pharmacological studies.

One hypothesis to address these questions is that the single copy of the OPRM1 gene creates multiple mu-opioid receptor splice variants or isoforms through alternative pre-mRNA splicing. Driven by this hypothesis, many efforts have been made to isolate alternatively spliced MOR variants in the past decades. We now know that the OPRM1 gene goes through extensive alternative splicing, generating an array of splice variants, which is far more complex than those suggested by the early pharmacological studies (see review: [17][18][19]). The OPRM1 alternative splicing is conserved from rodent to human. Interestingly, only the OPRM1 gene, but no other opioid receptor genes, underwent extensive and conserved alternative splicing, suggesting the evolutionary importance of the OPRM1 gene.

The OPRM1 splice variants can be categorized into three main types [18][19]: (1) the full-length 7 transmembrane (TM) *C*-terminal variants produced by alternative 3′ splicing (Figure 2). These 7TM *C*-terminal variants have identical receptor structures including the *N*-terminus, TM regions, intra-/extra-cellular loops and part of intracellular *C*-terminus, except for their differences at the *C*-terminal tails; (2) the truncated 6TM variants that lack the extracellular *N*-terminus and the first TM, generated by a combination of alternative promoter, exon skipping, alternative 5′ and/or 3′ splicing; (3) the truncated 1TM variants that contain only the extracellular *N*-terminus and the first TM, generated by exon skipping or insertion.

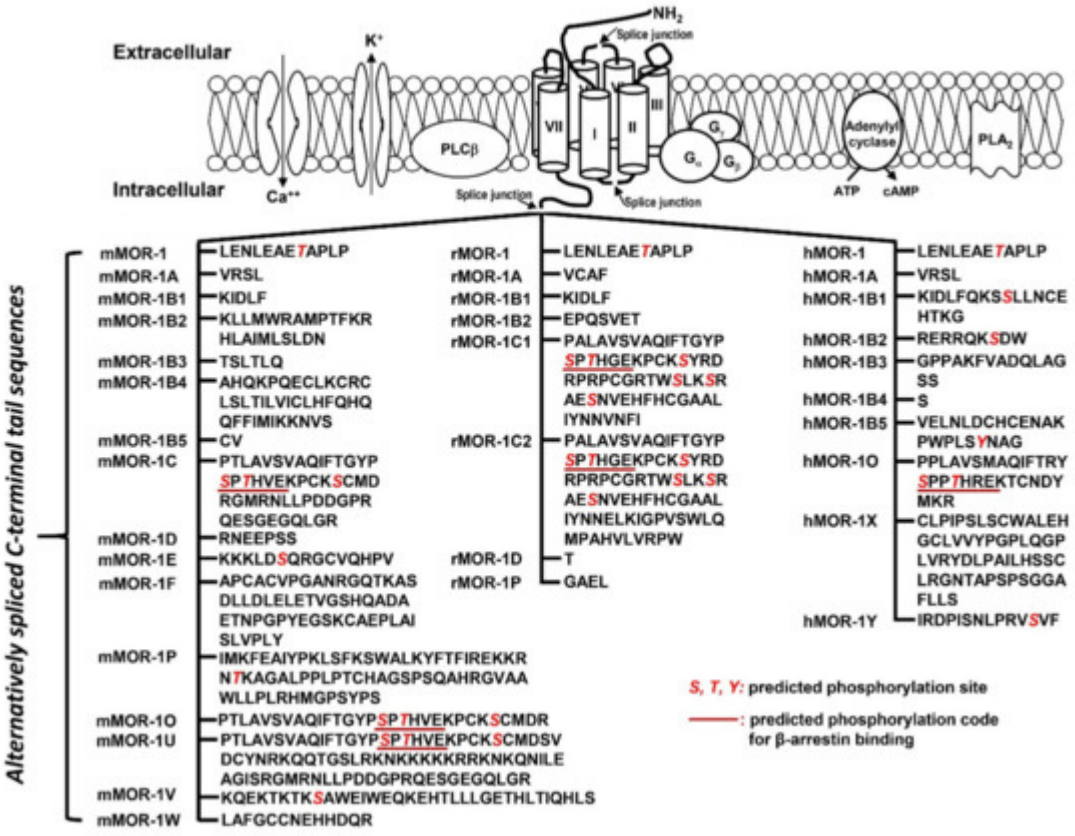

Figure 2. Predicted amino acid sequences from 7TM *C*-terminal variants (modified from [17]. The top panel is an animation that shows structures of MORs and adjacent proteins on membrane. TM domains are indicated by cylinders. Splice junctions are shown by arrows. Calcium (Ca++) and potassium (K+) channels are indicated by opened canals across membrane. Gα, Gβ and Gγ: G proteins; PLCβ: phospholipase Cβ; PLA2: phospholipase A2; The bottom panel listed predicted amino acid sequences encoded by downstream exons of exon 3 in mouse (mMOR), rat (rMOR) and human (hMOR) splice variants. Italic red S, T and Y are predicted phosphorylation sites. Underlined sequences are predicted phosphorylation codes, PxPxxE/D or PxxPxxE/D, for β-arrestin binding based on crystal G protein coupled receptors (GPCR) structures [72].

The functional relevance of the full-length 7TM *C*-terminal variants has been indicated by their differences in mu agonist-induced G protein coupling [73][74][75][76][77][78][79], β-arrestin2 recruitment [80][81], internalization [82][83], phosphorylation [82] and post-endocytic sorting [84] when expressed in cell lines. The 7TM *C*-terminal variants were differentially expressed in various brain regions or different inbred mouse strains at the mRNA level [85][86], and at

the protein level [87][88]. Dysregulation of these variant mRNAs was observed in the medial prefrontal cortex of human heroin abusers and heroin self-administering rats [89], multiple brain regions of morphine tolerant mice [85], and HIV patients [90][91]. Importantly, in vivo functions of these 7TM *C*-terminal variants were demonstrated in morphine-induced tolerance, dependence and reward using several *C*-terminal truncation mouse models [80]. For example, truncating exon 7-encoded *C*-terminal sequences reduced morphine tolerance and reward without the effect on morphine dependence. Conversely, truncating exon 4-encoded *C*-terminal sequences facilitated morphine tolerance and reduced morphine dependence without the effect on morphine reward. The mouse MOR-1D and human MOR-1Y involved morphine-induced itch (pruritus) [92][93].

The truncated 6TM variants mediated the analgesic actions of a subset of mu opioids including heroin, M6G [94], buprenorphine [95] and a novel class of opioid analgesics such as 3′-iodobenzoyl-6β-naltrexamide (IBNtxA) that are potent against a broad spectrum of pain models without many side-effects associated with traditional opiates [96][97]. The 1TM variants did not bind any opioids. However, the 1TM variants can increase expression of 7TM MOR-1 at the protein level as a molecular chaperon to enhance morphine analgesia [98]. The 6TM variants can also facilitate expression of 7TM MOR-1 at protein level through heterodimerization [99].

4. Binding Affinities of Endogenous Opioid Peptides in the Full-Length 7TM *C*-terminal Splice Variants

Soon after each 7TM variant cDNAs were cloned, the cell lines that stably expressed each of the individual 7TM variants in Chinese Hamster Ovary (CHO) and Human embryonic kidney (HEK) 293 cells were established [74][75][76][78][79][82][100] and initially used in opioid receptor binding assays to define their binding profiles. Saturation studies using [^{3}H][$_D$-Ala^2,N-MePhe^4,Gly-ol]-enkephalin (DAMGO), a synthetic opioid peptide and a full mu agonist, as indicated by the K_d values at subnanomolar range, suggest that [^{3}H]DAMGO has a high affinity to all these 7TM *C*-terminal variants. Competition studies using [^{3}H]DAMGO with various opioids, such as morphine, M6G and naloxone, further established their mu selectivity by the fact that all mu opioids competed the binding potently, as indicated by the K_i values at subnanomolar range, while delta or kappa drugs failed to compete at the concentration of over 500 nM. These results were not surprising because all these 7TM *C*-terminal variants contain the same binding pocket, which is mainly constituted by the transmembrane domains and extracellular loops. However, several endogenous opioid peptides displayed differential binding affinities among the 7TM *C*-terminal variants. Table 2 summaries the results of the K_i values of several endogenous opioid peptides against the mouse, rat and human 7TM *C*-terminal variants from several early studies [73][74][75][76][77][78][79][100]. Although these studies were performed at different times when the variants were isolated, the complied data provides reasonable comparisons regarding the binding affinities of the indicated endogenous opioid peptides among the 7TM *C*-terminal variants because all the competition assays were performed using [^{3}H]DAMGO with membranes isolated from the stable cell lines using the same parental CHO cells. The K_i values of DAMGO and morphine are also listed for the comparison.

Table 2. Competition of [^{3}H]DAMGO binding in Chinese Hamster Ovary (CHO) cells stably expressing mouse, rat and human Oprm1 7TM *C*-terminal variants.

K_i Value (nM)	Ligand DAMGO	Morphine	Fentanyl	Methadone	M6G	β-Endorphin	Dynorphin A	Endomorphin 1	Endomorphin 2	[Met]^{5}Enkephalin-Arg^6-Phe^7	Refs.
Mouse											
mMOR-1	1.8 ± 0.5	5.3 ± 2.0	2.3 ± 1.0	1.4 ± 0.1	5.2 ± 1.8	11 ± 2.9	11 ± 0.5	2.1 ± 0.8	4.2 ± 1.8	4.1 ± 1.0	[73] [100]
mMOR-1A	1.0 ± 0.3	3.1 ± 0.5	1.5 ± 0.6	0.7 ± 0.1	5.0 ± 1.5	4.3 ± 1.0	8.2 ± 2.8			3.5 ± 1.3	[73] [77]
mMOR-1C	0.93 ± 0.2	2.4 ± 0.6	1.2 ± 0.4	0.5 ± 0.1	4.1 ± 1.2	5.8 ± 0.5	5.6 ± 0.8	1.4 ± 0.4	1.6 ± 0.2	2.1 ± 0.7	[73] [100]
mMOR-1D	0.71 ± 0.1	1.5 ± 0.2	3.3 ± 1.5	1.4 ± 0.1	4.8 ± 0.8	1.7 ± 0.4	2.2 ± 0.8	1.8 ± 0.3	2.0 ± 0.3	3.7 ± 1.2	[73] [100]
mMOR-1E	1.2 ± 0.5	2.3 ± 0.4	1.2 ± 0.5	0.7 ± 0.3	5.6 ± 0.7	5.0 ± 1.2	8.9 ± 1.1	2.4 ± 0.1	4.4 ± 0.8	4.4 ± 0.9	[73] [100]
mMOR-1B1	1.4 ± 0.2	5.3 ± 1.0			10 ± 1.6	6.8 ± 3.2	15 ± 7.1	11 ± 5.6	12 ± 1.5		[75]
mMOR-1B2	1.3 ± 0.1	3.9 ± 0.4			8.4 ± 1.3	4.9 ± 1.7	34 ± 18	5.0 ± 1.8	8.4 ± 1.1		[75]
mMOR-1B3	1.8 ± 0.9	1.5 ± 0.5			3.9 ± 1.3	3.1 ± 1.4	8.7 ± 1.8	3.2 ± 0.6	3.2 ± 0.8		[75]
mMOR-1B5	1.0 ± 0.3	1.4 ± 0.6			5.2 ± 0.1	5.7 ± 1.2	8.9 ± 2.3	4.3 ± 0.8	11 ± 1.8		[75]
mMOR-1F	1.1 ± 0.2	2.9 ± 0.5	1.7 ± 0.5	1.3 ± 0.2	9.6 ± 0.8	6.0 ± 1.6	12 ± 1.0	2.9 ± 0.5	4.1 ± 1.3	3.9 ± 0.8	[73] [78]
mMOR-1O	3.3 ± 1.2	2.7 ± 0.6			17 ± 1.0	16 ± 5.3	58 ± 26				[77]
mMOR-1P	0.8 ± 0.3	1.2 ± 0.8			11 ±	5.9 ± 2.4	103 ± 23				[77]

K_i Value (nM)	Ligand DAMGO	Morphine	Fentanyl	Methadone	M6G	β-Endorphin	Dynorphin A	Endomorphin 1	Endomorphin 2	[Met]^{5}Enkephalin-Arg^6-Phe^7	Refs.
					3.4						
Rat											
rMOR-1	3.3 ± 0.6	5.6 ± 0.8			17 ± 2.2	3.7 ± 0.4	12 ± 3.0	4.1 ± 0.7	8.0 ± 2.0		[74]
rMOR-1A	6.0 ± 0.9	8.0 ± 0.4			26 ± 2.1	11 ± 0.6	23 ± 1.6	6.5 ± 0.3	12 ± 0.6		[74]
rMOR-1C1	4.5 ± 0.9	7.4 ± 0.3			25 ± 2.4	8.8 ± 0.5	13 ± 2.3	3.9 ± 0.1	10 ± 0.6		[74]
rMOR-1D	4.7 ± 1.2	7.4 ± 0.5			21 ± 1.8	8.5 ± 0.6	11 ± 1.7	3.9 ± 0.4	7.5 ± 0.4		[74]
Human											
hMOR-1	1.2 ± 0.2	2.2 ± 0.9			10 ± 0.3	15 ± 11.0	87 ± 14	4.2 ± 1.4	15 ± 7.1		[76]
hMOR-1B1	1.2 ± 0.4	2.4 ± 1.1			5.0 ± 0.2	7.8 ± 1.5	19 ± 6.6	3.8 ± 0.8	5.4 ± 0.6		[76]
hMOR-1B2	5.2 ± 1.4	11 ± 3.5			42 ± 7.9	25 ± 5.1	49 ± 22	12 ± 0.1	20 ± 1.3		[76]
hMOR-1B3	1.8 ± 0.5	3.2 ± 0.6			16 ± 1.2	8.2 ± 2.2	14 ± 2.3	4.9 ± 1.5	6.3 ± 1.5		[76]
hMOR-1B4	2.3 ± 0.6	5.5 ± 1.7			23 ± 7.4	16 ± 0.4	71 ± 30	9.9 ± 2.3	23 ± 2.0		[76]
hMOR-1B5	2.1 ± 0.4	3.9 ± 0.9			12 ± 2.6	10 ± 3.4	53 ± 23	4.6 ± 0.3	9.6 ± 3.0		[76]
hMOR-1O	2.2 ± 0.6	2.0 ± 0.7			16 ± 2.6		25 ± 8.5				[79]

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extraordinarily potent opioid peptide. Proc. Natl. Acad. Sci. USA 1979, 76, 6666–6670.

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K_i Value (nM)	DAMGO	Morphine	Fentanyl	Methadone	M6G	β-Endorphin	Ligand Dynorphin A	Endomorphin 1	Endomorphin 2	[Met]^{5}Enkephalin-Arg^6-Phe^7	Refs.
hMOR-1X	2.1 ± 0.2	2.7 ± 1.0			17 ± 5.3		187 ± 27				[79]
hMOR-1Y	2.5 ± 0.8	4.3 ± 1.7			8.3 ± 2.2	8.4 ± 1.8	25 ± 13	5.1 ± 1.1	9.4 ± 3.0		[76]

[^{3}H]DAMGO binding was performed with membranes prepared from CHO cells stably expressing indicated splice variants, as described in indicated references.

One intriguing observation from Table 2 is that the binding profiles of the endogenous opioid peptides among the 7TM C-terminal variants were different from those of DAMGO, morphine, fentanyl and methadone, all of which had similar K_i values against various 7TM C-terminal variants. For example, DAMGO's K_i values had a range of 0.7–3.3 nM among the mouse 7TM *C*-terminal variants, while fentanyl's K_i values arranged from 1.2–3.3 nM among the mouse variants. However, endogenous opioid peptides, particularly dynorphin A and β-endorphin, displayed versatile K_i value ranges toward the 7TM C-terminal variants. For example, in the mouse 7TM C-terminal variants, dynorphin A had higher affinities in mMOR-1C (5.6 nM) and mMOR-1D (2.2 nM), but showed lower affinities in mMOR-1O (58 nM) and mMOR-1P (103 nM), while it had intermediate K_i values in other variants. Similarly, there was a 13-fold difference in the K_i values of dynorphin A between hMOR-1X (186.8 nM) and hMOR-1B3 (13.8 nM). Similar scenarios were seen in β-endorphin and endomorphins. β-endorphin competed the binding more potent in mMOR-1D (1.7 nM) than in mMOR-1O (16 nM), an over 9-fold difference. Both endomorphin-1 and endomorphin-2 had higher affinities in mMOR-1C compared to lower affinities in mMOR-1B1. Furthermore, M6G showed moderately different K_i values, particularly against the human variants. All the 7TM *C*-terminal variants share the same opioid binding pocket but contain a different intracellular C-terminal tail sequence. Why can these C-terminal sequences away from the binding pocket modulate the binding affinities of the endogenous opioid peptides, but not DAMGO, morphine, fentanyl and methadone? The crystal structure of the MOR in both agonist and antagonist conformations has been resolved [101][102], providing the fundamental basis of our understanding on structural relationships of ligand-receptor interactions. However, these crystal structures were determined by using the *N*-terminal and C-terminal truncated receptor to allow for the establishment of the stabilized crystal structures, offering no information on the role of the C-terminal sequences on overall MOR structure. Although future structural determination of various C-terminal tails' role on the ligand binding would give an ideal answer to the question, we speculate two possible mechanisms: (1) the intracellular loops, especially the intracellular loop II and III, can impact G protein coupling or receptor agonist conformation. Potential interactions of the C-terminal tail sequences with these intracellular loop regions could differentially modulate the receptor agonist conformation especially for the endogenous opioid peptides; (2) several known proteins such as G proteins and β-arrestins or unknown proteins can associate with the MORs at basal or active states, influencing ligand binding. The C-terminal tail sequences could alter the receptor agonist conformation mainly for the endogenous opioid peptides by interacting with these associated proteins.

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5. G Protein Coupling Induced by Endogenous Opioid Peptides in the Full-Length 7TM C-terminal Splice Variants

Intracellular location of the alternative *C*-termini raises apparent questions regarding their roles on mu agonist-induced G protein coupling. [^{35}S]GTPγS binding assays have commonly been used for measuring ligand-induced G protein coupling in G protein coupled receptors (GPCRs) [103][104]. Using unhydrolyzable GTPγS nature, [^{35}S]GTPγS binding assays provide an accurate and sensitive tool to quantify the total amount of G proteins coupled with receptors, although the assays cannot determine which G proteins are involved. The abilities of various mu ligands, including mu opioids and endogenous opioid peptides, in the stimulation of G protein coupling on different 7TM *C*-terminal variants were extensively studied using the same plasma membranes isolated from the CHO cells stably expressing individual variants that were used for opioid receptor binding assays. Table 3 puts together the data from endogenous opioid peptides, as well as DAMGO and morphine, from several published papers [73][74][75][76][77]. Concentration-response curves for each ligand on individual variants were used to determine the potency, indicated by EC_{50} values, and efficacy, indicated by % maximum stimulation (% Max) that was normalized to that of DAMGO for comparisons.

Table 3. Mu agonist-induced [^{35}S]GTPγS binding in Chinese Hamster Ovary (CHO) cells stably expressing mouse, rat and human mu opioid receptor gene (Oprm1) 7TM *C*-terminal variants.

	Ligand														
	DAMGO		Morphine		β-Endorphin		Dynorphin A		Endomorphin 1		Endomorphin 2		[Met]^{5}Enkephalin-Arg^6-Phe^7		Ref.
	EC_{50} (nM)	%Max	EC_{50} (nM)	%Max	EC_{50} (nM)	%Max	EC_{50} (nM)	%Max	EC_{50} (nM)	%Max	EC_{50} (nM)	%Max	EC_{50} (nM)	%Max	
Mouse															
mMOR-1	68 ± 4	100	23 ± 2	102 ± 5	64 ± 7	97 ± 2	34 ± 9	109 ± 7	26 ± 4	98 ± 8	72 ± 11	124 ± 8	53 ± 3	118 ± 15	[73]
mMOR-1A	70 ± 3	100	19 ± 4	91 ± 2	111 ± 27	83 ± 3	150 ± 36	73 ± 6	42 ± 13	69 ± 2	97 ± 28	76 ± 3	133 ± 9	75 ± 4	[73]
mMOR-1C	62 ± 4	100	23 ± 5	75 ± 4	123 ± 19	44 ± 3	140 ± 19	76 ± 10	83 ± 20	68 ± 15	122 ± 46	62 ± 15	60 ± 17	51 ± 2	[73]
mMOR-1D	62 ± 6	100	82 ± 34	99 ± 3	73 ± 18	105 ± 6	100 ± 41	102 ± 6	47 ± 21	94 ± 8	137 ± 24	92 ± 5	170 ± 16	94 ± 3	[73]
mMOR-1E	48 ± 4	100	41 ± 13	116 ± 4	113 ± 25	130 ± 3	113 ± 9	129 ± 9	80 ± 4	85 ± 9	52 ± 26	86 ± 8	131 ± 19	94 ± 10	[73]

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	Ligand														
	DAMGO		Morphine		β-Endorphin		Dynorphin A		Endomorphin 1		Endomorphin 2		[Met]^{5}Enkephalin-Arg^6-Phe^7		Ref.
	EC_{50} (nM)	%Max	EC_{50} (nM)	%Max	EC_{50} (nM)	%Max	EC_{50} (nM)	%Max	EC_{50} (nM)	%Max	EC_{50} (nM)	%Max	EC_{50} (nM)	%Max	
mMOR-1B1	39 ± 8	100	100 ± 38	104 ± 38	113 ± 47	69 ± 21	137 ± 69	83 ± 23	57 ± 23	68 ± 19	197 ± 95	90 ± 0			[75]
mMOR-1B2	85 ± 18	100	76 ± 13	82 ± 8	163 ± 22	84 ± 5	210 ± 25	81 ± 6	126 ± 29	82 ± 8	187 ± 23	92 ± 4			[75]
mMOR-1B3	100 ± 14	100	51 ± 6	91 ± 3	75 ± 19	93 ± 2	147 ± 56	90 ± 6	99 ± 1	97 ± 2	110 ± 6	80 ± 3			[75]
mMOR-1B5	89 ± 13	100	53 ± 4	87 ± 7	83 ± 27	80 ± 4	197 ± 32	75 ± 3	89 ± 13	86 ± 7	155 ± 8	82 ± 4			[75]
mMOR-1F	50 ± 6	100	44 ± 17	76 ± 13	26 ± 6	74 ± 7	40 ± 8	73 ± 3	44 ± 18	113 ± 5	68 ± 18	107 ± 4	29 ± 9	94 ± 16	[73]
mMOR-1O	60 ± 19	100	85 ± 31	66 ± 23	6 ± 1	141 ± 8									[77]
mMOR-1P	133 ± 23	100	58 ± 9	115 ± 23	24 ± 5	55 ± 3									[77]
Rat															
rMOR-1	12 ± 3	100			4 ± 2	105.58			14 ± 4	137.34					[74]
rMOR-1A	13 ± 5	100			13 ± 5	100.57			15 ± 3	116.48					[74]
rMOR-1C1	74 ± 22	100			48 ± 4	154.94			54 ± 8	161.80					[74]
rMOR-1D	125 ± 26	100			91 ± 14	146.02			100 ± 26	128.32					[74]
Human															

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Ligand															
	DAMGO		Morphine		β-Endorphin		Dynorphin A		Endomorphin 1		Endomorphin 2		[Met]^{5}Enkephalin-Arg^6-Phe^7		Ref.
	EC_{50} (nM)	%Max	EC_{50} (nM)	%Max	EC_{50} (nM)	%Max	EC_{50} (nM)	%Max	EC_{50} (nM)	%Max	EC_{50} (nM)	%Max	EC_{50} (nM)	%Max	
hMOR-1	120 ± 17	100	21 ± 4	97.57	4 ± 1	68.75	296 ± 16	36.46							[76]
hMOR-1A	161 ± 21	100	30 ± 2	121.31	8 ± 2	71.31	36 ± 1	63.93							[76]
hMOR-1B1	255 ± 46	100	41 ± 5	64.41	25 ± 6	57.97	63 ± 17	50.51							[76]
hMOR-1B2	1028 ± 68	100	77 ± 9	80.00	73 ± 10	97.84	292 ± 66	97.84							[76]
hMOR-1B3	549 ± 86	100	86 ± 19	65.44	33 ± 11	61.78	98 ± 27	39.38							[76]
hMOR-1B4	341 ± 65	100	38 ± 5	71.68	19 ± 2	65.32	58 ± 14	40.75							[76]
hMOR-1B5	936 ± 233	100	90 ± 18	61.46	55 ± 2	92.01	158 ± 15	81.60							[76]
hMOR-1Y	571 ± 255	100	100 ± 20	88.05	43 ± 3	73.18	100 ± 21	77.26							[76]

[^{35}S]GTPγS binding assay was performed with membranes from CHO cells stably expressing indicated splice variants, as described in indicated references. The percentage of maximum stimulation (% Max) of the agonists was normalized with that of DAMGO.

The results revealed marked differences in [^{35}S]GTPγS binding by endogenous opioid peptides in both potency (EC_{50} value) and efficacy (% Max), particularly β-endorphin. For example, the EC_{50} values of β-endorphin differed over 28-fold between mMOR-1O with a 30-amino acid (aa) *C*-terminal tail encoded by exon 7 and mMOR-1B2 with a different 23-aa *C*-terminal tail encoded by exon 5b. mMOR-1C has an identical exon 7-encoded 30-aa as mMOR-1O, but contains an additional 22-aa *C*-terminal sequence encoded by exons 8/9 (see the sequences in Figure 2). Interestingly, the 22-aa sequences in mMOR-1C increased β-

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endorphin's EC_{50} value by 20-fold. β-endorphin was more potent in mMOR-1 with its 12-aa tail encoded by exon 4 (4 nM) than in mMOR-1B2 with a totally different 7-aa tail encoded by exon 5b (73 nM). Furthermore, dynorphin A EC_{50} values varied over 6-fold between mMOR-1 and mMOR-1B2 and over 7-fold between hMOR-1A and hMOR-1B2. One intriguing observation is that there was no correlation between the potency (EC_{50} value) of mu agonists, including endogenous opioid peptides, to activate [^{35}S]GTPγS binding and their binding affinity (K_i value) (Figure 3). For example, [Met]^{5}Enkephalin-Arg^6-Phe^7 had a wide range of EC_{50} values (29–170 nM) among the mouse 7TM *C*-terminal variants, despite that its K_i values from the binding assays were very similar (Table 2). The binding affinity of β-endorphin in mMOR-1O was over 9-fold lower than in mMOR-1D. Contrastingly, β-endorphin was more potent in stimulating [^{35}S]GTPγS binding in mMOR-1O (EC_{50}: 6 nM) than in mMOR-1D (EC_{50}: 73 nM). The mismatch between the K_i and EC_{50} values suggests that different *C*-terminal sequences can impact on the potency of endogenous opioid peptides to stimulate [^{35}S]GTPγS binding independent of their binding affinity.

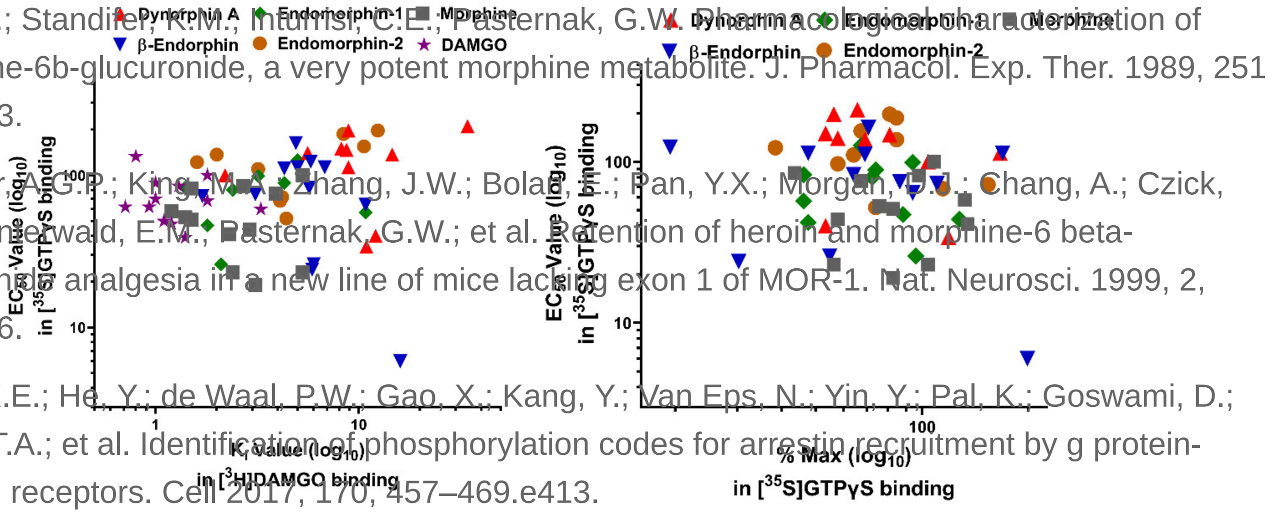

Figure 3. Correlation of the EC_{50} values with % maximum stimulation (% Max) in [^{35}S]GTPγS binding and with the K_i values in receptor binding among mouse Oprm1 7TM *C*-terminal variants. A). Correlations of the K_i values in receptor binding from Table 2 with the EC_{50} values in [^{35}S]GTPγS binding from Table 3. Correlation coefficients (r^2) were calculated from linear regression analysis by (Prism 8, GraphPad). There was no significant correlation between the binding affinity (K_i) and potency (EC_{50}) in the [^{35}S]GTPγS binding. DAMGO, r^2 = 0.03; Morphine, r^2 = 0.01; β-endorphin, r^2 = 0.24; Dynorphin A, r^2 = 0.16; Endomorphin-1, r^2 = 0.01; Endomorphin-2, r^2 = 0.44. B). Correlation of the EC_{50} values and % maximum stimulation (% Max) in the [^{35}S]GTPγS binding. No significant correlation between the EC_{50} and % Max was observed. Morphine, r^2 = 0.00; β-endorphin, r^2 = 0.05; Dynorphin A, r^2 = 0.16; Endomorphin-1, r^2 = 0.04; Endomorphin-2, r^2 = 0.07.

The relationship between the K_i and EC_{50} values can be further indicated by the EC_{50}/K_i ratio, which represents both an assessment of the ability of an agonist to stimulate the receptor in [^{35}S]GTPγS binding relative to its receptor occupancy or binding affinity, and an indirect indication of intrinsic activity (Table 4). Again, we observed a wide range of the EC_{50}/K_i ratios among 7TM *C*-terminal variants for endogenous opioid peptides, particularly β-endorphin, consistent with no correlation between the K_i and EC_{50} values. What is most striking is that the ratios

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between mMOR-1O and mMOR-1D differed over 113-fold. Additionally, there was an 18-fold difference of β-endorphin EC_{50}/K_i ratio between hMOR-1 and hMOR-1Y, and a 10-fold difference between rMOR-1 and rMOR-1D. The EC_{50}/K_i ratios of dynorphin A varied over 14-fold between mMOR-1 and mMOR-1D and 8-fold between hMOR-1B3 and hMOR-1B4. These results suggest that the *C*-terminal tail sequences have significant impact on the intrinsic activity of mu agonists including endogenous opioid peptides.

Table 4. EC_{50}/K_i value ratios of mu agonists among the mouse, rat, and human mu opioid receptor gene (OPRM1) 7TM C-terminal variants.

	Ligand							
	DAMGO	Morphine	β-Endorphin	Dynorphin A	Endomorphin-1	Endomorphin-2	$[Met]^5$Enkephalin-Arg^6-Phe^7	Refs.
	EC_{50}/K_i	EC_{50}/K_i	EC_{50}/K_i	EC_{50}/K_i	EC_{50}/K_i	EC_{50}/K_i	EC_{50}/K_i	
Mouse								
mMOR-1	38	4	6	3	12	17	13	[73] [100]
mMOR-1A	70	6	26	18			38	[73] [77]
mMOR-1C	67	10	21	25	59	76	29	[73] [100]
mMOR-1D	87	55	43	45	26	69	46	[73] [100]
mMOR-1E	40	18	23	13	33	12	30	[73] [100]
mMOR-1B1	28	19	17	9	5	16		[75]
mMOR-1B2	65	19	33	6	25	22		[75]
mMOR-1B3	56	34	24	17	31	34		[75]
mMOR-1B5	89	38	15	22	21	15		[75]
mMOR-1F	45	15	4	3	15	17	7	[73] [78]
mMOR-1O	18	31	0.4					[77]

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	Ligand							
	DAMGO	Morphine	β-Endorphin	Dynorphin A	Endomorphin-1	Endomorphin-2	[Met]^{5}Enkephalin-Arg^6-Phe^7	Refs.
	EC_{50}/K_i	EC_{50}/K_i	EC_{50}/K_i	EC_{50}/K_i	EC_{50}/K_i	EC_{50}/K_i	EC_{50}/K_i	
mMOR-1P	166	48	4					[77]
Rat								
rMOR-1	4		1		3			[74]
rMOR-1A	2		1		2			[74]
rMOR-1C1	16		5		14			[74]
rMOR-1D	27		11		26			[74]
Human								
hMOR-1	100	10	0.3	3				[76] [79]
hMOR-1B1	213	17	3	3				[76]
hMOR-1B2	198	7	3	6				[76]
hMOR-1B3	305	27	4	7				[76]
hMOR-1B4	148	7	1	0.8				[76]
hMOR-1B5	4	23	6	3				[76]
hMOR-1Y	228	23	5	4				[76]

The relative efficacy or % maximum stimulation (% Max) of endogenous opioid peptides in stimulation of [^{35}S]GTPγS binding varied markedly among the 7TM *C*-terminal variants (Table 3). For example, β-endorphin was a full agonist in mMOR-1D (105%), mMOR-1E (130%) and mMOR-1O (141%), while it became a partial agonist in mMOR-1C (44%) and mMOR-1P (55%). Interestingly, both β-endorphin and dynorphin A were a partial agonist in hMOR-1, hMOR-1B3 and hMOR-1B4, but a full agonist in hMOR-1B2. Similarly, the efficacy of endomorphin-1 and endomorphin-2 differed among the mouse 7TM variants. Just as there was no correlation between the K_i and

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EC_{50} values, there was no correlation between the EC_{50} values and %Emax of endogenous opioid peptides among the 7TM C-terminal variants.

Together, these results suggest that different intracellular *C*-terminal tails greatly impact Receptor-G protein coupling induced by the endogenous opioid peptides. It should be pointed out that the influence of the *C*-terminal tails on G protein coupling was also observed by most mu agonists such as DAMGO, morphine, fentanyl, and methadone [73][74][75][76][77][78][79][81], in contract to their unchanged binding affinity (Table 2). These results suggest the differential effects of the *C*-terminal tails on ligand binding and G protein activation between endogenous opioid peptides and other mu agonists. The above-mentioned studies were all performed using CHO cells and the results may be irrelevant in vivo. However, it is difficult to determine the impact of endogenous opioid peptide-induced Receptor-G protein coupling or their binding affinity on individual 7TM variants in vivo since they co-exist in the brain. It would be interesting to further explore in vivo functional relevance of these differentially expressed 7TM *C*-terminal variants in the Receptor-G protein coupling induced by endogenous opioid peptides using new gene targeting animal models in which only one individual 7TM *C*-terminal variant is expressed. Region-specific, cell-specific, or strain-specific expression of the OPRM1 splice variants including the 7TM *C*-terminal variants were observed at both mRNA [85][86][89] and protein levels [87][88][105] in animals and humans, raising questions whether their roles in mu agonist-induced G protein coupling are region-specific or cell-specific.

6. Biased Signaling of Endogenous Opioid Peptides in the Full-Length 7TM *C*-Terminal Splice Variants

Originally, G protein coupled receptors (GPCRs) were defined to signal through interactions with G proteins that translate their downstream signaling cascades. However, GPCRs have been found to couple to non-G protein transducers, such as β-arrestins, to produce G protein-independent signaling, leading to the concept of biased signaling, or biased agonism, or functional selectivity in which different agonists can trigger divergent signaling pathways via the same receptor and produce distinct behavioral responses [106][107][108]. G protein and β-arrestin2 signaling through various mu agonists are mostly studied in the original mu opioid receptor, MOR-1. Various mu agonists can differentially induce receptor-β-arrestin interactions that block Receptor-G protein coupling and/or produce β-arrestin-dependent signaling. The hypothesis that G protein signaling produces analgesic responses while β-arrestin2 signaling is responsible for common side-effects has led to the effort to develop novel analgesic drugs that are G protein-biased and/or non-β-arrestin-biased [109][110]. Discovery of multiple OPRM1 7TM *C*-terminal variants raises questions about the roles of these variants in biased signaling via various mu opioids, including endogenous opioid peptides.

There are four arrestin subtypes encoded by four different genes: SAG, ARRB1, ARRB2 and ARR3. The SAG was isolated as Arrestin1 or a visual arrestin. The ARR3 was cloned as Arrestin4 or X-arrestin or a cone arrestin. The ARRB1 (Arrestin1) and ARRB2 (Arrestin2) were identified as non-visual arrestins, and also named as Arrestin2 and Arrestin3, respectively, which often cause confusion about their gene or protein identity in literature. Both Arrestin1 (ARRB1) and Arrestin2 (ARRB2) have been widely studied in GPCR field. Here we refer to arrestin2 as the gene product of the ARRB2, which was sometimes called arrestin3 in literature.

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mMOR-1O (-2). Similar scenarios were seen in endomorphin-1 and β-endorphin. When the bias factors of individual agonists were normalized to mMOR-1 (Figure 4B), all endogenous opioid peptides and mu opioids excluding endomorphin-2 clearly displayed β-arrestin2 bias toward mMOR-1O, an exon 7-associated 7TM variant, compared to mMOR-1. Similarly, all endogenous opioid peptides and mu opioids except for [Met]^{5}Enkephalin-Arg^6-Phe^7 showed greater β-arrestin2 bias in mMOR-1B1. Interestingly, [Met]^{5}Enkephalin-Arg^6-Phe^7 exhibited G protein bias toward all 7TM variants with the exception of mMOR-1O. These results underline the functional importance of these 7TM *C*-terminal variants on biased signaling induced by not only various mu opioids but also by endogenous opioid peptides.

A. Normalized to DAMGO at MOR-1

	MOR-TR	MOR-1	MOR-1A	MOR-1B1	MOR-1C	MOR-1E	MOR-1O
DAMGO	-3.1	1.0	-1.9	-3.5	-1.9	1.6	-10.2
Morphine	-1.1	-1.5	-1.7	-4.3	-1.3	-7.6	-5.0
B-Endorphin	-54.8	-2.1	-1.9	-2.4	-1.3	1.7	-9.2
Endomorphine-1	-14.0	-3.3	-1.5	-8.1	-4.8	1.1	-19.4
Endomorphine-2	-6.5	-4.5	-2.2	-9.5	-5.7	-4.1	-4.1
Met-Enk-Arg-Phe	-9.6	3.1	7.7	5.7	20.5	24.5	-2.0
Methadone	1.1	2.1	1.3	-6.5	-18.6	2.9	-6.0
Fentanyl	-12.8	-4.4	-4.9	-21.0	-9.8	-1.9	-44.3

B. Normalized to each drug at MOR-1

	MOR-TR	MOR-1	MOR-1A	MOR-1B1	MOR-1C	MOR-1E	MOR-1O
DAMGO	-3.1	1.0	-1.9	-3.5	-1.9	1.6	-10.2
Morphine	1.4	1.0	-1.1	-2.8	1.2	-5.0	-3.3
B-Endorphin	-25.6	1.0	1.1	-1.1	1.6	3.7	-4.3
Endomorphine-1	-4.3	1.0	2.2	-2.5	-1.5	3.7	-6.0
Endomorphine-2	-1.4	1.0	2.1	-2.1	-1.3	1.1	1.1
Met-Enk-Arg-Phe	-29.7	1.0	2.5	1.9	6.7	8.0	-6.1
Methadone	-1.9	1.0	-1.6	-13.4	-38.5	1.4	-12.4
Fentanyl	-2.9	1.0	-1.1	-4.7	-2.2	2.3	-10.0

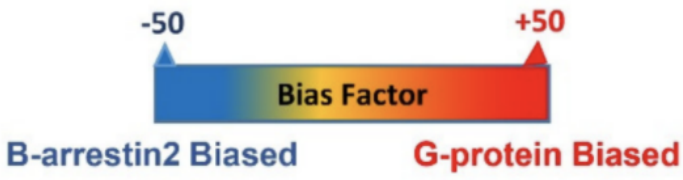

Figure 4. Heatmap of biased factors (adopted from [81]). Biased factors were calculated using the Black and Leff Operational Model by using different normalization methods, as described in [81]. (**A**). Normalized with respect to DAMGO at MOR−1 for a comparison between drugs and variants. (**B**). Normalized with respect to each drug at mMOR−1 for a comparison across variants. The negative (blue) values indicate β-arrestin2 bias whereas the positive bias (red) values indicate G protein bias.

Why do the *C*-terminal sequences have marked impact on biased signaling by endogenous opioid peptides and mu opioids in terms of G protein coupling and β-arrestin2 recruitment? One possible mechanism is that different *C*-terminal sequences contain various potential phosphorylation sites and differential phosphorylation induced by mu agonists can modulate G protein and/or β-arrestin2 signaling. The *C*-terminal tails encoded by exon 7 have a consensus phosphorylation code, PxPxxE/D or PxxPxxE/D, for high affinity arrestin binding that was predicted from the crystal studies of GPCRs [72]. When this code was mutated, mMOR-1O, an exon 7-associated 7TM variant was unable to recruit β-arrestin2 by mu agonists (unpublished data). This may explain why mMOR-1O had most β-arrestin2 bias toward most mu agonists including endogenous opioid peptides. Another possibility is that the *C*-terminal sequences can interact with intracellular loops of the receptor that are important for G protein or β-arrestin2 signaling or with other receptor-associated signaling proteins, a similar mechanism for the differences in the binding affinity of the endogenous opioid peptides among 7TM variants as mentioned above. Finally, different *C*-terminal tails may modulate receptor conformations favoring either G protein coupling or β-arrestin2 recruitment particularly induced by endogenous opioid peptides. Biased signaling has been referred to different signaling pathways produced by various agonists on a single GPCR. The results from the 7TM *C*-terminal variants offer another meaning of biased signaling in which a single agonist can stimulate divergent signaling pathways via multiple 7TM *C*-terminal variants.

7. Conclusions

Extensive alternative splicing of the OPRM1 gene creates multiple splice variants or receptor isoforms that are conserved from rodent to human, providing new insights into our understanding of the complex actions of various mu agonists, including endogenous opioid peptides. Like most mu opioids such as morphine and fentanyl, endogenous opioid peptides can differentially induce G protein coupling, β-arrestin2 recruitment, and biased signaling through various 7TM *C*-terminal splice variants. Variable binding affinities of endogenous opioid peptides toward the 7TM *C*-terminal variants indicate the influence of *C*-terminal tail sequences on overall receptor structure and/or ligand binding pockets for the endogenous opioid peptides. Future structural determination of such influences by the *C*-terminal sequences using new technologies such as high-resolution cryogenic electron microscopy would greatly advance our knowledge on the role of the 7TM *C*-terminal variants, especially in the pharmacology of endogenous opioid peptides. Although all the results presented in this review were obtained from in vitro cell models, they suggest the functional relevance of these 7TM *C*-terminal variants in mediating the actions of endogenous opioid peptides and mu opioids in vivo where they are co-expressed. The in vivo pharmacological function of an endogenous opioid peptide or a mu opioid should be considered as its combinational effects on different 7TM *C*-terminal variants. Region-specific or cell-specific expression of the 7TM *C*-terminal variants also

raises questions on whether the 7TM *C*-terminal variants have distinct roles in a region-specific or cell-specific manner. It will be interesting to further investigate in vivo functions of each individual 7TM *C*-terminal variant using novel gene targeting animal models in which only one individual 7TM *C*-terminal variant is expressed.