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REVIEW ARTICLES

Opioid receptor subtypes: fact or artifact?

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Editor's key points

- Traditional opioid receptor classification conflicts with recent genetic knockout evidence, casting doubt over previous pharmacological evidence.
- In this review, Dietis, Rowbotham, and Lambert examine the evidence for the existence of receptors and their subtypes and illustrate challenges and opportunities for future research.

Summary. There is a vast amount of pharmacological evidence favouring the existence of multiple subtypes of opioid receptors. In addition to the primary classification of μ (mu: MOP), δ (delta: DOP), κ (kappa: KOP) receptors, and the nociceptin/orphanin FQ peptide receptor (NOP), various groups have further classified the pharmacological μ into μ_{1-3}, the δ into $\delta_{1-2}/\delta_{complexed/non-complexed}$, and the κ into κ_{1-3}. From an anaesthetic perspective, the suggestions that μ_1 produced analgesia and μ_2 produced respiratory depression are particularly important. However, subsequent to the formal identification of the primary opioid receptors (MOP/DOP/KOP/NOP) by cloning and the use of this information to produce knockout animals, evidence for these additional subtypes is lacking. Indeed, knockout of a single gene (and hence receptor) results in a loss of all function associated with that receptor. In the case of MOP knockout, analgesia and respiratory depression is lost. This suggests that further sub-classification of the primary types is unwise. So how can the wealth of pharmacological data be reconciled with new molecular information? In addition to some simple misclassification (κ_3 is probably NOP), there are several possibilities which include: (i) alternate splicing of a common gene product, (ii) receptor dimerization, (iii) interaction of a common gene product with other receptors/signalling molecules, or (iv) a combination of (i)–(iii). Assigning variations in ligand activity (pharmacological subtypes) to one or more of these molecular suggestions represents an interesting challenge for future opioid research.

Keywords: dimerization; opioid receptors; pharmacological classification; splice variants; subtypes

The existence of receptors for opiate drugs was first proposed in 1954 based on pharmacological studies with synthetic opiates.[1] In the early 1970s, high-affinity stereospecific binding sites for different opiate drugs were discovered in the brain using naloxone,[2] etorphine,[3] and dihydromorphine,[4] among others. In 1976, Martin and colleagues[5] presented the first definitive evidence that the opioid receptor was not homogeneous, implying the existence of opioid receptor types. They proposed two opioid receptors named after the prototypic drugs used in their studies, i.e. the μ receptor (mu for morphine) and the κ receptor (kappa for ketocyclazocine). In 1977, pharmacological analysis of the effects of opioid peptides in the mouse vas deferens led to the discovery of the third or δ receptor (delta for deferens).[6] In parallel, a search for the endogenous ligands for these receptors led to the discovery of the enkephalins by Hughes and colleagues[7] as natural ligands for δ, β-endorphins by Cox and colleagues[8] as natural ligands with activity at μ, and dynorphins by Goldstein and colleagues[9] as natural ligands for κ receptors. The search for selective endogenous μ ligands intensified in 1997 with the identification of the endomorphins,[10] but the precursors for these small peptides remain elusive. In 1992, the groundbreaking opioid studies of Kieffer and colleagues[11] and Evans and colleagues[12] led to the cloning of the δ receptor, with the μ,[13–16] the κ,[17 18] and the nocicepin/orphanin FQ peptide receptor GPCR[19 20] soon to follow. The three primary receptor types (μ/δ/κ) are naloxone-sensitive.

In the years up to the time of formal molecular identification of single MOP, DOP, KOP, and NOP receptor genes by cloning, multiple additional subtypes have been proposed. This, along with an attempt to reconcile the conflicting pharmacological and molecular evidence, is the focus of this review. Basic classification, distribution, function, and pharmacology are summarized in Table 1.

In this review, we will be referring to the four opioid receptors as 'opioid receptor types' and to further proposed opioid receptors as 'putative receptor subtypes'. We will also describe, wherever possible, pre-cloning pharmacology using the μ/δ/κ terminology and the post-cloning using the IUPHAR standard terminology of MOP/DOP/KOP and NOP (for the nociceptin/orphanin FQ peptide receptor).

Table 1 Proposed distribution, pharmacology and function of putative receptor subtypes of the MOP, DOP, and KOP receptors. TRIMU-5, [Tyr-D-Ala-Gly-$NHC_2H_4CH(CH_3)]_2$; β-FNA, β-funaltrexamine; M6G, morphine-6-glucuronide; BNTX, 7-benzylidenenaltrexone; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; DSLET, D-Ser^2-Leu-enkephalin-Thr^6; NalBzOH, naloxone-benzoyl-hydrazone; DALCE, [D-Ala^2,Leu^5,Cys^6]enkephalin; NO, nitric oxide; nor-BNI, norbinaltorphimine; 3-MTX, 3-methoxynaltrexone; 5-NTII, naltrindole-5′-isothiocyanate. * ↓ shows lower affinity compared with the 'main' subtype. #Probable NOP; no evidence for NOP subtypes so not included in this table

Pharmacological subtypes	No. of genes	IUPHAR classification	Distribution	Possible discriminatory ligands	Other relevant ligands	Function/effect
μ_1	One	MOP	Brain, spinal cord, periphery	Naloxonazine (antagonist)	Morphine (agonist), TRIMU-5 (antagonist), β-FNA (antagonist), Dihydromorphine (agonist), Naloxone (antagonist), Nalorphine (anagonist) Codeine (agonist), Oxycodone (↓agonist)	Analgesia
μ_2		MOP	Brain, spinal cord, periphery	TRIMU-5 (agonist), M6G (agonist)	Morphine (↓ agonist)*, naloxone (↓ antagonist)*, Dihydromorphine (↓ agonist)*, β-FNA (antagonist), M6G (agonist), heroin (agonist), Naloxonazine (↓ antagonist)	Analgesia, GI transit, respiratory depression, itching
μ_3		MOP	Immune cells, amygdala, peripheral neural, CV endothelial cells	(opioid peptide insensitivity)	Morphine (↓agonist)*, naloxone (↓ antagonist)*, dihydromorphine (↓ agonist)*, β-FNA (antagonist), M6G (↓agonist)*	Various including NO release
δ_1	One	DOP	Brain, periphery	DPDPE (agonist), BNTX (antagonist), DALCE (antagonist)	Enkephalin (agonist), deltorphin-D (agonist), naltrexone (antagonist)	Analgesia, cardioprotection
δ_2		DOP	Brain and spinal	Deltorphin-II (agonist), DSLET (agonist) 5-NTII (antagonist), Naltriben (antagonist)	Enkephalin (agonist), deltorphin-D (agonist), naltrexone (antagonist), deltorphin-II (agonist)	Analgesia, cardioprotection, thermoregulation
κ_{1a}	One	KOP	Brain (nucleus accumbens, neocortex, cerebellum)	Dynorphin A (agonist), U50,488H (agonist)	nor-BNI (antagonist), U69,593 (agonist),	Analgesia, feeding
κ_{1b}		KOP		Dynorphin B (agonist), α-neoendorphin (agonist)		
κ_{2a}		KOP	Brain (hippocampus, thalamus, brainstem)	*Leu*-enkephalin (antagonist), oxycodone (agonist)	nor-BNI (↓antagonist)*, bremazocine (agonist)	Analgesia, diuresis, neuroendocrine
κ_{2b}		KOP				
$\kappa_3^{\#}$		KOP	Brain	NalBzOH	Nalorphine (agonist), nor-BNI (↓antagonist)*	Spinal analgesia, peripheral effects

Pharmacologically defined opioid receptor subtypes

As early as 1965, Portoghese[21] suggested that it may be necessary to propose the existence of more than one opioid receptor type or that multiple modes of interaction of ligands with opioid receptors were possible. Since then, and based on the pharmacology of a large number of opioid ligands, an equally large number of putative opioid receptor subtypes have been proposed. The first direct suggestions for the existence of opioid subtypes started with the μ receptor, the main target for the production of clinical analgesia,[22 23] and this came about from the observations that some μ ligands could differentially affect the analgesic response and the unwanted respiratory depression. These sites were named μ_1 and μ_2 receptors and are discussed in detail below. There is a vast amount of literature on pharmacological classification, thus we have been necessarily selective in coverage of the main ligands.

Pharmacologically defined μ-receptor subtypes

Naloxonazine, β-funaltrexamine, TRIMU-5, and μ_1/μ_2

Some of the first antagonists developed that could discriminate pharmacological μ subtypes were naloxazone and naloxonazine (derivatives of naloxone), with the latter being more potent and more long acting.[24 25] *In vivo*, naloxonazine blocks morphine analgesia but does not significantly alter respiratory depression or gastrointestinal transit[26 27] or facial scratching.[28] Naloxonazine binding to the μ_1 was irreversible and the μ_2 reversible.[29 30] It was suggested that morphine bound with higher affinity to μ_1 receptors to produce analgesia with lower affinity to a second site μ_2 to produce side-effects typical of μ agonists.[26–28] Moreover, there is evidence for site-related differences where Paul and colleagues[31] have provided evidence that naloxonazine blocks only the analgesia produced by supraspinally but not spinally administered morphine. On the other hand, the alkylating agent β-funaltrexamine (β-FNA) completely blocks spinal and supraspinal morphine analgesia.[31 32] A number of studies have suggested that the μ opioid receptor is either sensitive or non-sensitive to β-FNA[33 34] and this may reveal μ receptor heterogeneity. Indeed, we have used this compound to suggest that there were two populations of μ receptor in the human neuroblastoma cell line SH-SY5Y.[35]

TRIMU-5 (Tyr-D-Ala-Gly-$NHC_2H_4CH(CH_3)_2$) has been a particularly interesting ligand with proposed antagonist activity at the putative μ_1 and agonist activity at the putative μ_2 receptor subtype.[36 37] The interest in TRIMU-5 arises from the fact that it provides some evidence for communication between supraspinal and spinal μ systems. A supraspinal, non-analgesic dose of TRIMU-5 antagonizes the analgesia produced by supraspinal morphine, an action that is thought to result from antagonism at μ_1. However, when the same dose of supraspinal TRIMU-5 was co-administered with spinal and supraspinal morphine in a synergy model, it potentiated analgesia, implying that (i) supraspinal μ_1 receptor sites mediate supraspinal morphine analgesia and (ii) supraspinal μ_2 receptor sites mediate interactions with spinal μ opioid receptor systems.[37]

A series of pharmacogenetic studies in mice of different strains (i.e. the same species with some genetic variation) have added weight to putative μ receptor subtypes. Moskowitz and Goodman[38] have shown that the CXBK mice are deficient in putative μ_1 sites. In later studies, different mice strains treated with a fixed dose of morphine (5 mg kg^{-1} s.c.) have shown wide differences in their analgesic sensitivity from 90% in BALB strain mice to 0% in CXBK mice.[39 40] The analgesic insensitivity of s.c. and supraspinal morphine in CXBK mice agrees with the μ_1 deficiency seen by Moskowitz and Goodman. Pick and colleagues[37] have expanded this suggestion by showing a significant analgesic sensitivity of spinally administered morphine in CXBK mice. This implies that supraspinal and s.c. morphine do not have significant analgesic effects when putative μ_1 receptor subtypes are not expressed in the brain.

Pharmacological μ_3 receptor subtype

A third putative MOP subtype, μ_3, has also been described pharmacologically in various human tissues (neural, immune, vascular, gastrointestinal), where it is associated with a nitric oxide release pathway. The receptor is insensitive to opioid peptides but sensitive to opioid alkaloids.[41–44] Morphine and naloxone display 20 and 10 times lower affinity for μ_3 over μ_1 sites, whereas the peptides Leu-enkephalin, Met-enkephalin, DAMGO, and DADLE did not interact with μ_3.[43 45] The putative μ_3 cDNA, cloned in Cos-1 cells, expressed a receptor where a dose-dependent release of NO was produced in the presence of morphine.[43] Davis and colleagues[46] showed that β-FNA blocks the inducible nitric oxide synthase (iNOS) activity in human astroglial cells, by inhibiting its expression but the model expressed all opioid receptors. The exact mechanism of that inhibition is not clear.

Morphine-6-glucuronide receptor?

Morphine undergoes glucuronidation to morphine-6-glucuronide (M6G) and morphine-3-glucuronide (M3G), with M6G being a potent antinociceptive ligand.[28 47] Andoh and colleagues have shown that analgesia is produced in mice after administration of intracisternal morphine and M6G, but not by M3G. In an interesting study, also in mice, Rossi and colleagues[48] showed that morphine-tolerant animals exhibited cross-tolerance to codeine but not to M6G, diamorphine or L-methadone and in rats, antisense oligonucleotides directed against the 5′-untranslated region of μ_1 blocks morphine but not M6G analgesia.[48 49]

Knock-out of the single gene encoding MOP removes μ receptor function.

Pharmacologically defined δ-receptor subtypes

Pharmacological subtypes of the δ receptor have been suggested with the main classification for putative subtypes being δ_1, δ_2 and $\delta_{complexed}$, $\delta_{non\text{-}complexed}$. Most evidence is spinal and comes from mice. Of particular interest are the antagonists naltrindole 5′-isothiocynate (5′-NTII), naltriben, and 7-benzylidenenaltrexone (BNTX). 5′-NTII reversed the analgesic effects of the δ-agonist deltorphin-II but not the archetypal synthetic [D-Pen^2 ^{5}]enkephalin (DPDPE).[50] Naltriben and BNTX selectively blocked the effects of [D-Ser^2]-Leu-Enkephalin-Th (DSLET) and DPDPE, respectively.[51] Subsequently, δ_1 receptor sites were classified as those activated by DPDPE and sensitive to BNTX where the δ_2 receptor sites were activated by deltorphin-II and DSLET and sensitive to 5′-NTII and naltriben.[52] More recently, Maslov[53] showed that the cardioprotective effects of *deltorphin-II* (putative δ_2 agonist) were abolished by naltriben (putative δ_2 antagonist), whereas putative δ_1 antagonists were ineffective. Interestingly, Hirose and colleagues showed that DPDPE (putative δ_1 agonist) and DSLET (putative δ_2 agonist) significantly enhanced dopamine release from the nucleus accumbens of the rat in a dose-related

manner. The effect of the former ligand was abolished by BNTX and the effect of the latter was abolished by naltriben.[51] In a study by Rawls and colleagues,[54] the DOP receptor agonist SNC-80 produced hypothermia that was blocked by naltriben but not BNTX, which, on face value, implicates putative δ_2 receptor subtypes in thermoregulation. There is evidence (some covered here and also in our previous work)[55] to show an interaction of δ with μ (a receptor heterodimer); these are the putative $\delta_{complexed}$ receptor subtypes.[56]

Knock-out of the single gene encoding DOP removes δ receptor function.

Pharmacologically defined κ-receptor subtypes

From a pharmacological perspective, the κ receptor is more complex because there is evidence for κ_1, κ_2, and κ_3 receptor subtypes[57] and further sub-division into κ_{1a} and κ_{1b} receptor sites[58] and also κ_{2a} and κ_{2b} sites.[59] There are a number of κ ligands that have been used to come to these pharmacological conclusions. The main ligands include U69,593, U50,488H, and naloxone benzoylhydrazone (NalBzOH). Consensus seems to be that U69,593 discriminates κ_1 sites,[60 61] whereas κ_2 receptor subtypes are discriminated by difference[59 62 63] and κ_3 sites are discriminated by NalBzOH and insensitivity to U50,488H.[58] Soon after the cloning of the κ receptor, Pan and colleagues[64] used antisense oligonucleotide directed at the second extracellular loop of κ_3 receptor subtypes in order to block NalBzOH-induced analgesia in mice. NalBzOH was initially thought to be a prototypic selective putative κ_3 agonist[58] along with other actions at classical and non-classical opioid receptors.[65] Moreover, this is also questioned by Paul and colleagues, where they used nalorphine to selectively activate the κ_3 receptor subtype.[66] Paul and colleagues showed peculiar nalorphine pharmacology, where in low doses, it antagonizes morphine analgesia, and at larger doses manifests analgesia by full agonism. This analgesia was naloxone-sensitive but β-FNA, naltrindole, and nor-BNI insensitive. However, this useful distinction has been questioned, and it is generally accepted that the κ_3 receptor subtype is likely to be the NOP.[65 67]

The pharmacological κ_1 has been further subdivided based on differences seen with dynorphin A and U50,488H (proposed to bind to both κ_{1a} and κ_{1b} putative subtypes), and dynorphin-B and α-neoendorphin (proposed to be selective for the putative κ_{1b} receptor).[58] Rothman and colleagues[61] investigated the pharmacological subdivision of the κ_2 receptor subtype using leu-enkephalin as a selective antagonist of the κ_{2b} site. Moreover, Nielsen and colleagues[59] proposed that oxycodone is a putative κ_{2b} agonist. In this study, rats pretreated with i.c.v. nor-BNI (κ antagonist) prevented i.c.v. oxycodone but not morphine antinociception. The opposite was shown with naloxonazine (putative μ_1 antagonist), suggesting an oxycodone analgesic effect through KOP receptor binding. In addition, leu-enkephalin (proposed κ_{2b} antagonist) prevented the displacement of [^{3}H]bremazocine by oxycodone. Further, in animals tolerant to i.v. morphine, Nielsen and colleagues[68] showed that there is an absence of antinociceptive cross-tolerance to i.c.v. oxycodone. Interestingly, Nozaki and Kamei[69] showed that the antinociceptive effect of s.c. oxycodone in mice was completely antagonized by s.c. naloxonazine and only partially antagonized by s.c. nor-BNI. These studies imply a marked difference in the distribution of the putative κ_2 receptor subtypes and possibly an interaction with μ receptors at different sites (see later).

Knock-out of the single gene encoding KOP removes κ receptor function.

Evidence for putative NOP receptor subtypes

The least amount of data are available regarding subtypes of NOP and this is not surprising as deorphanization of the receptor did not occur until 1995.[70 71] There were early suggestions of putative subtypes (central and peripheral), but most of these can be adequately explained by the nature of the ligands used, that is, they were partial agonists and central tissues in general had higher expression than peripheral tissues facilitating full agonist activity centrally.[72–74] Interestingly, and in contrast to the classical opioid story described above, pharmacological and molecular (cloning) evidence were gathered side by side. There are published data for splice variants of NOP. For example, the short (truncated) form displays an unsurprising marked reduction in binding affinity for a range of NOP ligands and this is coupled with loss of function.[75–78] There are some interesting new data in the periaqueductal grey (PAG) of the rat (which expresses NOP) where the Roche agonist Ro64–6198 mimics the effects of N/OFQ in some but not all N/OFQ-sensitive neurones. However, we feel that these data can adequately be explained by the observation that Ro64-6198 is likely a partial agonist and that there is heterogeneous expression of NOP in the PAG.[79 80] The overriding piece of data (in complete agreement with the classical opioid receptors) is that in NOP-knockout animals, NOP pharmacology/function is absent.[81–85]

Can we reconcile the differences between the pharmacological and molecular evidence?

A simple count of the possible receptor subtypes gives 12 (classical and non-classical) (Fig. 1). Yet, knockout of each of the individual receptor subtypes removes all of the receptor function associated with that particular receptor.[81 82 84 86–90] The logical conclusion of this rather simple statement is that there are only four opioid receptor types (μ, δ, κ, and NOP), recognized by IUPHAR as MOP, DOP, KOP, and NOP (which, unless referring to the pharmacological subtypes, we will use for the remainder of this review). We would like to discuss three possible reconciliatory strategies for the pharmacological opioid receptor subtypes observed: (i) alternative splicing-splice variants, (ii) receptor dimerization,

and (iii) a speculative interaction of a common opioid gene product with other proteins and ligand directed signalling.

Alternative splicing

A gene comprises introns (non-transcriptional genomic sequence) and exons (transcriptional sequence), where mRNA is produced by excluding the introns and placing the exons *in tandem* (Fig. 2, top). Translation of the mRNA produces a functional protein. The MOP, DOP, and KOP mRNA include highly conserved regions, but also have differences in the exons translated for receptor production.[91] MOP mRNA is composed of exons 1, 2, 3, and 4; DOP mRNA is composed of exons 1, 2, and 3, whereas KOP mRNA is composed of exons 2, 3, and 4.[91] Alternative splicing occurs when, by various modes, the mRNAs produced from a single gene have differences in their exon composition and thus make up a different mRNA that will eventually produce a different (alternative) protein.[92] Alternative splicing is considered a mechanism used by cells in order to enhance protein (thus receptor) diversity, by simply using a single gene-template. Abnormal regulation of alternative splicing is also implicated in disease.[93]

This mode of producing different proteins from a common gene product has been used in the opioid field in an attempt to explain subtypes.[94] The first real data came from the MOP gene where Zimprich and colleagues[95] identified an additional splice variant of the rat MOP (then called rMOR1B) which produced a receptor truncated at the C-terminus. In the absence of C-terminal phosphorylation sites, this receptor was relatively resistant to desensitization and hence functionally different from the 'normal' MOP receptor. Unfortunately, both variants bound naloxonazine equally and the authors concluded that these receptors

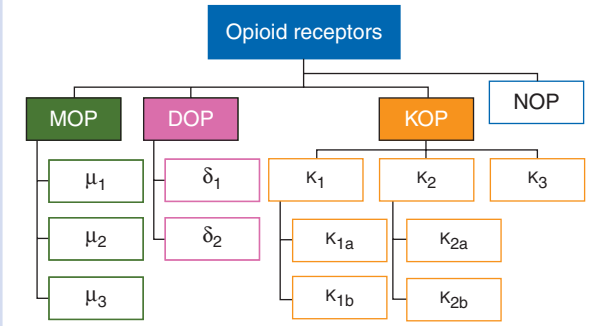

Fig 1 Pharmacological classification of the opioid receptor family.

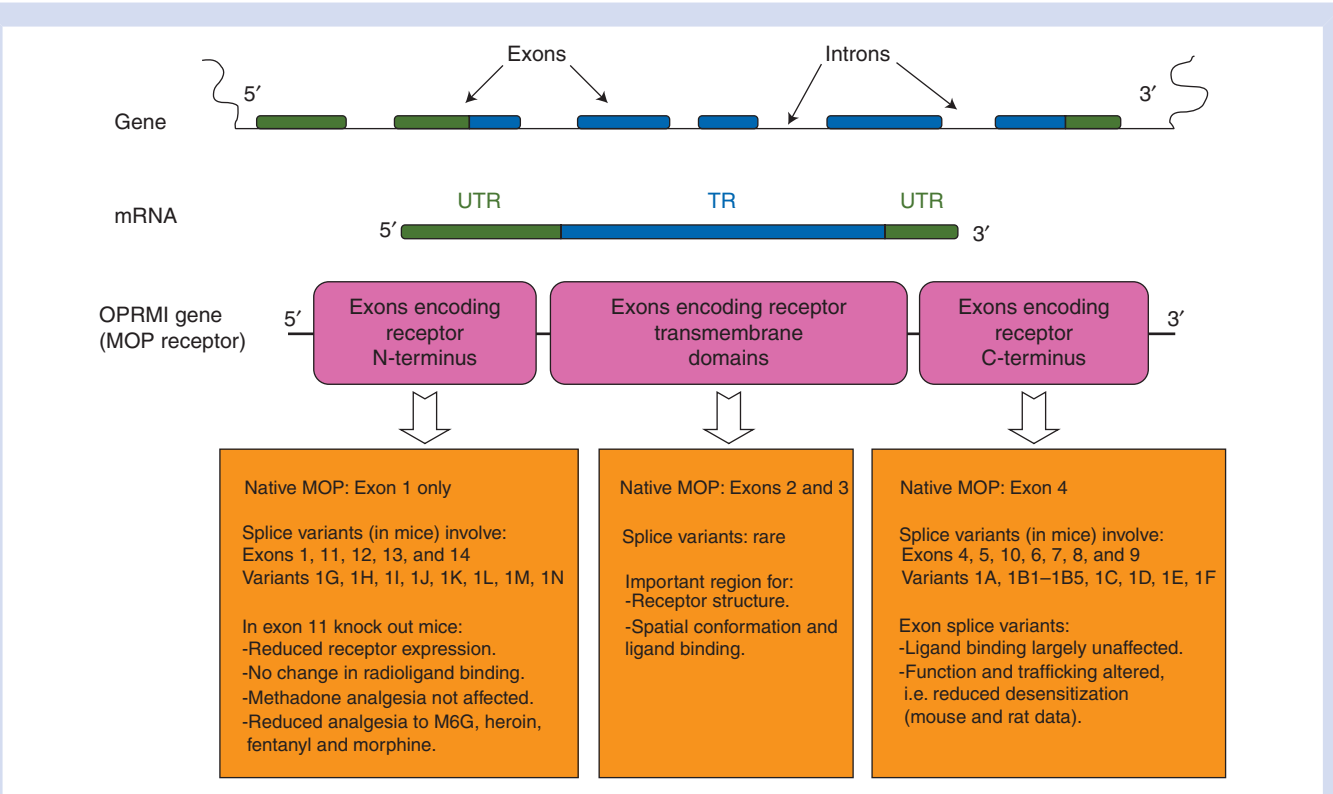

Fig 2 Schematic representation of the important MOP-receptor gene (OPRM1) splice variants. A gene contains a series of exons (seen as blocks) and introns (seen as gaps between exons). Transcription of the gene to a messenger RNA (mRNA) retains the exons together and excludes the intermediate introns. Translation will process the mRNA to a functional protein (i.e. a receptor). The region of the mRNA that will or will not translate into a part of the protein is called the untranslated (UTR; shown as green) and translated region (TR; shown as blue) respectively. *For source references, see text.*

were not μ_1 and μ_2. Since the publication of this paper, the MOP receptor gene (OPRM1) has provided a large number of splice variants[32 42 96–98] and it is beyond the scope of this review to cover all of these in detail. We have considered the OPRM1 gene in more detail in terms of three very general regions at the 5′ end coding the N-terminus in the 'middle' encoding the trans-membrane regions of the receptor and at the 3′ end encoding the C-terminus (Fig. 2).

Considering first the N-terminus (i.e. targeted deletion of exon 11), this reduces opioid receptor density but does not affect ligand recognition at the expressed receptor. The analgesic response to M6G, heroin, fentanyl, and morphine, but not methadone, is reduced.[99] Splice variants affecting the transmembrane portion of the receptor are rare and we have already described that C-terminally truncated variants show loss of function and reduced desensitisation[95] (Fig. 2).

Cadet and colleagues[41] proposed a correlation of an OPRM1 splice variant with the μ_3 putative subtype. Compared with the OPRM1 mRNA, the variant contained a truncated mRNA 5′ end (hence a truncated exon-1 and different receptor N-terminus), and a unique exon at the mRNA 3′ end (hence longer receptor C-terminus), followed by a 202-nucleotides fragment of the OPRM1 untranslated region. When expressed in a heterologous system, the pharmacology of this variant was the same as that of the μ_3.

Interestingly, Schuller and colleagues[100] showed that although morphine analgesia was completely abolished in exon-1 knockouts, diamorphine and M6G analgesia were still present. These results strengthened the idea that variants of the MOP receptor lacking exon-1 are responsible for the residual activity of M6G and diamorphine. Antisense oligonucleotide targeting studies for exon-1 and -2 (i.e. down-regulation of mRNA) did not show similar effects for morphine, but they blocked the analgesic effects of M6G.[32] This is consistent with the suggestion that M6G and diamorphine may act through different receptor subtypes when compared with morphine (or bind with different affinities), or conversely, these receptors are splice variants.

In addition to receptor variations in terms of density, function, and desensitization profiles, regional differences should also be mentioned. Xu and colleagues[96] showed that in mouse, there is a differential expression of the receptor variants among brain regions. However, this expression could be at low levels. These data may be in line with regional differences seen in opioid receptor binding and activation, and also degrees of opioid tolerance seen in different tissues as observed by Xu and others. Pasternak[32] reports that some receptor variants differ greatly in distribution and localization with respect to the regular MOP receptor. For example, in the dorsal horn of the mouse spinal cord, there are cells expressing either MOP1 or MOP1C, but not both. Also, MOP1 is equally distributed pre- and post-synaptically, whereas MOP1C is distributed only presynaptically. Finally, MOP1C is always co-localized with calcitonin gene-related peptide (CGRP), whereas MOP1 is not. This example is characteristic for the distribution and localization of different splice variants and, although consistent with histological data, its biological significance is not entirely understood.

Opioid heterodimerization

Data that support the interaction between two opioid receptors have been the focus of intense activity since the late 1990s, in particular heterodimerization (i.e. the interaction of two different opioid receptor types). This interest was partially triggered by the large number of possible combinations arising from the four main (or primary) opioid subtypes (MOP, DOP, KOP, and NOP), and also by initial studies that proposed the behaviour of the heterodimer as a receptor entity with distinct pharmacology and differences in signalling mechanisms.[101–105] Jordan and Devi[101] studied the DOP/KOP heterodimer, presenting the first pharmacological data of an opioid dimer and evidence for distinct pharmacology from the monomeric KOP or DOP receptor. Knock-out studies by Simonin and colleagues[90] have suggested that the putative κ_2 receptor may represent mixed populations of MOP, DOP, and KOP receptors. Nielsen and colleagues[59] also suggested that the pharmacology seen with oxycodone may represent the binding and activation of an opioid receptor dimer, like the DOP/KOP.

However, some less clear data have been generated when studying MOP/DOP dimers. Gomes and colleagues[106] provided evidence that MOP/DOP dimers possess functional and ligand binding synergy, whereas George and colleagues[102] showed a distinct binding profile of opioid ligands at MOP/DOP dimers. van Rijn[107] recently reviewed data for opioid receptor dimer trafficking, and some may be used to correlate with the properties seen of putative pharmacological subtypes. In another interesting recent study, Chakrabarti and colleagues[108] reported *in vivo* data indicating that MOP/KOP dimers are vastly more prevalent in the spinal cord of proestrous *vs* diestrous females and *vs* males and suggested MOP/KOP dimers as a female-specific pain target.

In Hirose and colleagues'[51] study of the inhibition of dopamine release via opioid receptor stimulation, mentioned in a previous section of this review, it was suggested that stimulation of MOP receptors activates putative δ_1 receptor subtypes which in turn activate putative δ_2 sites in nucleus accumbens, a suggestion that attempts to explain the gradual rise of extracellular dopamine after MOP activation. However, this implies either cross-communication of MOP and DOP systems or a direct interaction (i.e. dimerization).

There is also evidence for dimerization of the NOP receptor (described as κ_3 in some of these papers). Pan and colleagues[109] demonstrated the presence of NOP–MOP dimers where some MOP ligands could displace [^{3}H]N/OFQ binding. Similar NOP–MOP dimers were described by Wang and colleagues[110] in which MOP signalling (cAMP formation) was reduced, perhaps providing a cellular basis for the anti-opioid actions of NOP. In a very recent and elegant study, Evans and colleagues[111] showed that NOP could dimerize with all (MOP, DOP, KOP) receptors and that activation of NOP causes

internalization of all receptor types and interestingly MOP and NOP co-localize/internalize with Cav2.2 calcium channel.

Interaction of a common opioid gene product with other proteins

It is possible that the pharmacological subtypes could be explained based on differences in coupling to effector systems. There is evidence that opioid receptors are capable of coupling to $G_{i/o}$ and G_s[108 112–114] and we showed that MOP, DOP, and DOP were capable of coupling to phospholipase C to increase the production of $Ins(1,4,5)P_3$.[115 116] However, the pharmacology of these responses did not yield many clues as to distinctly different receptor populations. There are differences in the way peptide and non-peptide MOP ligands induce receptor internalization,[117] but these have not been reconciled against putative μ_1 or μ_2 sites. Receptor dimerization between opioid and non-opioid receptors has also shown a number of combinations (KOP and β_2-adrenoceptor,[104] DOP and α_{1A}-adrenoceptor),[118] and these may produce differences in pharmacological behaviour but are outside the scope of this review.

A further potential explanation for existence of pharmacological receptor subtypes comes from the concept of functional selectivity or more correctly biased agonism.[119] This stems from the observations that ligands active at the same receptor are capable of producing different responses (i.e. the end response is biased depending on the ligand and signalling repertoire of the cell/tissue under consideration); there are compelling data in this context for the β-adrenoceptor.[120] So what about the opioid family? The seminal work of Whistler and von Zastrow[117] showed that etorphine but not morphine desensitized the MOP receptor (a biased response). Etorphine (and other ligands like fentanyl) produce high levels of phosphorylation and coupling to the arrestin pathway to produce desensitization; on the other hand, morphine appears to produce little MOP phosphorylation and couples to PKCε to enhance ERK phosphorylation and hence desensitization.[121] In an elegant study using MOP mutants that blocked phosphorylation, Zheng and colleagues[122] recently demonstrated that etorphine (and fentanyl) now behaved like morphine. Similar agonist biased responses have been reported for the DOP receptor where SNC80 and ARM390 (DOP ligands with similar antinociceptive actions) produced different desensitization responses; SNC80 desensitized but ARM390 did not. Following chronic treatment, SNC80 reduced receptor density and ARM390 resulted in uncoupling of Ca^{2+} channels.[123] So can this give any clues to subtypes? The work in this area is still at an early stage and we cannot be firm here, but it will be interesting to use some of the discriminatory ligands in Table 1 (especially antagonists) to probe for differential antagonism of any biased agonist response.

We would like to end this section with some very speculative thoughts. Consider the calcitonin receptor-like receptor (CRLR—a class B GPCR; opioids are class A). When CRLR associates with receptor activity modifying protein (RAMP) isoform-1, it becomes a CGRP receptor but when it interacts with RAMP-2 or -3, it becomes an adrenomedullin receptor.[124] As a provocative thought what would common MOP (or DOP/KOP) gene product behave like if associated with similar modifying proteins?

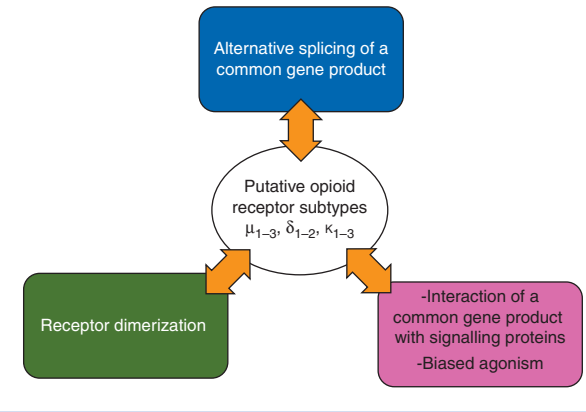

Fig 3 Suggestions to reconcile the differences between pharmacological subtypes and the result of molecular cloning.

Conclusions

Putative opioid receptor subtypes are suggested mainly by: (i) different modulation by pharmacological agents of functional responses (*in vivo*, *ex vivo*, and *in vitro*); (ii) an incomplete cross-tolerance profile between different receptor agonists; (iii) complex binding characteristics including: shallow ligand displacement curves and differential irreversibility of ligand binding. However, these suggestions must be set beside the molecular evidence of (i) only single receptor encoded by a single gene and (ii) genetic knockout of the single receptor gene results in a loss of ligand binding and function associated with that receptor. That there are physiological, pharmacological, and behavioural differences with ligands of different classes is not in doubt, but we do not believe that, based on these differences, receptor subtype status can be ascribed. Moreover, there is at least one example of possible misclassification (putative κ_3 is likely to be NOP).

We believe that these pharmacological subtypes can be reconciled with the molecular data by considering (i) alternative splicing, (ii) receptor dimerization, (iii) interaction with other proteins and biased agonism, and (iv) combinations of (i)–(iii) (Fig. 3).

We will finish by trying to answer the question 'Opioid receptors: fact or artifact?'; variations in ligand activity along with MOP, DOP, KOP, and NOP are a fact. As for the artifact, that is for the reader to decide, but we do not believe that subtype status can be ascribed. What *is* clear is that assigning variations in ligand activity to one or more of the molecular suggestions represents one of the future directions for opioid research.

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Conflict of interest

The authors have collaborative links with *University of Ferrara Peptides* (UFPeptides) that is involved in the development of opioid ligands. D.G.L. holds a consultancy with Grunenthal GmbH.

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References

1 Beckett AH, Casy AF. Synthetic analgesics: stereochemical considerations. *J Pharm Pharmacol* 1954; **6**: 986–1001

2 Pert CB, Snyder SH. Opiate receptor: demonstration in nervous tissue. *Science* 1973; **179**: 1011–4

3 Simon EJ, Hiller JM, Edelman I. Stereospecific binding of the potent narcotic analgesic (3H) Etorphine to rat-brain homogenate. *Proc Natl Acad Sci USA* 1973; **70**: 1947–9

4 Terenius L. Characteristics of the 'receptor' for narcotic analgesics in synaptic plasma membrane fraction from rat brain. *Acta Pharmacol Toxicol (Copenh)* 1973; **33**: 377–84

5 Martin WR, Eades CG, Thompson JA, Huppler RE, Gilbert PE. The effects of morphine- and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog. *J Pharmacol Exp Ther* 1976; **197**: 517–32

6 Lord JA, Waterfield AA, Hughes J, Kosterlitz HW. Endogenous opioid peptides: multiple agonists and receptors. *Nature* 1977; **267**: 495–9

7 Hughes J, Smith TW, Kosterlitz HW, Fothergill LA, Morgan BA, Morris HR. Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature* 1975; **258**: 577–80

8 Cox BM, Goldstein A, Hi CH. Opioid activity of a peptide, beta-lipotropin-(61–91), derived from beta-lipotropin. *Proc Natl Acad Sci USA* 1976; **73**: 1821–3

9 Goldstein A, Tachibana S, Lowney LI, Hunkapiller M, Hood L. Dynorphin-(1–13), an extraordinarily potent opioid peptide. *Proc Natl Acad Sci USA* 1979; **76**: 6666–70

10 Zadina JE, Hackler L, Ge LJ, Kastin AJ. A potent and selective endogenous agonist for the mu-opiate receptor. *Nature* 1997; **386**: 499–502

11 Kieffer BL, Befort K, Gaveriaux-Ruff C, Hirth CG. The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. *Proc Natl Acad Sci USA* 1992; **89**: 12048–52

12 Evans CJ, Keith DE, Morrison H, Magendzo K, Edwards RH. Cloning of a delta opioid receptor by functional expression. *Science* 1992; **258**: 1952–5

13 Zastawny RL, George SR, Nguyen T, *et al.* Cloning, characterization, and distribution of a mu-opioid receptor in rat brain. *J Neurochem* 1994; **62**: 2099–105

14 Thompson RC, Mansour A, Akil H, Watson SJ. Cloning and pharmacological characterization of a rat mu opioid receptor. *Neuron* 1993; **11**: 903–13

15 Chen Y, Mestek A, Liu J, Hurley JA, Yu L. Molecular cloning and functional expression of a mu-opioid receptor from rat brain. *Mol Pharmacol* 1993; **44**: 8–12

16 Wang JB, Imai Y, Eppler CM, Gregor P, Spivak CE, Uhl GR. mu opiate receptor: cDNA cloning and expression. *Proc Natl Acad Sci USA* 1993; **90**: 10230–4

17 Li S, Zhu J, Chen C, *et al.* Molecular cloning and expression of a rat kappa opioid receptor. *Biochem J* 1993; **295**: 629–33

18 Meng F, Xie GX, Thompson RC, *et al.* Cloning and pharmacological characterization of a rat kappa opioid receptor. *Proc Natl Acad Sci USA* 1993; **90**: 9954–8

19 Mollereau C, Parmentier M, Mailleux P, *et al.* ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. *FEBS Lett* 1994; **341**: 33–8

20 Bunzow JR, Saez C, Mortrud M, *et al.* Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a mu, delta or kappa opioid receptor type. *FEBS Lett* 1994; **347**: 284–8

21 Portoghese PS. A new concept on the mode of interaction of narcotic analgesics with receptors. *J Med Chem* 1965; **8**: 609–16

22 Wolozin BL, Pasternak GW. Classification of multiple morphine and enkephalin binding sites in the central nervous system. *Proc Natl Acad Sci USA* 1981; **78**: 6181–5

23 Pasternak GW, Childers SR, Snyder SH. Opiate analgesia: evidence for mediation by a subpopulation of opiate receptors. *Science* 1980; **208**: 514–6

24 Pasternak GW, Hahn EF. Long-acting opiate agonists and antagonists: 14-hydroxydihydromorphinone hydrazones. *J Med Chem* 1980; **23**: 674–6

25 Pasternak GW, Childers SR, Snyder SH. Naloxazone, a long-acting opiate antagonist: effects on analgesia in intact animals and on opiate receptor binding in vitro. *J Pharmacol Exp Ther* 1980; **214**: 455–62

26 Ling GS, Spiegel K, Lockhart SH, Pasternak GW. Separation of opioid analgesia from respiratory depression: evidence for different receptor mechanisms. *J Pharmacol Exp Ther* 1985; **232**: 149–55

27 Paul D, Pasternak GW. Differential blockade by naloxonazine of two mu opiate actions: analgesia and inhibition of gastrointestinal transit. *Eur J Pharmacol* 1988; **149**: 403–4

28 Andoh T, Yageta Y, Konno M, *et al.* Evidence for separate involvement of different mu-opioid receptor subtypes in itch and analgesia induced by supraspinal action of opioids. *J Pharmacol Sci* 2008; **106**: 667–70

29 Hahn EF, Carroll-Buatti M, Pasternak GW. Irreversible opiate agonists and antagonists: the 14-hydroxydihydromorphinone azines. *J Neurosci* 1982; **2**: 572–6

30 Chaijale NN, Aloyo VJ, Simansky KJ. A naloxonazine sensitive (mu1 receptor) mechanism in the parabrachial nucleus modulates eating. *Brain Res* 2008; **1240**: 111–8

31 Paul D, Bodnar RJ, Gistrak MA, Pasternak GW. Different mu receptor subtypes mediate spinal and supraspinal analgesia in mice. *Eur J Pharmacol* 1989; **168**: 307–14

32 Pasternak GW. Molecular biology of opioid analgesia. *J Pain Symptom Manage* 2005; **29**: S2–9

33 Rothman RB, Jacobson AE, Rice KC, Herkenham M. Autoradiographic evidence for two classes of mu opioid binding sites in rat brain using [125I]FK33824. *Peptides* 1987; **8**: 1015–21

34 Franklin TG, Traynor JR. Alkylation with beta-funaltrexamine suggests differences between mu-opioid receptor systems in guinea-pig brain and myenteric-plexus. *Br J Pharmacol* 1991; **102**: 718–22

35 Elliott J, Smart D, Lambert DG, Traynor JR. Characterisation of mu-opioid receptors on SH-SY5Y cells using naloxonazine and beta-funaltrexamine. *Eur J Pharmacol* 1994; **268**: 447–50

36 Tive LA, Pick CG, Paul D, Roques BP, Gacel GA, Pasternak GW. Analgesic potency of TRIMU-5: a mixed mu 2 opioid receptor agonist/mu 1 opioid receptor antagonist. *Eur J Pharmacol* 1992; **216**: 249–55

37 Pick CG, Roques B, Gacel G, Pasternak GW. Supraspinal mu 2-opioid receptors mediate spinal/supraspinal morphine synergy. *Eur J Pharmacol* 1992; **220**: 275–7

38 Moskowitz AS, Goodman RR. Autoradiographic distribution of mu1 and mu2 opioid binding in the mouse central nervous system. *Brain Res* 1985; **360**: 117–29

39 Chang A, Emmel DW, Rossi GC, Pasternak GW. Methadone analgesia in morphine-insensitive CXBK mice. *Eur J Pharmacol* 1998; **351**: 189–91

40 Pick CG, Nejat RJ, Pasternak GW. Independent expression of two pharmacologically distinct supraspinal mu analgesic systems in genetically different mouse strains. *J Pharmacol Exp Ther* 1993; **265**: 166–71

41 Cadet P, Mantione KJ, Stefano GB. Molecular identification and functional expression of mu 3, a novel alternatively spliced variant of the human mu opiate receptor gene. *J Immunol* 2003; **170**: 5118–23

42 Cadet P. Mu opiate receptor subtypes. *Med Sci Monit* 2004; **10**: MS28–32

43 Stefano GB, Hartman A, Bilfinger TV, *et al.* Presence of the mu3 opiate receptor in endothelial cells. Coupling to nitric oxide production and vasodilation. *J Biol Chem* 1995; **270**: 30290–3

44 Stefano GB, Goumon Y, Bilfinger TV, Welters ID, Cadet P. Basal nitric oxide limits immune, nervous and cardiovascular excitation: human endothelia express a mu opiate receptor. *Prog Neurobiol* 2000; **60**: 513–30

45 Stefano GB, Digenis A, Spector S, *et al.* Opiate-like substances in an invertebrate, an opiate receptor on invertebrate and human immunocytes, and a role in immunosuppression. *Proc Natl Acad Sci USA* 1993; **90**: 11099–103

46 Davis RL, Buck DJ, Saffarian N, *et al.* Beta-funaltrexamine inhibits inducible nitric-oxide synthase expression in human astroglial cells. *J Neuroimmune Pharmacol* 2008; **3**: 150–3

47 Lambert DG, Atcheson R, Hirst RA, Rowbotham DJ. Effects of morphine and its metabolites on opiate receptor binding, cAMP formation and [3H]noradrenaline release from SH-SY5Y cells. *Biochem Pharmacol* 1993; **46**: 1145–50

48 Rossi GC, Brown GP, Leventhal L, Yang K, Pasternak GW. Novel receptor mechanisms for heroin and morphine-6 beta-glucuronide analgesia. *Neurosci Lett* 1996; **216**: 1–4

49 Rossi GC, Standifer KM, Pasternak GW. Differential blockade of morphine and morphine-6 beta-glucuronide analgesia by antisense oligodeoxynucleotides directed against MOR-1 and G-protein alpha subunits in rats. *Neurosci Lett* 1995; **198**: 99–102

50 Vanderah T, Takemori AE, Sultana M, *et al.* Interaction of [D-Pen2,D-Pen5]enkephalin and [D-Ala2,Glu4]deltorphin with delta-opioid receptor subtypes in vivo. *Eur J Pharmacol* 1994; **252**: 133–7

51 Hirose N, Murakawa K, Takada K, *et al.* Interactions among mu- and delta-opioid receptors, especially putative delta1- and delta2-opioid receptors, promote dopamine release in the nucleus accumbens. *Neuroscience* 2005; **135**: 213–25

52 Jiang Q, Takemori AE, Sultana M, *et al.* Differential antagonism of opioid delta antinociception by [D-Ala2,Leu5,Cys6]enkephalin and naltrindole 5′-isothiocyanate: evidence for delta receptor subtypes. *J Pharmacol Exp Ther* 1991; **257**: 1069–75

53 Maslov LN, Lishmanov YB, Oeltgen PR, *et al.* Activation of peripheral delta2 opioid receptors increases cardiac tolerance to ischemia/reperfusion injury Involvement of protein kinase C, NO-synthase, KATP channels and the autonomic nervous system. *Life Sci* 2009; **84**: 657–63

54 Rawls SM, Hewson JM, Inan S, Cowan A. Brain delta2 opioid receptors mediate SNC-80-evoked hypothermia in rats. *Brain Res* 2005; **1049**: 61–9

55 Dietis N, Guerrini R, Calo G, Salvadori S, Rowbotham DJ, Lambert DG. Simultaneous targeting of multiple opioid receptors: a strategy to improve side-effect profile. *Br J Anaesth* 2009; **103**: 38–49

56 Traynor JR, Elliott J. delta-Opioid receptor subtypes and cross-talk with mu-receptors. *Trends Pharmacol Sci* 1993; **14**: 84–6

57 Wollemann M, Benyhe S, Simon J. The kappa-opioid receptor: evidence for the different subtypes. *Life Sci* 1993; **52**: 599–611

58 Clark JA, Liu L, Price M, Hersh B, Edelson M, Pasternak GW. Kappa opiate receptor multiplicity: evidence for two U50,488-sensitive kappa 1 subtypes and a novel kappa 3 subtype. *J Pharmacol Exp Ther* 1989; **251**: 461–8

59 Nielsen CK, Ross FB, Lotfipour S, Saini KS, Edwards SR, Smith MT. Oxycodone and morphine have distinctly different pharmacological profiles: radioligand binding and behavioural studies in two rat models of neuropathic pain. *Pain* 2007; **132**: 289–300

60 Zukin RS, Eghbali M, Olive D, Unterwald EM, Tempel A. Characterization and visualization of rat and guinea pig brain kappa opioid receptors: evidence for kappa 1 and kappa 2 opioid receptors. *Proc Natl Acad Sci USA* 1988; **85**: 4061–5

61 Rothman RB, Bykov V, Xue BG, *et al.* Interaction of opioid peptides and other drugs with multiple kappa receptors in rat and human brain. Evidence for species differences. *Peptides* 1992; **13**: 977–87

62 Su X, Sengupta JN, Gebhart GF. Effects of kappa opioid receptor-selective agonists on responses of pelvic nerve afferents to noxious colorectal distension. *J Neurophysiol* 1997; **78**: 1003–12

63 Childers SR, Xiao R, Vogt L, Sim LJ. Lack of evidence of kappa2-selective activation of G-proteins: kappa opioid receptor stimulation of [35S] GTPgammaS binding in guinea pig brain. *Biochem Pharmacol* 1998; **56**: 113–20

64 Pan YX, Cheng J, Xu J, *et al.* Cloning and functional characterization through antisense mapping of a kappa 3-related opioid receptor. *Mol Pharmacol* 1995; **47**: 1180–8

65 Lambert DG. The nociceptin/orphanin FQ receptor: a target with broad therapeutic potential. *Nat Rev Drug Discov* 2008; **7**: 694–710

66 Paul D, Pick CG, Tive LA, Pasternak GW. Pharmacological characterization of nalorphine, a kappa 3 analgesic. *J Pharmacol Exp Ther* 1991; **257**: 1–7

67 Connor M, Kitchen I. Has the sun set on kappa3-opioid receptors? *Br J Pharmacol* 2006; **147**: 349–50

68 Nielsen CK, Ross FB, Smith MT. Incomplete, asymmetric, and route-dependent cross-tolerance between oxycodone and

morphine in the Dark Agouti rat. *J Pharmacol Exp Ther* 2000; **295**: 91–9

69 Nozaki C, Kamei J. Involvement of mu1-opioid receptor on oxycodone-induced antinociception in diabetic mice. *Eur J Pharmacol* 2007; **560**: 160–2

70 Meunier JC, Mollereau C, Toll L, *et al.* Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* 1995; **377**: 532–5

71 Reinscheid RK, Nothacker HP, Bourson A, *et al.* Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science* 1995; **270**: 792–4

72 McDonald J, Barnes TA, Okawa H, *et al.* Partial agonist behaviour depends upon the level of nociceptin/orphanin FQ receptor expression: studies using the ecdysone-inducible mammalian expression system. *Br J Pharmacol* 2003; **140**: 61–70

73 Mason SL, Ho M, Nicholson J, McKnight AT. In vitro characterization of Ac-RYYRWK-NH(2), Ac-RYYRIK-NH(2) and [Phe1Psi(CH(2)-NH)Gly2] nociceptin(1–13)NH(2) at rat native and recombinant ORL(1) receptors. *Neuropeptides* 2001; **35**: 244–56

74 Okawa H, Nicol B, Bigoni R, *et al.* Comparison of the effects of [Phe1psi(CH2-NH)Gly2]nociceptin(1–13)NH2 in rat brain, rat vas deferens and CHO cells expressing recombinant human nociceptin receptors. *Br J Pharmacol* 1999; **127**: 123–30

75 Xie G, Ito E, Maruyama K, *et al.* An alternatively spliced transcript of the rat nociceptin receptor ORL1 gene encodes a truncated receptor. *Brain Res Mol Brain Res* 2000; **77**: 1–9

76 Currò D, Yoo JH, Anderson M, Song I, Del Valle J, Owyang C. Molecular cloning of the orphanin FQ receptor gene and differential tissue expression of splice variants in rat. *Gene* 2001; **266**: 139–45

77 Xie GX, Meuser T, Pietruck C, Sharma M, Palmer PP. Presence of opioid receptor-like (ORL1) receptor mRNA splice variants in peripheral sensory and sympathetic neuronal ganglia. *Life Sci* 1999; **64**: 2029–37

78 Pan YX, Xu J, Wan BL, Zuckerman A, Pasternak GW. Identification and differential regional expression of KOR-3/ORL-1 gene splice variants in mouse brain. *FEBS Lett* 1998; **435**: 65–8

79 Liao YY, Teng SF, Lin LC, *et al.* Functional heterogeneity of nociceptin/orphanin FQ receptors revealed by (+)-5a Compound and Ro 64–6198 in rat periaqueductal grey slices. *Int J Neuropsychopharmacol* 2010; Oct 29 [Epub ahead of print] 1–13

80 Chiou LC, Chuang KC, Wichmann J, Adam G. Ro 64–6198 [(1S,3aS)-8-(2,3,3a,4,5,6-Hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one] acts differently from nociceptin/orphanin FQ in rat periaqueductal gray slices. *J Pharmacol Exp Ther* 2004; **311**: 645–51

81 Rizzi A, Molinari S, Marti M, Marzola G, Calo' G. Nociceptin/orphanin FQ receptor knockout rats: in vitro and in vivo studies. *Neuropharmacology* 2011; **60**: 572–9

82 Ueda H, Yamaguchi T, Tokuyama S, Inoue M, Nishi M, Takeshima H. Partial loss of tolerance liability to morphine analgesia in mice lacking the nociceptin receptor gene. *Neurosci Lett* 1997; **237**: 136–8

83 Chung S, Pohl S, Zeng J, Civelli O, Reinscheid RK. Endogenous orphanin FQ/nociceptin is involved in the development of morphine tolerance. *J Pharmacol Exp Ther* 2006; **318**: 262–7

84 Burmeister MA, Ansonoff MA, Pintar JE, Kapusta DR. Nociceptin/orphanin FQ (N/OFQ)-evoked bradycardia, hypotension, and diuresis are absent in N/OFQ peptide (NOP) receptor knockout mice. *J Pharmacol Exp Ther* 2008; **326**: 897–904

85 Noda Y, Mamiya T, Nabeshima T, Nishi M, Higashioka M, Takeshima H. Loss of antinociception induced by naloxone benzoylhydrazone in nociceptin receptor-knockout mice. *J Biol Chem* 1998; **273**: 18047–51

86 Nitsche JF, Schuller AG, King MA, Zengh M, Pasternak GW, Pintar JE. Genetic dissociation of opiate tolerance and physical dependence in delta-opioid receptor-1 and preproenkephalin knock-out mice. *J Neurosci* 2002; **22**: 10906–13

87 Kitchen I, Slowe SJ, Matthes HW, Kieffer B. Quantitative autoradiographic mapping of mu-, delta- and kappa-opioid receptors in knockout mice lacking the mu-opioid receptor gene. *Brain Res* 1997; **778**: 73–88

88 Kieffer BL. Opioids: first lessons from knockout mice. *Trends Pharmacol Sci* 1999; **20**: 19–26

89 Zhu Y, King MA, Schuller AG, *et al.* Retention of supraspinal delta-like analgesia and loss of morphine tolerance in delta opioid receptor knockout mice. *Neuron* 1999; **24**: 243–52

90 Simonin F, Slowe S, Becker JA, *et al.* Analysis of [3H]bremazocine binding in single and combinatorial opioid receptor knockout mice. *Eur J Pharmacol* 2001; **414**: 189–95

91 Wei LN, Loh HH. Transcriptional and epigenetic regulation of opioid receptor genes: present and future. *Annu Rev Pharmacol Toxicol* 2011; **51**: 75–97

92 Black DL. Mechanisms of alternative pre-messenger RNA splicing. *Annu Rev Biochem* 2003; **72**: 291–336

93 Matlin AJ, Clark F, Smith CW. Understanding alternative splicing: towards a cellular code. *Nat Rev Mol Cell Biol* 2005; **6**: 386–98

94 Tanowitz M, Hislop JN, von Zastrow M. Alternative splicing determines the post-endocytic sorting fate of G-protein-coupled receptors. *J Biol Chem* 2008; **283**: 35614–21

95 Zimprich A, Simon T, Höllt V. Cloning and expression of an isoform of the rat mu opioid receptor (rMOR1B) which differs in agonist induced desensitization from rMOR1. *FEBS Lett* 1995; **359**: 142–6

96 Xu J, Xu M, Rossi GC, Pasternak GW, Pan YX. Identification and characterization of seven new exon 11-associated splice variants of the rat mu opioid receptor gene, OPRM1. *Mol Pain* 2011; **7**: 9

97 Pan YX, Xu J, Mahurter L, Bolan E, Xu M, Pasternak GW. Generation of the mu opioid receptor (MOR-1) protein by three new splice variants of the Oprm gene. *Proc Natl Acad Sci USA* 2001; **98**: 14084–9

98 Pan YX. Diversity and complexity of the mu opioid receptor gene: alternative pre-mRNA splicing and promoters. *DNA Cell Biol* 2005; **24**: 736–50

99 Pan YX, Xu J, Xu M, Rossi GC, Matulonis JE, Pasternak GW. Involvement of exon 11-associated variants of the mu opioid receptor MOR-1 in heroin, but not morphine, actions. *Proc Natl Acad Sci USA* 2009; **106**: 4917–22

100 Schuller AG, King MA, Zhang J, *et al.* Retention of heroin and morphine-6 beta-glucuronide analgesia in a new line of mice lacking exon 1 of MOR-1. *Nat Neurosci* 1999; **2**: 151–6

101 Jordan BA, Devi LA. G-protein-coupled receptor heterodimerization modulates receptor function. *Nature* 1999; **399**: 697–700

102 George SR, Fan T, Xie Z, *et al.* Oligomerization of mu- and delta-opioid receptors. Generation of novel functional properties. *J Biol Chem* 2000; **275**: 26128–35

103 Cvejic S, Devi LA. Dimerization of the delta opioid receptor: implication for a role in receptor internalization. *J Biol Chem* 1997; **272**: 26959–64

104 Ramsay D, Kellett E, McVey M, Rees S, Milligan G. Homo- and hetero-oligomeric interactions between G-protein-coupled

receptors in living cells monitored by two variants of bioluminescence resonance energy transfer (BRET): hetero-oligomers between receptor subtypes form more efficiently than between less closely related sequences. *Biochem J* 2002; **365**: 429–40

105 McVey M, Ramsay D, Kellett E, *et al.* Monitoring receptor oligomerization using time-resolved fluorescence resonance energy transfer and bioluminescence resonance energy transfer. The human delta-opioid receptor displays constitutive oligomerization at the cell surface, which is not regulated by receptor occupancy. *J Biol Chem* 2001; **276**: 14092–9

106 Gomes I, Jordan BA, Gupta A, Trapaidze N, Nagy V, Devi LA. Heterodimerization of mu and delta opioid receptors: a role in opiate synergy. *J Neurosci* 2000; **20**: RC110

107 van Rijn RM, Whistler JL, Waldhoer M. Opioid-receptor-heteromer-specific trafficking and pharmacology. *Curr Opin Pharmacol* 2010; **10**: 73–9

108 Chakrabarti S, Liu NJ, Gintzler AR. Formation of mu-/kappa-opioid receptor heterodimer is sex-dependent and mediates female-specific opioid analgesia. *Proc Natl Acad Sci USA* 2010; **107**: 20115–9

109 Pan YX, Bolan E, Pasternak GW. Dimerization of morphine and orphanin FQ/nociceptin receptors: generation of a novel opioid receptor subtype. *Biochem Biophys Res Commun* 2002; **297**: 659–63

110 Wang HL, Hsu CY, Huang PC, *et al.* Heterodimerization of opioid receptor-like 1 and mu-opioid receptors impairs the potency of micro receptor agonist. *J Neurochem* 2005; **92**: 1285–94

111 Evans RM, You H, Hameed S, *et al.* Heterodimerization of ORL1 and opioid receptors and its consequences for N-type calcium channel regulation. *J Biol Chem* 2010; **285**: 1032–40

112 Chakrabarti S, Regec A, Gintzler AR. Biochemical demonstration of mu-opioid receptor association with Gsalpha: enhancement following morphine exposure. *Brain Res Mol Brain Res* 2005; **135**: 217–24

113 Connor M, Christie MD. Opioid receptor signalling mechanisms. *Clin Exp Pharmacol Physiol* 1999; **26**: 493–9

114 Crain SM, Shen KF. Modulation of opioid analgesia, tolerance and dependence by Gs-coupled, GM1 ganglioside-regulated opioid receptor functions. *Trends Pharmacol Sci* 1998; **19**: 358–65

115 Smart D, Smith G, Lambert DG. mu-Opioid receptor stimulation of inositol (1,4,5)trisphosphate formation via a pertussis toxin-sensitive G protein. *J Neurochem* 1994; **62**: 1009–14

116 Smart D, Lambert DG. delta-Opioids stimulate inositol 1,4,5-trisphosphate formation, and so mobilize Ca^{2+} from intracellular stores, in undifferentiated NG108–15 cells. *J Neurochem* 1996; **66**: 1462–7

117 Whistler JL, von Zastrow M. Morphine-activated opioid receptors elude desensitization by beta-arrestin. *Proc Natl Acad Sci USA* 1998; **95**: 9914–9

118 Ramsay D, Carr IC, Pediani J, *et al.* High-affinity interactions between human alpha1A-adrenoceptor C-terminal splice variants produce homo- and heterodimers but do not generate the alpha1L-adrenoceptor. *Mol Pharmacol* 2004; **66**: 228–39

119 Kenakin T. Functional selectivity through protean and biased agonism: who steers the ship? *Mol Pharmacol* 2007; **72**: 1393–401

120 Evans BA, Sato M, Sarwar M, Hutchinson DS, Summers RJ. Ligand-directed signalling at beta-adrenoceptors. *Br J Pharmacol* 2010; **159**: 1022–38

121 Chu J, Zheng H, Zhang Y, Loh HH, Law PY. Agonist-dependent mu-opioid receptor signaling can lead to heterologous desensitization. *Cell Signal* 2010; **22**: 684–96

122 Zheng H, Chu J, Zhang Y, Loh HH, Law PY. Modulating {micro}-opioid receptor phosphorylation switches agonist-dependent signaling as reflected in PKC{epsilon} activation and dendritic spine stability. *J Biol Chem* 2011; **286**: 12724–33

123 Pradhan AA, Walwyn W, Nozaki C, *et al.* Ligand-directed trafficking of the δ-opioid receptor in vivo: two paths toward analgesic tolerance. *J Neurosci* 2010; **30**: 16459–68

124 Brain SD, Grant AD. Vascular actions of calcitonin gene-related peptide and adrenomedullin. *Physiol Rev* 2004; **84**: 903–34