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# Therapeutic potential of opioid receptor heteromers in chronic pain and associated comorbidities

Running title: **Opioid receptor heteromers** in chronic pain

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## **NOMENCLATURE OF TARGETS AND LIGANDS**

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander *et al.*, 2019).

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## **CONFLICT OF INTEREST STATEMENT**

The authors declare no conflict of interest

## **DATA AVAILABILITY**

Data sharing is not applicable to this article because no new data were created or analysed in this study

## **ABSTRACT**

Chronic pain affects 20 to 45% of the global population and is often associated with the development of anxiodepressive disorders. Treatment of this debilitating condition remains particularly challenging with opioids prescribed to alleviate moderate to severe pain. However, despite strong antinociceptive properties, numerous adverse effects limit opioid use in the clinic. Moreover, opioid misuse and abuse have become a major health concern worldwide. This prompted efforts to design original strategies that would efficiently and safely relieve pain. Targeting of opioid receptor heteromers is one of these. This review summarizes our current knowledge on the role of heteromers involving opioid receptors in the context of chronic pain and anxiodepressive comorbidities. It also examines how heteromerization in native tissue affects ligand binding, receptor signalling and trafficking properties. Finally, the therapeutic potential of ligands designed to specifically target opioid receptor heteromers is considered.

## **KEYWORDS**

Neuropharmacology, pain, drug discovery/target validation, GPCR, ligands, opioids, heteromers

## **ABBREVIATIONS**

5'-GNTI, 5'-guanidinonaltrindole; 6'-GNTI, 6'-guanidinonaltrindole; Acb, nucleus accumbens; BLA, basolateral amygdala; CCL5= chemokine (C-Cmotif) ligand 5; Co-IP, co-immunoprecipitation; CPP, conditioned place preference; CREB, cAMP response element-binding protein; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH<sub>2</sub>; CYM51010, 1-4-(Acetylamino)phenylmethyl-4-(2-phenylethyl)-4-

Piperidinecarboxylic acid ethyl ester; DAMGO, [D-Ala<sup>2</sup>, N-MePhe<sup>4</sup>, Gly-ol]-enkephalin; DAPTA, D-Ala-peptide T-amide; DPDPE, D-Pen<sup>2,5</sup>-Enkephalin; DRG, dorsal root ganglia; ER, endoplasmic reticulum; FCS, fluorescence correlation spectroscopy; FDA, american food and drug administration; IASP, international association for the study of pain; HU210, (6aR-trans-3-(1, 1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzob,dpyran-9-methanol; Iba1= Allograft inflammatory factor 1; ICI-199441, 2-(3,4-Dichlorophenyl)-N-methyl-N-[(1S)-1-phenyl-2-(1-pyrrolidinyl)ethyl]acetamide hydrochloride; ISH, in situ hybridization; i.pl., intraplantar; JWH-018, (1-pentyl-3-(1-naphthoyl)indole); LC, locus coeruleus; M40, 1R,2S)-2-({N-[(benzyloxy)carbonyl]-3-cyclohexyl-L-alanyl}amino)-1-hydroxy-3-[(3S)-2-oxopyrrolidin-3-yl]propane-1-sulfonic acid; na, not applicable; MCL101 (-)-3-hydroxy-N-cyclobutylmethylmorphinan S(+)-mandelate; NMR, nuclear magnetic resonance; NNTA, N-naphthoyl-β-naltrexamine; nor-BNI, norbinaltorphimine; PAG, periaqueductal gray; PBMC, peripheral blood mononuclear cells; PD-PALM, photoactivatable dyes photo-activated localization microscopy; PF-514273, 2-(2-Chlorophenyl)-3-(4-chlorophenyl)-7-(2,2-difluoropropyl)-6,7-dihydro-2H-pyrazolo[3,4-f][1,4]oxazepin-8(5H)-one; PFC, prefrontal cortex; PLA, proximity ligation assay; RVM, rostral ventromedial medulla; SC, spinal cord; Sol, nucleus of the solitary tract; SNC80, (+)-4-[(αR)-α-((2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide; SRI22141=5'-(4-Chlorophenyl)-6,7-didehydro-4,5α-epoxy-3-hydroxy-17-methyl-14-(3-phenylpropoxy)pyrido[2',3':6,7]morphinan; STED, stimulated emission depletion; TAK-220, 1-acetyl-N-[3-[4-[[4-(aminocarbonyl)phenyl]methyl]-1-piperidinyl]propyl]-N-(3-chloro-4-methylphenyl)-4-piperidinecarboxamide; TAT, transactivating transcriptional activator; TIPPP<sub>ψ</sub>, (2S)-2-[[[(2S)-2-[[[(3S)-2-[(2S)-2-amino-3-(4-hydroxyphenyl)propanoyl]-3,4-dihydro-1H-isoquinolin-3-yl]methylamino]-3-phenylpropanoyl]amino]-3-phenylpropanoic acid; TG, trigeminal ganglia; TM, transmembrane domain; UFP512, (3S)-3-[[[(3S)-2-[(2S)-2-amino-3-(4-hydroxy-2,6-dimethylphenyl)propanoyl]3,4-dihydro-1H-isoquinoline-3-carbonyl]amino]-3-(1H-benzimidazol-2-yl)propanoic acid; VTA, ventral tegmental area.

## 1. INTRODUCTION

According to the International Association for the Study of Pain (IASP), pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage (Raja *et al.*, 2020). This complex phenomenon involves sensory, emotional and cognitive dimensions that can be influenced by factors such as emotional state, attention, culture or personal experience. In physiological conditions, pain serves a protective role but becomes pathological when persisting or recurring for more than 3 months (Treede *et al.*, 2019). Chronic pain is thus a debilitating condition interfering with daily life (Breivik *et al.*, 2013) and often coincides with the development of major depressive disorder with a comorbid prevalence around 50% (reviewed in (Bair *et al.*, 2003)). Because it affects between 20 to 45% of the global population (Breivik *et al.*, 2013), treating chronic pain is of primary importance. Treatments commonly prescribed to treat acute pain are often minimally effective. Tricyclic antidepressants (e. g. amitryptiline) or selective serotonin-noradrenaline recapture inhibitors (e.g. duloxetine, venlafaxine), but also antiepileptics such as gabapentinoids (e.g. pregabalin or gabapentin) are recommended as first-line treatments. Second- and third-line treatments include weak (e. g. Tramadol) and strong (e. g. oxycodone and morphine) opioids (reviewed in (Kremer *et al.*, 2016)), respectively. However, opioid treatments frequently produce unwanted side effects, resulting from both acute (e. g. respiratory depression, nausea, dizziness, sedation, constipation) and long-term (tolerance, hyperalgesia and dependence) use. In the USA and Canada, the growing need to treat chronic pain increased prescription opioid use, leading to a dramatic rise in misuse, abuse, and drug overdose mortality (reviewed in (Busserolles *et al.*, 2020; Kremer *et al.*, 2016)). This so-called opioid crisis (or epidemic) prompted numerous efforts to design innovative strategies that would ally efficient pain relief and safe usage.

Studies using mice deficient in the mu opioid ( $\mu$ ) receptor (Alexander *et al.*, 2019b) revealed that this receptor is the primary molecular target of opioids, mediating both analgesic and unwanted side effects (Matthes *et al.*, 1996). This G protein-coupled receptor (GPCR) not only activates G protein dependent signalling cascades, but also  $\beta$ -arrestin dependent ones. In  $\beta$ -arrestin 2 knockout animals, morphine-induced analgesia was enhanced with a diminution of tolerance, constipation and respiratory depression (Bohn *et al.*, 2000; Raehal *et al.*, 2005) raising hopes that biased agonists favouring G-protein signalling would improve morphine analgesia while decreasing unwanted effects. However, this strategy proved deceptive and the concept of G protein biased strategy and its therapeutic relevance are currently debated. Indeed, constipation and respiratory depression were observed in genetically modified mice with impaired activation of the  $\beta$ -arrestin pathway indicating that these opioid adverse effects depend on activation of G protein dependent signalling cascades (Kliwer *et al.*, 2019). For example, oliceridine (TRV-130), recently approved by the American Food and Drug Administration (FDA N° 210730) for severe acute pain in medical environments, shows analgesic potency similar to morphine but retains most of the adverse effects (Altarifi *et al.*, 2017). Recent studies evidenced that G protein biased agonists such as oliceridine behave as  $\mu$  partial agonists suggesting that the biased activation of G protein signalling reflects the low intrinsic efficacy of these ligands (reviewed in (Gillis *et al.*, 2020; Singleton *et al.*, 2021)).

In response to the urgent need for alternative therapeutic strategies, several lines of research are explored such as selective activation of peripheral receptors or receptor splice variants, single molecules targeting multiple opioid receptors, or strengthening of endogenous opioid peptide action (reviewed in (Gunther *et al.*, 2018; Machelska and Celik, 2018)). In addition, opioid receptor heteromers have also gained attention. These macromolecular complexes are formed by association of at least two functional receptors with ligand binding, receptor signalling and/or trafficking properties different from those of the individual components. Accordingly, disruption of the close physical proximity between receptors in native tissue drastically alters the specific properties of heteromers (Massotte, 2015). Here, we review current knowledge on the contribution of opioid receptor heteromers to chronic pain states and associated comorbidities, morphine analgesia and side effects. We conclude by examining the therapeutic potential of these compounds.

## 2. IN VIVO HETEROMERIZATION

Physical proximity has been demonstrated for a limited number of receptor pairs in the spinal cord, dorsal root ganglia (DRG) and supraspinal structures but also in immune or cancer cells using co-immunoprecipitation, disruption of physical interaction using TAT fused peptides, proximity ligation assay and/or antibodies selective for receptor heteromers (Table 1, Figure 1). In addition, physical association of the  $\mu$  and vasopressin 1B ( $V_{1B}$ ) receptors is supported by a loss of functional outcome in mice expressing a C-terminal truncated form of the  $V_{1B}$  receptor (Koshimizu *et al.*, 2018). Also, physical proximity of the  $\mu$  and cannabinoid  $CB_1$  ( $CB1$ ) receptors was detected by electron microscopy in striatal synapses (Rodriguez *et al.*, 2001). These two receptor pairs will therefore be discussed here. Other receptor pairs have been reported in co-transfected cells but will not be considered here as, to the best of our knowledge, no information is available regarding *in vivo* physical proximity (reviewed in (Ugur *et al.*, 2018)).

The heteromerization process remains largely unknown in native environments but involves constitutive association taking place in the endoplasmic reticulum (ER) in co-transfected cells (Décaillot *et al.*, 2008; Fujita *et al.*, 2019; Hasbi *et al.*, 2007; Xie *et al.*, 2009). Biochemical studies based on the use of interfering peptides or mutant receptors pointed to a contact interface involving the  $\mu$  receptor transmembrane domain (TM) TM1 in heteromers between  $\mu$  and delta opioid ( $\delta$ ) receptors (He *et al.*, 2011) or TM5 in  $\mu$ -galanin 1 ( $GAL_1$ ) heteromers (Moreno *et al.*, 2017). In addition, the C-terminal tail appeared critical in the heteromerization process of  $\mu$ - $\delta$  (Fan *et al.*, 2005; Kabli *et al.*, 2013; Law *et al.*, 2005; O'Dowd *et al.*, 2012; Walwyn *et al.*, 2009) or  $\mu$ -nociceptin (NOP) receptors (Wang *et al.*, 2005) and the third intracellular loop of the  $\mu$  or  $\delta$  receptors in  $\mu$ - $\delta$  heteromerization (Law *et al.*, 2005). Physical association with ion channels has also been identified between  $\mu$  or dopamine  $D_1$  and the GluN1 subunit of the NMDA receptors or between NOP receptors and N-type Calcium channels ( $Ca_v2.2$ ) (Alexander *et al.*, 2019a) pointing to more complex functional interactions (see section 6)(Table 1).

## 3. HETEROMERS AND CHRONIC PAIN

Only four heteromers have been clearly identified as participating in the reduction of the nociceptive signal in chronic pain conditions (Figure 2). Three ( $\delta$ - kappa opioid ( $\kappa$ ),  $\delta$ - $CB_1$ ,  $\mu$ - $\delta$ ) involve the  $\delta$  receptor whose expression is increased in neuropathic or inflammatory conditions and whose selective targeting alleviates mechanical allodynia (Gendron *et al.*, 2015; Nadal *et al.*, 2013). The fourth heteromer involves association of the  $\mu$  and CCR5 chemokine receptors.

### 3.1 $\mu$ - $\delta$ heteromers

$\mu$ - $\delta$  close physical proximity was evidenced in the DRG (Xie *et al.*, 2009), spinal cord (Gomes *et al.*, 2004; He *et al.*, 2011), hippocampus (Erbs *et al.*, 2015) and nucleus accumbens (Acb) (Kabli *et al.*, 2013). However, brain mapping of the receptors using fluorescent knockin mice also revealed co-expression in discrete neuronal populations located in subcortical networks essential for the perception and processing of aversive stimuli (Erbs *et al.*, 2015), suggesting that  $\mu$ - $\delta$  heteromerization could be more widely distributed. The impact of  $\mu$ - $\delta$  heteromerization on ligand binding, receptor signalling, and trafficking has been mostly studied in co-transfected cells (reviewed in (Fujita *et al.*, 2014a)). However, increasing evidence indicates that  $\mu$ - $\delta$  heteromerization indeed affects ligand binding properties and modifies  $\mu$  receptor signalling and trafficking in native environments. Positive cooperativity reflecting bidirectional positive allosteric modulation was reported in SKNSH neuroblastoma cells where occupation of the binding site of either receptor by an agonist, an antagonist or an inverse agonist increased the affinity of the other receptor for agonists (Gomes *et al.*, 2000; Gomes *et al.*, 2004; Gomes *et al.*, 2011). This suggests that the interface between the interacting receptors is dynamic as reported for the neurotensin 1 (NTS1) receptor (Dijkman *et al.*, 2018) and that ligand binding would stabilize the receptor conformation and possibly favour receptor association as observed for class C metabotropic receptors (Moller *et al.*, 2018). In mouse membrane preparations, this positive-cooperativity led to a significant increase in the potency and efficacy of  $\mu$  agonists in terms

of G protein signalling (Gomes *et al.*, 2004), MAPK phosphorylation (Gomes *et al.*, 2000) or inhibition of voltage dependent calcium channels (Walwyn *et al.*, 2009). These effects were lost in membranes from mice lacking the  $\delta$  receptor (Gomes *et al.*, 2004; Walwyn *et al.*, 2009) or in the presence of antibodies specific for  $\mu$ - $\delta$  heteromers (Gupta *et al.*, 2010). Bidirectional positive allosteric modulation in  $\mu$ - $\delta$  heteromers could also underlie the increased neuronal hyperpolarisation observed in neurons of the ventral tegmental area (VTA) upon co-application of an antagonist of one receptor and an agonist for the other (Margolis *et al.*, 2017).

Ligand selective co-internalization and co-targeting to the lysosomal compartment of  $\mu$  and  $\delta$  receptors were observed in primary hippocampal neurons, suggesting that  $\mu$ - $\delta$  heteromerization modifies the  $\mu$  receptor intracellular fate (Derouiche *et al.*, 2020). However, phosphorylation by second messenger recruited kinases cannot be entirely excluded. In SKNSH neuroblastoma cells, treatment with DAMGO or deltorphan II led to sustained ERK1/2 phosphorylation that was absent in cells pretreated with a  $\mu$  siRNA or with a  $\mu$  or a  $\delta$  antagonist (Rozenfeld and Devi, 2007).  $\beta$ -arrestin 2 knockdown also abolished sustained ERK1/2 phosphorylation (Rozenfeld and Devi, 2007). Because  $\beta$ -arrestin recruitment initiates receptor internalization in clathrin coated pits, sustained ERK phosphorylation may reflect signalling by co-internalized receptors in agreement with recent advances in the subcellular organization of GPCR activity (Eichel and von Zastrow, 2018).

At the behavioural level,  $\mu$ - $\delta$  heteromers may contribute to both thermal and mechanical nociception. In keeping with this, intrathecal (i.t.) administration of DPDPE, deltorphan II or DAMGO induced thermal antinociception and reduced mechanical sensitivity in wild type mice but not in mice deficient for the  $\mu$  receptor (van Rijn *et al.*, 2012). Moreover, the enhanced thermal analgesia induced by a submaximal dose of the  $\mu$  agonist morphine in the presence of the  $\delta$  antagonist TIPPV is in line with the positive allosteric modulation in ligand binding and receptor signalling (Gomes *et al.*, 2004). Co-administration of equal amounts of the  $\mu$  agonist DAMGO and  $\delta$  agonist deltorphan II in the rostral ventromedial medulla (RVM) of naive rats resulted in a synergistic increase in paw withdrawal latencies (Sykes *et al.*, 2007). This effect may result from  $\mu$ - $\delta$  heteromer activation, although interactions taking place at the cellular or circuit levels cannot be excluded. In chronic morphine treated animals, co-administration of DAMGO and deltorphan II directly in the RVM synergistically increased the GABAergic inhibitory control via a phospholipase A<sub>2</sub> dependent mechanism that may, or not, involve  $\mu$ - $\delta$  activation (Zhang and Pan, 2010). A recent study also showed increased pain hypersensitivity in a cisplatin-induced neuropathic pain model and in a post-surgical inflammatory pain model following i.t. injections of a disrupting peptide corresponding to the  $\delta$  C-terminus, indicating that  $\mu$ - $\delta$  heteromers may control latent pain sensitization (Inyang *et al.*, 2021). An antinociceptive role of  $\mu$ - $\delta$  heteromers is also supported by data collected using ligands designed to selectively target  $\mu$ - $\delta$  heteromers (see below section 7). Of note, functional interactions between  $\mu$  and  $\delta$  also take place in the enteric nervous system and may include a  $\mu$ - $\delta$  contribution as the two receptors are co-localized in a subset of neurons (DiCello *et al.*, 2020) and can be detected in close physical proximity (Fujita *et al.*, 2014b).

### **3.2 $\delta$ -CB<sub>1</sub> heteromers**

Similar to  $\delta$  receptors, CB<sub>1</sub> receptors are critically involved in pain, anxiety and depression (reviewed in (Nadal *et al.*, 2013; Yin *et al.*, 2019)). The abundance of  $\delta$ -CB<sub>1</sub> heteromers is increased in the cortex, hypothalamus and midbrain of neuropathic rats following spinal nerve ligation (Bushlin *et al.*, 2012) or in the spinal cord of mice in a model of chemotherapy-induced peripheral neuropathy (Sierra *et al.*, 2019). Increased  $\delta$ -CB<sub>1</sub> heteromerization was also observed in postmortem spinal cords of patients suffering from chemotherapy-induced peripheral neuropathy (Sierra *et al.*, 2019).

In cortical membranes from naïve mice deficient for the  $\delta$  receptor, basal and agonist induced G protein signalling of the CB<sub>1</sub> receptor increased, suggesting that  $\delta$  receptors act as negative allosteric modulators of the CB<sub>1</sub> activity (Rozenfeld *et al.*, 2012). Reciprocally, activation of the CB<sub>1</sub> receptor allosterically inhibited  $\delta$  ligand binding (Kathmann *et al.*, 2006; Vaysse *et al.*, 1987). However, in cortical membranes from neuropathic rats, DPDPE binding and signalling were increased by a non-signalling dose of the CB<sub>1</sub> agonist Hu-210 or by the CB<sub>1</sub> antagonist PF-514273. The effect was blocked by  $\delta$ -CB<sub>1</sub>

heteromer selective antibodies, suggesting positive allosteric modulation of  $\delta$  in  $\delta$ -CB<sub>1</sub> heteromers in these conditions (Bushlin *et al.*, 2012). In a mouse model of chemotherapy-induced peripheral neuropathy, a combination of non-effective doses of agonists for the CB<sub>1</sub> (Hu210) and  $\delta$  (SNC80) receptors, reduced mechanical allodynia. The increase in mechanical thresholds was counteracted by the use of  $\delta$ -CB<sub>1</sub> selective antibodies, supporting the involvement of  $\delta$ -CB<sub>1</sub> heteromers in this anti-allodynic effect (Sierra *et al.*, 2019).

### **3.3 $\delta$ - $\kappa$ heteromers**

$\delta$ - $\kappa$  physical proximity was established in trigeminal ganglia (Berg *et al.*, 2012) whereas knockout subtraction autoradiography suggested that  $\delta$ - $\kappa$  heteromerization in the brain is restricted to the periaqueductal gray (PAG), amygdala, and thalamus; all three are part of the nociceptive circuit (Yoo *et al.*, 2014). In cultures from rat trigeminal ganglia,  $\kappa$  antagonists differentially regulated  $\delta$  dependent adenylate cyclase activity (Berg *et al.*, 2012), suggesting allosteric modulation between the two receptors. At the behavioural level, the  $\kappa$  antagonist nor-BNI potentiated the analgesic effect of a non-effective dose of DPDPE. This effect was enhanced in the presence of  $\delta$ - $\kappa$  specific antibodies (Berg *et al.*, 2012) pointing to a contribution of  $\delta$ - $\kappa$  heteromers. Similarly, the  $\delta$  antagonist naltrindole, allosterically increased the analgesic potency of a non-effective dose of the  $\kappa$  agonist ICI-199441 in a model of inflammatory pain (Jacobs *et al.*, 2019), indicating bidirectional positive allosteric modulation.

### **3.4 $\mu$ -CCR5 heteromers**

Close physical proximity between the  $\mu$  and the chemokine CCR5 receptors was first established by co-immunoprecipitation in human lymphocytes (Suzuki *et al.*, 2002). Neuronal co-expression of  $\mu$  and CCR5 receptors was then detected in the PAG (Heinisch *et al.*, 2011), a key region with both inhibitory and facilitatory functions in the descending modulation of pain. Furthermore, increased  $\mu$  receptor expression was observed in neurons from the PAG of mice deficient for the CCR5 receptor (Lee *et al.*, 2013), suggesting that physical interaction could also take place in the brain and contribute to pain processing. In both neuronal and immune cells, activation by either the CCL5 chemokine or a  $\mu$  agonist induced phosphorylation and desensitization of the other receptor, respectively (Szabo *et al.*, 2002) (Grimm *et al.*, 1998). These observations are consistent with a bidirectional allosteric modulation leading to functional pronociceptive  $\mu$ -CCR5 heteromers, but heterologous desensitization involving the recruitment of PKC $\zeta$  by second messengers has also been evidenced (Song *et al.*, 2011). At the behavioural level, acute analgesia induced by the  $\mu$  agonist DAMGO in the rat PAG was decreased in the presence of CCL5 (Szabo *et al.*, 2002). In addition, chemically induced visceral and inflammatory pain were decreased in mice lacking the CCR5 receptor or following injection of the CCR5 antagonist DAPTA (Lee *et al.*, 2013), both consistent with  $\mu$ -CCR5 heteromerization and/or heterologous desensitization. However, i.t. injections of the bivalent ligand MCC22 linking a  $\mu$  agonist and a CCR5 antagonist in models of inflammatory pain increased analgesia suggesting the presence of  $\mu$ -CCR5 heteromers in the spinal cord (see below section 7).

### **3.5 Other heteromers of potential relevance to chronic pain**

In proestrous female rats, spinal morphine antinociception necessitated  $\mu$  and  $\kappa$  co-activation for maximal efficiency and was strongly reduced by i.t. administration of the  $\kappa$  antagonist nor-BNI or anti-dynorphin antibodies, suggesting that co-activation of the two receptors was required in these animals for morphine to reach its maximal effect (Chakrabarti *et al.*, 2010; Liu *et al.*, 2007). These observations were paralleled with an estrogen-dependent increase of  $\mu$ - $\kappa$  heteromers in proestrous female rats compared to males or diestrous females (Chakrabarti *et al.*, 2010; Liu *et al.*, 2011a) pointing to the contribution of  $\mu$ - $\kappa$  heteromers to sex-based differences in the acute antinociceptive response to morphine. However, whether  $\mu$ - $\kappa$  heteromers play a role in chronic pain conditions or influence the development of morphine tolerance remains to be investigated.

Physical proximity has also been identified in pain related areas for 6 additional receptor pairs (Figure 1), which represent an additional pool of potential targets.  $\delta$ -somatostatin sst<sub>4</sub> heteromers

were observed in the striatum, cortex and spinal cord (Somvanshi and Kumar, 2014) and  $\kappa$ -NTS<sub>1</sub> heteromers were detected in primary striatal neurons (Liu *et al.*, 2016). These heteromers deserve further investigation because  $\delta$  and sst<sub>4</sub> receptors have established roles in chronic pain (Kantas *et al.*, 2019; Nadal *et al.*, 2013) and activation of  $\kappa$  or NTS<sub>1</sub> (Brouillette *et al.*, 2020) receptors in the spinal cord improves the nociceptive threshold in chronic pain conditions. Heteromers formed by  $\mu$  and somatostatin sst<sub>2</sub> receptors were identified in human pancreatic and breast cancerous cells (Jorand *et al.*, 2016; Kharmate *et al.*, 2013) and could also play a role in migraine as both receptors are critically involved in this chronic trigeminal pain condition (Lambert and Zagami, 2018; Menon *et al.*, 2012). Hypertension is a frequent comorbidity of chronic pain (Sacco *et al.*, 2013) and hypertensive conditions increase expression of  $\mu$ -adrenoceptor  $\alpha_{2A}$  and  $\mu$ -AT<sub>1</sub>angiotensin heteromers (Sun *et al.*, 2015; Sun *et al.*, 2019) in the nucleus of the tractus solitarius (Sol). This region is an important relay in the transmission of somatic and visceral nociceptive information (reviewed in (Berthoud and Neuhuber, 2000)) pointing to their relevance in the context of chronic pain. However, the functional consequences of  $\mu$ -  $\alpha_{2A}$  adrenoceptor heteromerization are difficult to anticipate because they seem to differ across regions. Bidirectional allosteric desensitization was reported in the spinal cord (Jordan *et al.*, 2003) and in primary DRG cultures (Tan *et al.*, 2009) but unidirectional allosteric activation of the  $\mu$  receptor was described in neurons from the locus coeruleus (LC) (Illes and Norenberg, 1990). Finally, heteromers involving  $\delta$  and the chemokine CXCR<sub>4</sub> receptors were identified in primary monocytes and brain tissue and could therefore play a role in inflammation and pain sensitization (Burbassi *et al.*, 2010; Pello *et al.*, 2008).

#### 4. HETEROMERS AND ANXIO-DEPRESSION

Chronic pain is associated with a high prevalence of anxiodepressive symptoms. The  $\mu$  receptor is critically involved in the rewarding properties of natural stimuli and anhedonia is a hallmark of depression (Bair *et al.*, 2003). Therefore, the role of  $\mu$  receptor heteromers in the emotional processing of pain has been investigated in the Acb and VTA, two key regions of the reward system where the  $\mu$  receptor is highly expressed (Figure 2). In the rat VTA, bidirectional negative allosteric modulation took place upon coadministration of the  $\mu$  agonist endomorphin 1 and galanin. It reduced ERK1/2, Akt and cAMP response element-binding protein (CREB) phosphorylation and was abrogated in the presence of a disrupting peptide corresponding to the  $\mu$  TM5 supporting the existence of functional  $\mu$ -GAL<sub>1</sub> heteromers (Moreno *et al.*, 2017). In addition, a unilateral negative allosteric modulation of the GAL<sub>1</sub> receptor by the opioid antagonist CTOP was also abolished in the presence of the TM5 peptide whereas the galanin antagonist M40 did not affect endomorphin 1 activity (Moreno *et al.*, 2017). Reduced ERK1/2 phosphorylation was associated with a decrease in dopamine release in the VTA (Moreno *et al.*, 2017) suggesting that activation of  $\mu$ -GAL<sub>1</sub> heteromers would be associated with less reinforcing properties than activation of  $\mu$  receptors. Activation of  $\mu$ -GAL<sub>1</sub> heteromers might therefore contribute to the anhedonic state.

Functional interactions between the  $\mu$  and cannabinoid CB<sub>1</sub> receptors in the Acb are known to modulate social play (Manduca *et al.*, 2016). Bidirectional negative allosteric modulation that reduced the generation of field-excitatory postsynaptic potentials was observed in Acb slices from rodents (Manduca *et al.*, 2016). In SKNSH neuroblastoma cells and rat striatal membranes, co-activation of the two receptors decreased G protein signalling (Rios *et al.*, 2006; Vaysse *et al.*, 1987). These data suggest that  $\mu$ -CB<sub>1</sub> heteromers could form in the Acb and contribute to the hedonic process. However heterologous desensitization and signalling cross talk between the two receptors cannot be ruled out. Because activation of  $\delta$  receptors has anxiolytic properties (Filliol *et al.*, 2000), the role of  $\mu$ - $\delta$  heteromers in anxiety and depression was also investigated. The use of a disrupting peptide corresponding to the C-terminus of the  $\delta$  receptor or pretreatment with the  $\mu$  antagonist CTOP or the  $\delta$  antagonist naltrindole reversed the anxiolytic and antidepressive properties resulting from the microinjection in the rat Acb of UFP-512, a ligand proposed to be  $\mu$ - $\delta$  selective (Kabli *et al.*, 2013). These observations are consistent with a possible involvement of  $\mu$ - $\delta$  heteromers in anxiodepressive behaviours. However, UFP-512 efficiently activates  $\delta$  receptors (Aguila *et al.*, 2007; Vergura *et al.*, 2008) suggesting possible alternative explanations.



## 5. HETEROMERS AND CHRONIC MORPHINE

Morphine analgesia is dependent on  $\mu_1$ , the most abundant  $\mu$  splice variant. Similarly, morphine side effects seem to engage heteromers of the  $\mu_1$  variant with the exception of morphine-induced itching (Figure 2). The latter is mediated by the  $\mu_{1D}$  variant heteromerized with the bombesin BB<sub>2</sub> (also called gastrin-releasing peptide receptor (GRPR)) receptor in the spinal cord (Liu *et al.*, 2011b) through a mechanism involving allosteric activation of the BB<sub>2</sub> receptor (Liu *et al.*, 2011b).

Inhibition or lack of functional  $\delta$  receptors reduces the development of morphine tolerance (reviewed in (Gendron *et al.*, 2015)). Moreover, chronic morphine administration enhances  $\delta$  expression at the plasma membrane in a  $\mu$  dependent manner (Erbs *et al.*, 2016; Gendron *et al.*, 2015) possibly through increased expression of the receptor transporter protein (RTP) 4 chaperone (Fujita *et al.*, 2019). This was associated with long-lasting increase in neuronal colocalization of  $\mu$  and  $\delta$  receptors (Pierre *et al.*, 2019) as well as  $\mu$ - $\delta$  heteromerization (Gupta *et al.*, 2010) in neuronal circuits that tightly connect autonomous/visceral functions with emotional/aversive processing. This indicates dynamic regulation of  $\mu$ - $\delta$  expression. In the presence of a peptide corresponding to the  $\delta$  2<sup>nd</sup> intracellular loop (Xie *et al.*, 2009) or to the  $\mu$  TM1 (He *et al.*, 2011) that both disrupt  $\mu$ - $\delta$  physical contact, morphine analgesia was increased, and morphine tolerance decreased, demonstrating the implication of  $\mu$ - $\delta$  heteromers. Activation of  $\mu$ - $\delta$  heteromers therefore appears to generate opposite effects. In animals chronically treated with morphine, they reduce morphine potency by mechanisms that remain to be determined whereas, in chronic pain conditions,  $\mu$ - $\delta$  activation exerts antinociceptive effects (see section 3).

Heteromerization of the  $\mu$  and V<sub>1B</sub> receptors in the RVM could also contribute to the development of morphine tolerance and physical dependence (Koshimizu *et al.*, 2018). Indeed, these side effects were decreased in mice deficient for the V<sub>1B</sub> receptor or upon injection of a V<sub>1B</sub> antagonist in the RVM, suggesting functional  $\mu$ -V<sub>1B</sub> interactions in this region (Koshimizu *et al.*, 2018). Accordingly, morphine tolerance was reduced in mutant mice expressing a dimerization-defective V<sub>1B</sub> receptor lacking C-terminal amino acids (Koshimizu *et al.*, 2018).

Finally, activation of  $\mu$ -cholecystokinin CCK<sub>2</sub> heteromers antagonizes morphine effects. Indeed, negative allosteric modulation of  $\mu$ , upon  $\mu$ -CCK<sub>2</sub> heteromerization, was observed in co-transfected cells and disruption of  $\mu$ -CCK<sub>2</sub> physical proximity in the spinal cord by i.t. injection of the  $\mu$  TM3 prevented CCK-8 antagonism of morphine analgesia (Yang *et al.*, 2018), consistent with a  $\mu$ -CCK<sub>2</sub> contribution to the pronociceptive action of the cholecystokinin system.

## 6. HETEROMERS INVOLVING ION CHANNELS

Physical association is not restricted to interactions between GPCRs but can also involve ion channels. This observation points to the need to adapt our current view to encompass more complex interactions taking place upon association of more than two proteins. Heterocomplexes involving the  $\mu$  and dopamine D<sub>1</sub> receptors together with the GluN1 subunit of the NMDA channels ( $\mu$ -D<sub>1</sub>-GluN1) represent a first example. In cotransfected cells, both  $\mu$ -D<sub>1</sub> and D<sub>1</sub>-GluN1 heteromers form in the ER through contacts involving their C-termini (Juhász *et al.*, 2008; Pei *et al.*, 2004). D<sub>1</sub>-GluN1 heteromerization enhanced D<sub>1</sub> signalling in the hippocampus (Pei *et al.*, 2004) whereas negative allosteric modulation of the  $\mu$  receptor activity by D<sub>1</sub> antagonists in  $\mu$ -D<sub>1</sub> heteromers located in the Acb decreased locomotor sensitization to morphine (Tao *et al.*, 2017). Physical proximity between  $\mu$  and GluN1 was identified in the mouse PAG where  $\mu$ -GluN1 activation by NMDA negatively regulated  $\mu$  activity by promoting dissociation of the heteromer and subsequent  $\mu$  receptor phosphorylation and desensitization (Rodríguez-Munoz *et al.*, 2012). In addition,  $\mu$ -GluN1 interaction was disrupted by morphine resulting in the development of tolerance (Rodríguez-Munoz *et al.*, 2012). Considering the broad distribution of  $\mu$ , D<sub>1</sub> and GluN1,  $\mu$ -D<sub>1</sub>-GluN1 complexes are likely to form in the nervous system and contribute to the modulation of nociception as well as anxi-depressive symptoms.

Heterocomplexes involving  $\mu$  and NOP receptors as well as voltage gated N-type calcium channels ( $\mu$ -NOP-Ca<sub>v</sub>2.2) have also been identified in DRGs where NOP-Ca<sub>v</sub>2.2 association resulted in a tonic inhibition of the channels likely due to the constitutive activity of the NOP receptor. The agonist

nociceptin also caused G protein dependent inhibition of Ca<sub>v</sub>2.2 and promoted receptor-channel co-internalization to the lysosomal compartment, both mechanisms contributing to reduce Ca<sup>2+</sup> entry (Altier *et al.*, 2006; Evans *et al.*, 2010). Physical proximity also exists between NOP and  $\mu$  in the DRGs (Evans *et al.*, 2010) where  $\mu$  activation led to co-internalization of Ca<sub>v</sub>2.2 and NOP (Evans *et al.*, 2010). The broad overlap in  $\mu$  and NOP expression (Ozawa *et al.*, 2015) and large distribution of Ca<sub>v</sub>2.2 (reviewed in (Kamp *et al.*, 2012)) in the nervous system suggest that such complexes likely form at the supraspinal level and contribute to the nociceptive response.

These examples suggest that the heteromerization process may initiate broader functional interactions than currently envisaged, by generating signalling platforms in which physical proximity between two or more receptor types and ion channels may constitute dynamic hot spots regulating neuronal activity.

## 7. LIGANDS TARGETING OPIOID RECEPTOR HETEROMERS

Exploiting the therapeutic potential of heteromers depends on the availability of selective ligands, whose design represents a major challenge (Table 2, Figure 3). Bivalent ligands consist of two ligands, each selective for one receptor type and linked together by a spacer of defined length. MDAN ( $\mu$   $\delta$  agonist antagonist) ligands bridging the  $\mu$  agonist oxycodone to the  $\delta$  antagonist naltrindole were the first to be designed. Acute and chronic intracerebroventricular (i.c.v.) administration of MDAN19 or MDAN21 showed increased thermal antinociceptive potency compared to morphine with no development of tolerance and low withdrawal in mice (Daniels *et al.*, 2005b). However, intramuscular injection of MDAN21 led to variable thermal antinociception in rhesus monkeys (Aceto *et al.*, 2012). Intravenous administration of MDAN19 or MDAN 21 failed to induce conditioned place preference and reinstatement in morphine treated mice suggesting no rewarding properties (Lenard *et al.*, 2007). The selectivity of these ligands, however, remains controversial because naltrindole only shows moderate selectivity with a 10-100-fold higher affinity for  $\delta$  compared to  $\mu$  receptors (Corbett *et al.*, 1993; Toll *et al.*, 1998). Therefore, binding of the MDAN compounds to two physically close  $\mu$  receptors exerting antagonistic effects could not be ruled out (Harvey *et al.*, 2012). More recently, D24M, a bivalent ligand linking the  $\delta$  antagonist Tyr-Tic-OH to the low affinity  $\mu$  antagonist (H-Tyr-Pro-Phe-D1Nal-NH<sub>2</sub>) with a 24-atom linker, showed about 100-fold increase in  $\mu$ - $\delta$  selectivity compared to  $\delta$  alone and had virtually no affinity for the  $\mu$  receptor in co-transfected cells (Olson *et al.*, 2018). *In vivo*, this compound dose-dependently blocked the thermal analgesia induced by CYM51010 or deltorphin II that both target endogenous  $\mu$ - $\delta$  heteromers (Derouiche *et al.*, 2020; Gomes *et al.*, 2013; Morgan *et al.*, 2021; Olson *et al.*, 2018; Tiwari *et al.*, 2020). D24M also reduced naloxone induced withdrawal symptoms associated with acute and chronic morphine administration (Olson *et al.*, 2018).

Two series of bivalent ligands were also generated to target  $\delta$ - $\kappa$  heteromers by bridging the  $\delta$  antagonist naltrindole with the  $\kappa$  agonist ICI-199441 in the KDAN ( $\kappa$   $\delta$  agonist antagonist) series (Daniels *et al.*, 2005a) or with the  $\kappa$  antagonist 5'-guanidinonaltrindole (5'-GNTI) in the KDN series (Bhushan *et al.*, 2004). KDAN18 exhibited thermal analgesic properties whereas KDN21 i.t. administration partially blocked DPDPE induced thermal analgesia (Ansonoff *et al.*, 2010). KDAN18 potency was reduced in  $\delta$  knockout mice and abolished in  $\kappa$  or  $\delta/\kappa$  knockout animals and KDN21 antagonistic properties were abolished in  $\kappa$  knockout mice (Ansonoff *et al.*, 2010) suggesting that these compounds bind, at least in part, to  $\delta$ - $\kappa$  heteromers.

Bivalent ligands targeting  $\mu$  and  $\kappa$  receptors that explored both the length of the spacer and the conformation of the ligand moieties were also generated. (-) (-) MCL 144 and (+) (-) MCL193 were synthesized by linking 2 molecules of MCL-101, an analogue of the agonist cyclorphan, with a linker corresponding to a 10 carbon chain ester. (-) (-) MCL 144 contained two active levorotary enantiomers whereas (+) (-) MCL193 combined one active (-) and one inactive (+) pharmacophores. Injection (i.c.v.) of either compound increased the threshold of thermal antinociception that was antagonized by a  $\mu$  or  $\kappa$  but not a  $\delta$  antagonist (Mathews *et al.*, 2008). Accordingly, these ligands did not affect morphine antinociception. However, selective binding to heteromers remains to be established.

Bivalent ligands were also developed to target receptors outside the opioid family by bridging the  $\mu$  receptor with a cannabinoid or a chemokine receptor. The  $\mu$  agonist  $\alpha$ -oxycodone tethered

to the CB<sub>1</sub> antagonist rimonabant by a 20-atom spacer induced potent thermal analgesia and weak tolerance after 24 hours when administered i.c.v. or i.t. (Le Naour *et al.*, 2013). Compound 19 linking the non-selective cannabinoid agonist JWH-018 with the  $\mu$  agonist oxycodone induced mechanical allodynia when injected i.t. in a chronic osteoarthritis pain model in rats (Dvoracko *et al.*, 2019). However, in both cases, selective targeting of  $\mu$ -CB<sub>1</sub> heteromers remains to be established.

When injected i.t., MCC22, a bivalent ligand bridging the  $\mu$  agonist oxymorphone with the chemokine CCR5 antagonist TAK-220 using a 22-atom linker, strikingly increased thermal antinociceptive potency in models of inflammatory pain with no tolerance measured after 24h (Akgun *et al.*, 2015). Similarly, MCC22 (i.p. or i.t.) reduced mechanical hypersensitivity without pharmacological tolerance in a model of spontaneous inflammatory arthritis (Dutta *et al.*, 2018) or in a model of chemotherapy induced peripheral neuropathy (Cataldo *et al.*, 2019). MCC22 systemic administration (i.p.) did not appear to have rewarding properties as it failed to induce conditioned place preference (Cataldo *et al.*, 2019). Expression of  $\mu$ -CCR5 in Iba1 positive cells of the spinal cord, and strong reduction of the antinociceptive effect in the presence of the inhibitor of microglial activation minocycline or of an antagonist of the complement system both support MCC22 binding to  $\mu$ -CCR5 heteromers expressed in glial cells (Akgun *et al.*, 2015; Akgun *et al.*, 2019; Cataldo *et al.*, 2019). Interestingly, the higher potency of i.t injection in inflammatory conditions compared to naïve mice may indicate higher level of  $\mu$ -CCR5 heteromers in the spinal cord, although this remains to be established.

Although bivalent ligands combining an agonist for one receptor with an antagonist of the other such as MDAN ( $\mu$ - $\delta$ ), KDAN ( $\mu$ - $\kappa$ ) or MCC22 ( $\mu$ -CCR5) induce antinociceptive responses, the molecular mechanisms remain elusive. Whether these ligands provide a mixed response corresponding to the effect of each ligand separately, i.e. activation of one receptor and inactivation of the other, or generate a different signalling output remains to be established. Moreover, occupancy of one receptor by an agonist or an antagonist results in positive ( $\mu$ - $\delta$ ,  $\mu$ - $\kappa$ ) or negative ( $\mu$ -CCR5) allosteric modulation of the other (see above) introducing additional levels of complexity. Instead, bivalent ligands bridging two antagonists, such as D24M, should offer less ambiguous interpretation and may be useful to reduce heteromer dependent adverse effects. Bivalent ligands share a limited capacity to cross the blood brain barrier. This likely explains the absence of rewarding effects when administered systemically, which can be viewed as an advantage over small opioid molecules. However, restricted targeting to peripheral receptor pairs may not provide maximal antinociception and limited central bioavailability hampers modulation of central sensory mechanisms involved in emotional and cognitive aspects, thereby limiting their potential in the clinic.

In contrast, small molecules efficiently cross the blood brain barrier. Among them, bifunctional or mixed ligands were designed that simultaneously target two receptors (reviewed in (Gunther *et al.*, 2018)). Cebranopadol for example is a mixed non selective opioid/NOP ligand in phase 3 clinical trial for the treatment of severe chronic pain (reviewed in (Kiguchi *et al.*, 2020)). It combines potent analgesia with reduced abuse liability and respiratory depression. SRI22141 is a dual agonist of  $\mu$  and  $\delta$  receptors. It shows efficient analgesic properties with reduced tolerance and dependence in two models of neuropathic pain (Lei *et al.*, 2020). Although of therapeutic interest, these ligands can bind to individual receptors as well as receptors engaged in heteromer formation. Attempts were thus made to develop small molecules that would selectively target heteromers. CYM51010 (Gomes *et al.*, 2013), and eluxadoline, recently approved by the FDA for the treatment of the irritable bowel syndrome (FDA N<sup>o</sup>206940) showed reduced signalling in the presence of  $\mu$ - $\delta$  selective antibodies (Fujita *et al.*, 2014b; Gomes *et al.*, 2013; Tiwari *et al.*, 2020; Wade *et al.*, 2012) and lower efficacy in  $\delta$  knockout mice (Fujita *et al.*, 2014b; Tiwari *et al.*, 2020) suggesting that these compounds bind to  $\mu$ - $\delta$  heteromers. However, both CYM51010 and eluxadoline can activate the  $\delta$  (Derouiche *et al.*, 2020; Fujita *et al.*, 2014b) or  $\mu$  (Gomes *et al.*, 2013; Tiwari *et al.*, 2020) receptors expressed alone. Nonetheless, CYM51010 induced efficient thermal analgesia with lower physical dependence and tolerance compared to morphine (Gomes *et al.*, 2013). In the spinal nerve ligation model, CYM51010 relieved mechanical and thermal allodynia even in animals chronically treated with morphine (Tiwari

*et al.*, 2020). Very recently, MP135, a derivative of the  $\mu$  agonist carfentanyl showed higher selectivity for  $\mu$ - $\delta$  heteromers and produced efficient acute thermal antinociception. Unfortunately, MP135 retained the side effects of the parent molecule as it exhibited rewarding properties and induced respiratory depression (Faouzi *et al.*, 2020).

N-naphthoyl- $\beta$ -naltrexamine (NNTA) produced strong thermal antinociception devoid of tolerance, physical dependence, or reinforcing properties upon i.t. injection that was abolished in  $\mu$  deficient mice (Yekkiralala *et al.*, 2011). Aversion was observed with doses 10 times higher than the antinociceptive ED50 consistent with the reported dysphoric effects of mixed  $\kappa$  agonist-  $\mu$  antagonist ligands. However, NNTA's strong  $\mu$  antagonism suggests limited selectivity towards  $\mu$ - $\kappa$  heteromers.

Intrathecal administration of 6-guanidinonaltrindole (6'GNTI) (Waldhoer *et al.*, 2005) or its local (i.pl.) administration in a model of inflammatory pain (Jacobs *et al.*, 2019) induced thermal antinociception. This effect was reduced by a pretreatment with a  $\delta$  antagonist or in mice deficient for the  $\delta$  or  $\kappa$  receptor (Berg *et al.*, 2012; Jacobs *et al.*, 2018) and was abolished in double knockout animals (Ansonoff *et al.*, 2010) or in the presence of a peptide corresponding to the  $\delta$  TM1 (Jacobs *et al.*, 2018). In addition, knocking down  $\delta$  or  $\kappa$  receptors blocked 6'GNTI signalling in cultures from rat trigeminal ganglia (Jacobs *et al.*, 2018) where  $\delta$  and  $\kappa$  receptors were co-immunoprecipitated (Berg *et al.*, 2012). When administered i.c.v., no thermal antinociception was observed, consistent with the reported low levels of  $\delta$ - $\kappa$  heteromers in the brain (Yoo *et al.*, 2014). Collectively, these data support 6'GNTI activation of  $\delta$ - $\kappa$  heteromers but the selectivity towards  $\delta$ - $\kappa$  heteromers appears only partial as this ligand is also a potent  $\kappa$  agonist (Rives *et al.*, 2012; Schmid *et al.*, 2013) and, can activate  $\mu$ - $\kappa$  heteromers although to a lesser extent (Waldhoer *et al.*, 2005).

To date, small molecules targeting heteromers retain affinity and activity for the receptor monomers, at least to some extent. Improving their selectivity constitutes a major pharmacological challenge to overcome.

## 8. PERSPECTIVES

More than 20 years after the first report in co-transfected cells (Jordan and Devi, 1999), studying opioid receptor heteromers *in vivo* remains extremely challenging due to discrete distribution in the nervous system, low expression level, co-existence with individual parent receptors, not to mention splice variants. In addition, physical association may also be dynamically regulated resulting in transient physical association. The limited amount of data collected so far to support the functional impact of heteromers *in vivo* highlights the lack of selective tools to identify their properties and distinguish them from receptor cross talk taking place at the level of signalling cascades or neural circuits.

Deciphering heteromer signalling and trafficking in native environments, however, is increasingly recognized as essential for improving our understanding of the molecular mechanisms governing their pathophysiological contribution. Indeed, information collected in co-transfected cells should be considered with caution because of the high receptor expression levels often achieved but also due to the difference in cellular contents. Specific antibodies or nanobodies, selective ligands, whether agonists or antagonists, are needed to probe receptor induced conformational changes and/or manipulate heteromer activity in native cellular environment (Che *et al.*, 2020; Livingston *et al.*, 2018). Crystal or NMR based 3D structure determination (Garcia-Recio *et al.*, 2020) and *in silico* approaches (reviewed in (Barreto *et al.*, 2020)) may also provide useful clues about contact interface and help in designing ligands. The reported allosteric modulation taking place within heteromers suggests that this interface is dynamic and sensitive to small conformational changes (Dijkman *et al.*, 2018; Manglik *et al.*, 2012), which may include a yet unrecognized contribution of the lipidic environment in shaping receptor conformation (Provasi *et al.*, 2015; Zheng *et al.*, 2012).

Better understanding of the dynamics of receptor association at the plasma membrane, but also during export and internalization, implies further technological developments. This could be achieved by combining recent approaches to tag receptors and improved imaging techniques. For example, selective tagging using ligand directed fluorescent labelling of endogenous receptors (Arttamangkul *et al.*, 2019), infrared fluorescent conjugated ligands (Ast *et al.*, 2020) and nanobodies (Gormal *et al.*,

2020; Moller *et al.*, 2020; Sungkaworn *et al.*, 2017), or (infra)red fluorescent receptor fusion proteins (Prangma *et al.*, 2020) represent new options. The latter could be used to visualize receptor close proximity by single molecule (dual-color photoactivatable dyes and localization microscopy (PD-PALM) (Jonas *et al.*, 2015), super-resolution (STED) (Mitronova *et al.*, 2017)) and/or dynamic (fluorescence intensity fluctuation spectrometry (Stoneman *et al.*, 2019)) imaging.

The increased expression and broader distribution reported for several heteromers in pathological conditions suggest that this aspect is essential when exploring the functional role of heteromers and their therapeutic relevance. Genetically modified animals such as inducible conditional knockin or knockout promoting or ablating expression of one receptor in a subset of cells expressing the other receptor would enable spatio-temporal control of their co-occurrence. This would make it possible to interrogate the contribution of heteromer formations in selected cell populations and/or neuronal circuits at various timepoints to better grasp their role in pathological states.

Heteromers should also be envisaged in the larger context of signalosomes as allosteric modulation of the receptor activity is likely to modify or engage novel interactions with intracellular partners. To date, only canonical signalling pathways have been explored, and only to a limited extent. Changes in  $\mu$  trafficking for example suggest modifications in interactions with scaffold proteins (Civciristov *et al.*, 2019). Identifying the interactome associated with heteromers by a proteomic approach would allow exploration of novel intracellular cascades. In addition, heteromerization may also reflect larger signalling platforms in which several receptor types and ion channels are present in physical proximity to dynamically regulate the cellular activity.

In conclusion, our understanding of endogenous opioid receptor based heteromers is still poor. Further technological developments will be instrumental for in-depth assessment of their functional role *in vivo*. In addition, highly selective ligands with good bioavailability are urgently needed to explore and exploit their potential as drug targets for improved therapeutic strategies in the clinic.

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## FIGURE LEGENDS

**FIGURE 1:** Distribution of opioid receptor heteromers in rodents.

Close physical proximity between receptors was established in basal (black) or in pathological (chronic pain or chronic morphine (blue) conditions. Other receptor pairs have been postulated for which information about the physical proximity in the region is still missing (red).

DRG, dorsal root ganglia; PAG, periaqueductal gray; RVM, rostral ventromedial medulla; Sol, nucleus of the tractus solitarius; TG, trigeminal ganglia; VTA, ventral tegmental area.

**FIGURE 2:** Opioid receptor heteromers modulate chronic pain and associated comorbidities.

Activation of some opioid receptor heteromers is associated with antinociceptive properties and potential improvement of anxiodepressive comorbidities (receptor pairs on blue background) whereas, for others, activation results in increased pain perception and side effects (receptor pairs on red background). Additional opioid receptor heteromers have been identified in structures relevant to nociception and/or involve receptors with known contribution in pain perception but their contribution has not yet been clearly identified (receptor pairs on grey background).

**FIGURE 3:** Chemical structures of ligands targeting opioid receptor heteromers. For bivalent ligands, the number of elementary units (n) defining the length of the linker and the receptor targeted by each moiety are indicated.

**Table 2** Ligands targeting opioid receptor heteromers: behavioural impact

Name	Target 1	Target 2	Linker length	Analgesia (chronic pain model)	Side effects	References
<b>Bivalent ligands</b>						
MDAN	$\mu$ agonist oxymorphone	$\delta$ antagonist naltrindole	19- 21	Increased thermal potency  No tolerance	Decreased withdrawal  no reward	(Aceto <i>et al.</i> , 2012; Daniels <i>et al.</i> , 2005b; Lenard <i>et al.</i> , 2007)
D24M	$\mu$ antagonist H-Tyr-Pro-Phe-D1Nal-NH <sub>2</sub>	$\delta$ antagonist Tyr-Tic-OH	24	Decreased thermal analgesia	Decreased morphine withdrawal	(Morgan <i>et al.</i> , 2021; Olson <i>et al.</i> , 2018)
KDAN	$\kappa$ agonist ICI-199, 441	$\delta$ antagonist naltrindole	18	Thermal		(Ansonoff <i>et al.</i> , 2010; Daniels <i>et al.</i> , 2005a)
KDN	$\kappa$ antagonist 5'-GNTI	$\delta$ antagonist naltrindole	21	Thermal		(Ansonoff <i>et al.</i> , 2010; Bhushan <i>et al.</i> , 2004)
MCL144 MCL193	MCL-101 $\mu$	MCL-101 $\kappa$	10	Thermal		(Mathews <i>et al.</i> , 2008)
Compound 5	$\mu$ agonist $\alpha$ -oxymorphone	Cannabinoid CB <sub>1</sub> antagonist rimonabant	20	Thermal Decreased tolerance		(Le Naour <i>et al.</i> , 2013)
Compound 19	$\mu$ agonist oxycodone	non-selective cannabinoid agonist JWH-018	4 amino acids	Mechanical (osteoarthritis)		(Dvoracsko <i>et al.</i> , 2019)
MCC22	$\mu$ agonist oxymorphone	chemokine CCR5 antagonist TAK-220	22	Increased thermal, mechanical (spontaneous inflammatory arthritis,	No tolerance  No reward	(Akgun <i>et al.</i> , 2015; Cataldo <i>et al.</i> , 2019; Dutta <i>et al.</i> , 2018)

				chemotherapy induced peripheral neuropathy)		
<b>Small molecules</b>						
CYM51010	μ	δ	na	Thermal, Mechanical (sciatic nerve ligation)  Reduced tolerance	Reduced withdrawal	(Gomes <i>et al.</i> , 2013; Tiwari <i>et al.</i> , 2020)
eluxadoline	μ	δ	na	Improved Gi transit (irritable bowel syndrome)		(Fujita <i>et al.</i> , 2014; Wade <i>et al.</i> , 2012)
MP135	μ	δ	na	Thermal	Reward (CPP)  Respiratory depression	(Faouzi <i>et al.</i> , 2020)
NNTA	μ	κ	na	Thermal  No tolerance	No withdrawal  No reward	(Yekkirala <i>et al.</i> , 2011)
6'GNTI	δ	κ	na	Thermal (prostaglandin E2, bradykinin, carrageenan)		(Ansonoff <i>et al.</i> , 2010; Jacobs <i>et al.</i> , 2018; Jacobs <i>et al.</i> , 2019) (Waldhoer <i>et al.</i> , 2005)

Abbreviations: CPP, conditioned place preference; δ, delta opioid; κ, kappa opioid; μ, mu opioid; na, non applicable

**Table 1:** Summary of *in vivo* distribution, functional and behavioural properties of opioid receptor heteromers

Receptor pair	<i>in vivo</i> physical proximity			Specific properties of native heteromers			
	Tissue	Technique	References	Tissue	Ligand binding, receptor signalling and trafficking	Functional outcome	References
$\mu$ - $\delta$	Mouse Acb, hippocampus, SC, DRG	Co-IP, disruptive peptide	(Erbs <i>et al.</i> , 2015; Kabli <i>et al.</i> , 2013; Xie <i>et al.</i> , 2009)	SKNSH, SC, VTA	Co-activation with an agonist, inverse agonist or antagonist for the other receptor induced bidirectional positive allosteric modulation		(Gomes <i>et al.</i> , 2000; Gomes <i>et al.</i> , 2004; Gomes <i>et al.</i> , 2011; Margolis <i>et al.</i> , 2017)
	Increased by chronic morphine in some brain areas	Specific $\mu$ - $\delta$ antibody  IHC	(Gupta <i>et al.</i> , 2010)  (Pierre <i>et al.</i> , 2019)	RVM	Co-activation in chronic morphine treated rats induces synergy	Increased analgesia	(Zhang and Pan, 2010)
				RVM	Co-activation induces synergy	Increased analgesia	(Sykes <i>et al.</i> , 2007)
				DRG, SC	$\mu$ - $\delta$ export to the surface	Increased analgesia	(Walwyn <i>et al.</i> , 2009; Xie <i>et al.</i> , 2009)
				SC, DRG		Increased morphine tolerance	(He <i>et al.</i> , 2011; Xie <i>et al.</i> , 2009)
				Striatum, hippocampus	Receptor co-internalization and co-degradation	Anxiolytic, anti-depressive, analgesic, decreased morphine tolerance and dependence	(Derouiche <i>et al.</i> , 2020; Gomes <i>et al.</i> , 2013; Kabli <i>et al.</i> , 2013)
				SKNSH	Increased $\beta$ -arrestin signalling		(Rozenfeld and Devi, 2007)

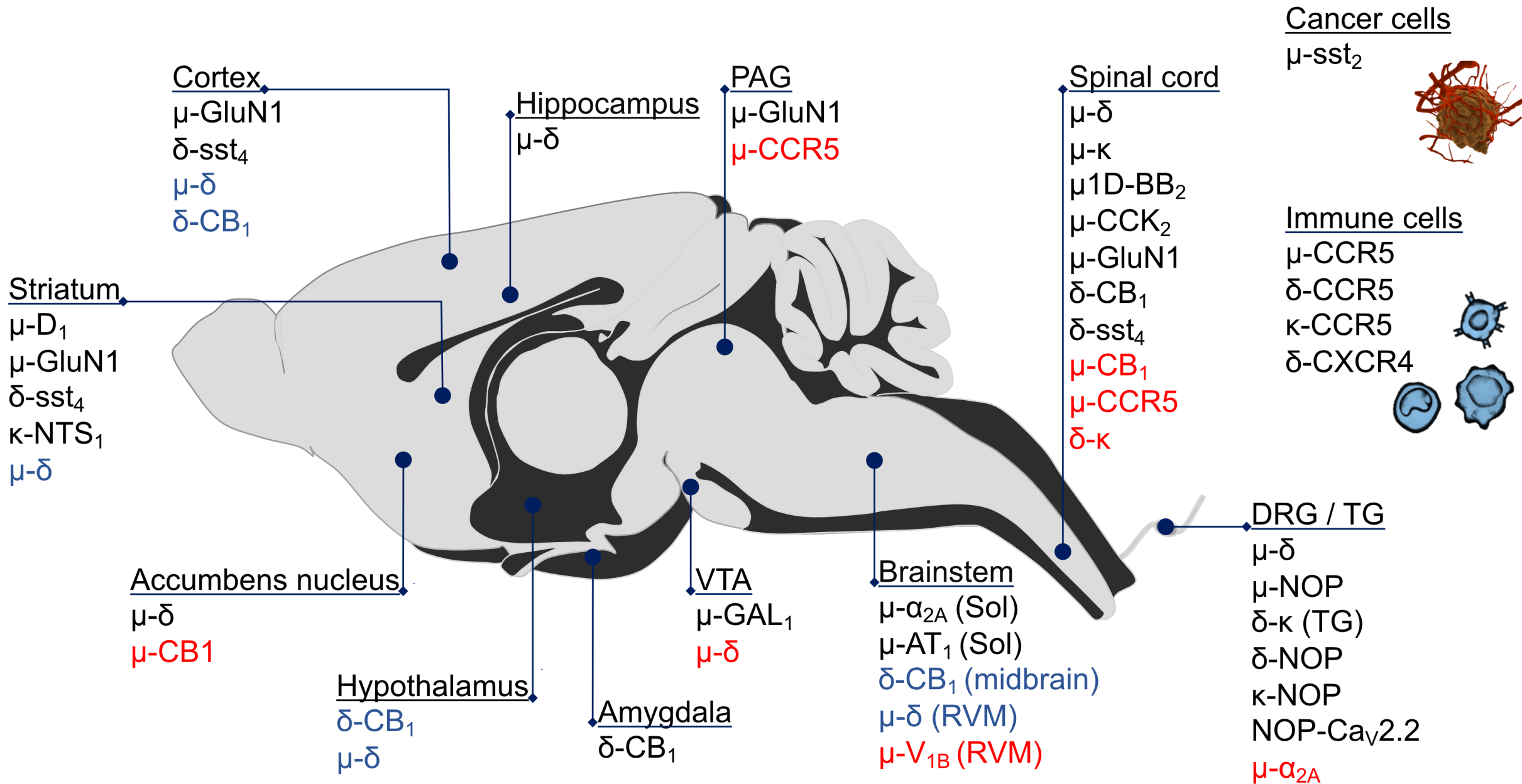
				SC		Decreased pain hypersensitivity after neuropathic pain remission	(Inyang <i>et al.</i> , 2021)
$\mu$ - $\alpha_{2A}$	Rat Sol; increased in hypertensive rats	Co-IP, PLA	(Sun <i>et al.</i> , 2015; Sun <i>et al.</i> , 2019)	SC or DRG primary cultures	Negative allosteric modulation. Receptor co-internalization.	Hypertension	(Jordan <i>et al.</i> , 2003; Sun <i>et al.</i> , 2015; Tan <i>et al.</i> , 2009)
$\mu$ -AT <sub>1</sub>	Rat Sol ; increased in hypertensive rats	PLA	(Sun <i>et al.</i> , 2019)				
$\mu$ -CB <sub>1</sub>	Rat striatum	Co-localization by electron microscopy	(Rodriguez <i>et al.</i> , 2001)	SKNSH, striatum, Acb	Bidirectional negative allosteric modulation	Neuritogenesis, social play	(Manduca <i>et al.</i> , 2016; Rios <i>et al.</i> , 2006; Vaysse <i>et al.</i> , 1987)
$\mu$ -CCK <sub>2</sub>	Rat SC	Disruptive peptide	(Yang <i>et al.</i> , 2018)	SC	Negative allosteric modulation	Increased morphine analgesia	(Yang <i>et al.</i> , 2018)
$\mu$ -CCR5	Human lymphocytes	Co-IP	(Suzuki <i>et al.</i> , 2002)	PAG, PMBC	Bidirectional allosteric modulation	Decreased nociception, HIV infection	(Lee <i>et al.</i> , 2013; Szabo <i>et al.</i> , 2002)
$\mu$ -D <sub>1</sub>	Mouse striatum	Co-IP	(Tao <i>et al.</i> , 2017)	striatum	Negative allosteric modulation	Opioid locomotor sensitization	(Tao <i>et al.</i> , 2017)
$\mu$ -GAL <sub>1</sub>	Rat VTA	Disruptive peptide	(Moreno <i>et al.</i> , 2017)	VTA	Co-activation induced bidirectional negative allosteric modulation, unidirectional negative	Opioid drug reward; DA release	(Moreno <i>et al.</i> , 2017)

					allosteric modulation on Gal1 signalling		
$\mu$ - $\kappa$	Rat SC proestrus females	Co-IP	(Chakrabarti <i>et al.</i> , 2010)		Co-activation morphine/dyn1:17 induced synergy	Increased morphine analgesia in females	(Chakrabarti <i>et al.</i> , 2010; Liu <i>et al.</i> , 2011a)
$\mu$ -NMDA	Mouse PAG, cortex, striatum, SC	Co-IP	(Rodriguez-Munoz <i>et al.</i> , 2012)	PAG	Unidirectional positive allosteric modulation on mu receptor and unidirectional negative allosteric modulation on NMDA CAMKII pathway	Decreased morphine analgesia, increased morphine tolerance	(Rodriguez-Munoz <i>et al.</i> , 2012)
$\mu$ -NOP	Rat DRG	Co-IP	(Evans <i>et al.</i> , 2010)	BE(2)-C neuroblastoma	Co-activation induced unidirectional negative allosteric modulation on NOP signalling	Nociception	(Mandyam <i>et al.</i> , 2002)
$\mu$ -sst <sub>2</sub>	Human pancreatic cancer cells	Co-IP, FCS	(Jorand <i>et al.</i> , 2016; Kharmate <i>et al.</i> , 2013)	Pancreatic cancer cells	Co-activation increased $\beta$ -arrestin signalling, decreased epithelial to mesenchymal transition	Increased cancer metastasis	(Jorand <i>et al.</i> , 2016)
$\mu$ -V <sub>1B</sub>	Mouse RVM	ISH, truncated V <sub>1B</sub> receptor	(Koshimizu <i>et al.</i> , 2018)	RVM	Increased $\beta$ -arrestin signalling	Increased morphine tolerance	(Koshimizu <i>et al.</i> , 2018)
$\mu$ 1D-BB <sub>2</sub>	Mouse SC, disruptive peptide	Co-IP	(Liu <i>et al.</i> , 2011b)	SC, DRG	Positive allosteric modulation on BB <sub>2</sub> signalling	Morphine induced itch	(Liu <i>et al.</i> , 2011b)
$\delta$ -CB <sub>1</sub>	Mouse BLA, mouse and human SC, increased by neuropathic pain in some brain areas	PLA, selective antibody, co-IP	(Bushlin <i>et al.</i> , 2012; Sierra <i>et al.</i> , 2019) (Degrandmaison <i>et al.</i> , 2020)	cortex	Positive allosteric modulation of CB <sub>1</sub> ligands (agonist or antagonist) on DOR G $\alpha$ signalling	Anti-allodynic effect	(Bushlin <i>et al.</i> , 2012; Sierra <i>et al.</i> , 2019)
				cortex	Negative allosteric modulation		(Rozenfeld <i>et al.</i> , 2012)



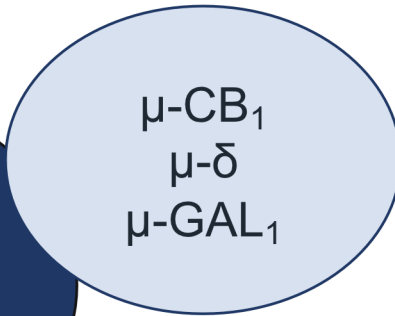
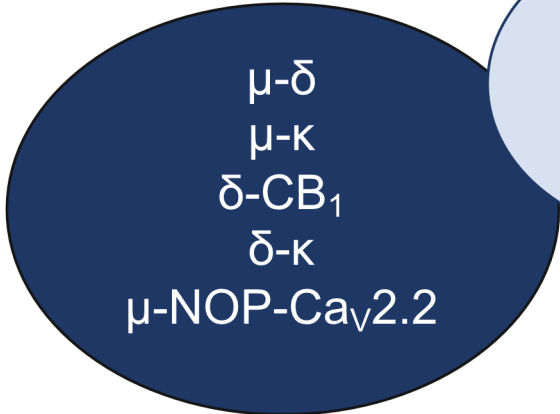
$\delta$ -CCR5	Human lymphocytes	Co-IP	(Suzuki <i>et al.</i> , 2002)				
$\delta$ -CXCR4	Human PBMC, mouse brain tissue	Co-IP	(Burbassi <i>et al.</i> , 2010; Pello <i>et al.</i> , 2008)				
$\delta$ - $\kappa$	Rat TG	Co-IP, disruptive peptide	(Berg <i>et al.</i> , 2012) (Jacobs <i>et al.</i> , 2018)	TG primary cultures		Inflammatory nociception, thermal anti-allodynia	(Berg <i>et al.</i> , 2012; Jacobs <i>et al.</i> , 2018; 2019)
$\delta$ -NOP	Rat DRG	Co-IP	(Evans <i>et al.</i> , 2010)				
$\delta$ -sst <sub>4</sub>	Rat striatum, SC, cortex	FRET, Co-IP	(Somvanshi and Kumar, 2014)				
$\kappa$ -CCR5	Human lymphocytes	Co-IP	(Suzuki <i>et al.</i> , 2002)				
$\kappa$ -NOP	Rat DRG	Co-IP	(Evans <i>et al.</i> , 2010)				
$\kappa$ -NTS <sub>1</sub>	Rat striatum	Co-IP, PLA	(Liu <i>et al.</i> , 2016)				
NOP-Cav2.2	Rat DRG, Brain	Co-IP	(Altier <i>et al.</i> , 2006; Beedle <i>et al.</i> , 2004)	DRG primary cultures	Nociceptin induced co-internalization		(Altier <i>et al.</i> , 2006)

Abbreviations: Acb, Nucleus Accumbens; BLA, Basolateral Amygdala; Co-IP, Co-immunoprecipitation;  $\delta$ , delta opioid; DRG, Dorsal Root Ganglia; FCS, fluorescence correlation spectroscopy; FRET, Fluorescence energy transfer; ISH, In Situ Hybridization;  $\kappa$ , kappa opioid;  $\mu$ , mu opioid; NMDA, N-methyl-D-aspartate; NOP, nociceptin; NTS, neurotensin; PAG, Periaqueductal Gray; PBMC, peripheral blood mononuclear cells; PFC, Prefrontal Cortex; PLA, Proximity Ligation Assay; RVM, Rostral Ventromedial Medulla; SC, Spinal Cord; Sol, nucleus of the solitary tract; sst, somatostatin; TG, trigeminal ganglia; VTA, Ventral Tegmental Area.

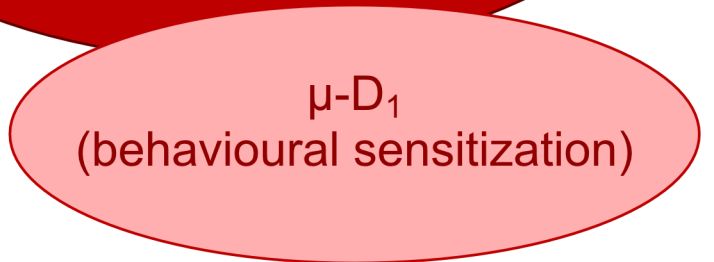
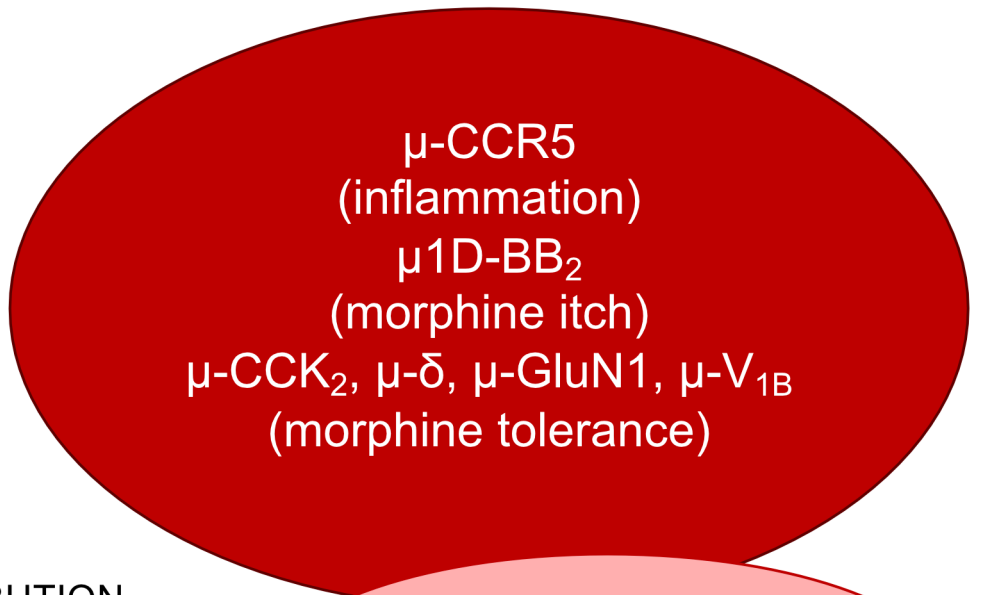


DECREASED COMORBIDITIES

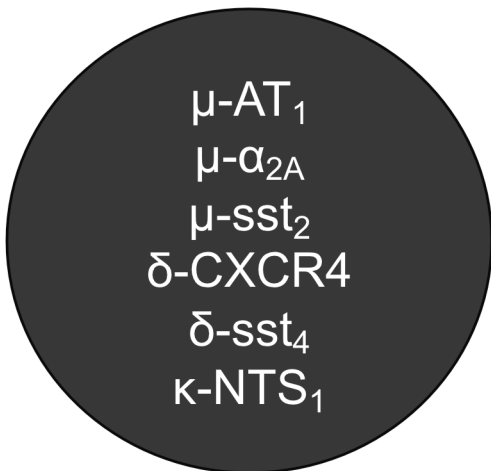
ANTINOCICEPTION



PRONOCICEPTION



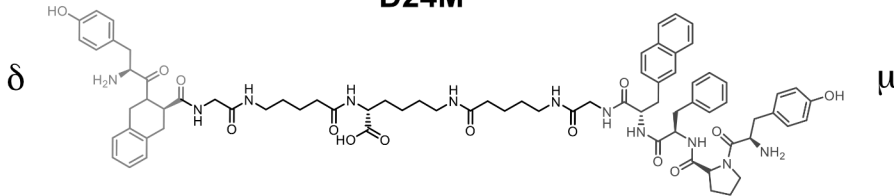
UNKNOWN CONTRIBUTION TO NOCICEPTION



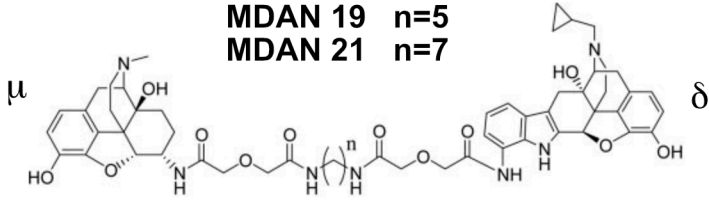
INCREASED COMORBIDITIES

## Bivalent molecules

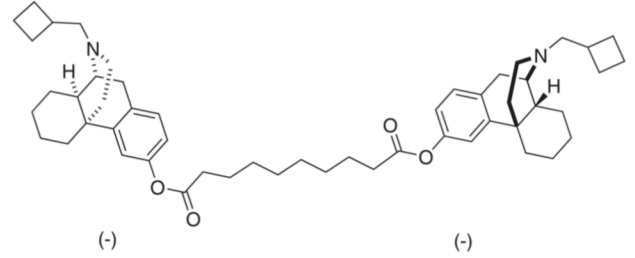
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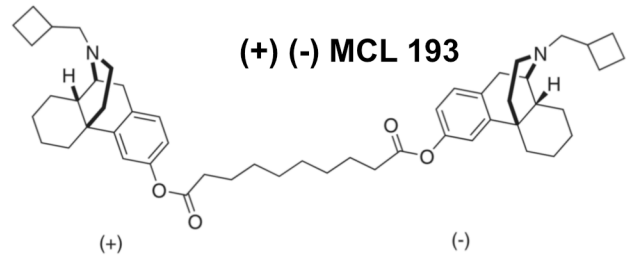
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**MDAN 21 n=7**



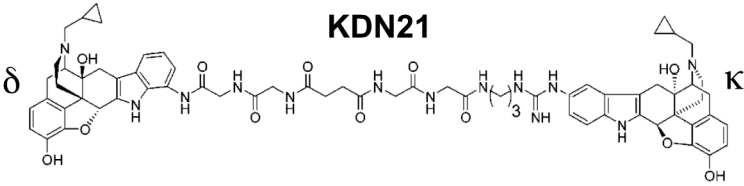
**(-) (-) MCL 144**



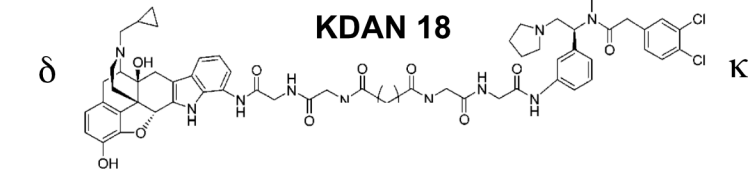
**(+) (-) MCL 193**



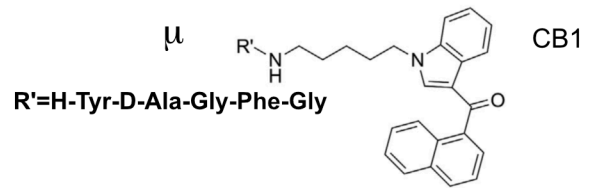
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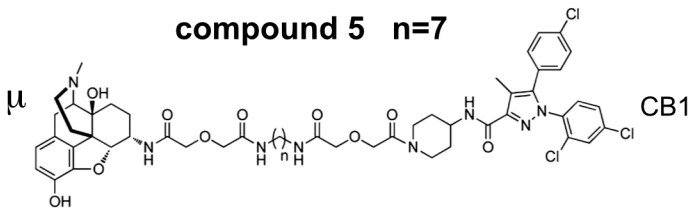
**KDAN 18**



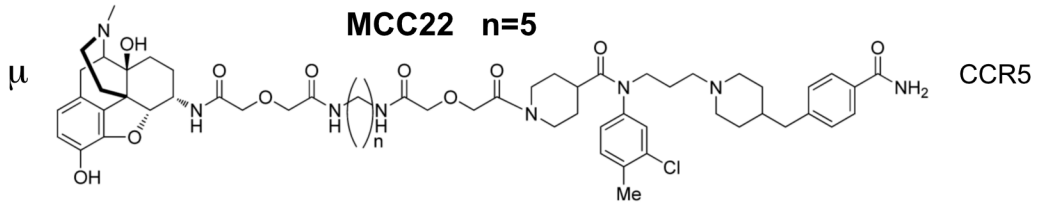
**compound 19**



**compound 5 n=7**

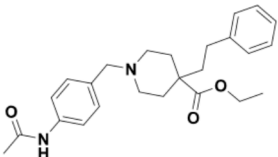


**MCC22 n=5**

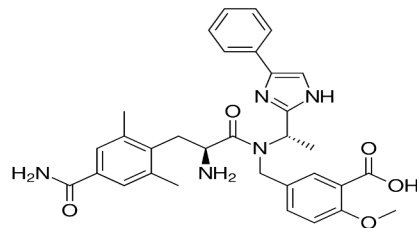


## Small molecules

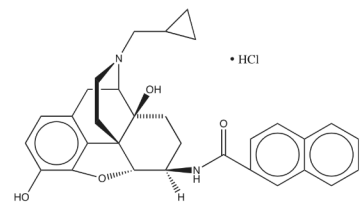
**CYM51010**



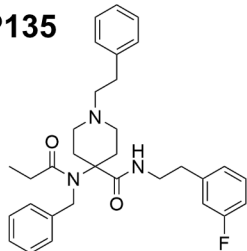
**eluxadoline**



**NNTA**



**MP135**



**6'GNTI**

