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SUPPLEMENTARY INFORMATION

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Opioid receptors: drivers to addiction?

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Supplementary Box 1 I Opioids receptor heterodimerization.

Today, the notion that G protein-coupled receptors (GPCRs) form functional heterodimers with specific pharmacological properties is well established (reviewed in Ref.[1]). To be considered heterodimers, (i) two GPCRs should colocalize and physically interact, (ii) the signalling should be unique or distinguishable from monomeric GPCR signalling and (iii) alteration of the heterodimer complex should disrupt heterodimer-specific biochemical and pharmacological properties[1]. Opioid receptors form homo- and heterodimers in transfected systems in vitro[2], and the first opioid receptor heterodimer formation was demonstrated between KOR and DOR in cells that co-expressed (myc- and flag-) tagged versions of these receptors to enable their detection and co-immunopreciptitation[3]. Since then, studies using recombinant receptors have revealed the formation of all possible combinations of opioid receptor heterodimers[1,4]. A next step, and one of the main challenges in GPCR research, will be to demonstrate the existence of heterodimers in tissues and *in vivo* and to understand their biological significance in physiology and behaviour. This goal is particularly difficult, as receptors are not abundant and suitable tools are lacking for biochemistry approaches.

Among all the proposed GPCR heterodimers, the MOR-DOR combination is best described[1], and recent evidence suggests the possibility of this receptor dimerization *in vivo*[4,5]. First, to identify potential anatomical sites for MOR-DOR heterodimerization, co-localization of the two receptors was mapped using double knockin mice expressing functional, fluorescently tagged receptors (MOR-mCherry[6] and DOR-eGFP[7,8]). Through this approach, MOR-DOR heterodimers were detected mainly in midbrain and hindbrain regions[6]. Recently, MOR and DOR anatomy was fine-mapped in these mice with a focus on neurons belonging to pain pathways. This mapping showed highly localized co-expression in the pons, as well as in the dorsal horn (which carries fibres involved in nociception, touch) and ventral horn (involved in motor control) of the spinal cord[9].

However, the two receptors did not co-internalize and independently activated GIRK channels in the spinal cord[9], suggesting that anatomical co-localization does not necessarily mean dimerization in the case of MOR and DOR.

Next, physical MOR–DOR interaction was tested by co-immunoprecipitation experiments in tissue extracts. MOR-mCherry/DOR-eGFP complexes were detected in samples from hippocampus but not cortex of double mutant mice[6]. Furthermore, native MOR–DOR heterodimers were observed in the mouse spinal cord using antibodies against the native receptors (reviewed in[10]). Disruption of this direct interaction, using an interfering peptide comprising the first MOR transmembrane domain, reduced DOR agonist-induced endocytosis of both DOR and MOR in the mouse spinal cord, enhanced morphine analgesia and reduced tolerance to this effect [11], supporting biological significance of the physical interaction in cells and *in vivo*. Relevant to addiction and mood disorders, a MOR–DOR heterodimer was proposed to reduce anxiety and depressive-like behaviours at the level of the nucleus accumbens in rats[12], but the receptor complex has yet to be detected in this brain region.

Finally, compounds that selectively activate heterodimers, but not monomeric receptors, have been developed, and conferred analgesia with less tolerance in rodents[1]. The most promising of these compounds are CYM51010, a small molecule identified by ligand screening[13], MDAN-21 (in which oxymorphone was linked to naltrindole)[14], and eluxadoline, which succeeded in phase III trials for the treatment of diarrhoea-predominant irritable bowel syndrome[1,10].

Taken together, these findings suggest that opioid receptor heterodimers may form *in vivo,* and that targeting these dimers for drug design is feasible. However, whether these drugs will ultimately act via receptor complexes *in vivo* to produce better analgesics remains an open question.

Supplementary Table 1 | Effects of opioid receptor knockout in behavioural models of addiction

Main phenotypes of constitutive knockouts (KO) and conditional knockout mice (Cre driver indicated) for MOR, KOR and DOR are shown, in behaviours modelling each stages of the addiction cycle (Fig. 2b). Table summarises studies reviewed in Refs.[15] and animal models categorized within each stage of the addiction cycle[16]. Complete references could be found in [15].

Stages of addiction cycle	Animal model	MOR	KOR	DOR
Binge or intoxication	SA	**Morphine:** decreased or abolished in KO **Heroin:** increased seeking in DLX-MOR KO [17] **Remifentanil:** restored upon MOR rescue in striatal *Pdyn*-positive neurons [18] **Cocaine:** decreased in KO **Alcohol:** decreased or abolished in KO; decreased in DLX-MOR KO[19]	**Alcohol:** decreased in KO	**Morphine:** unchanged KO **Nicotine:** decreased or unchanged in KO **Alcohol:** increased in KO
	CPP	**Morphine and heroin:** abolished in KO; unchanged in DLX-MOR KO[17]; restored upon MOR rescue in striatal *Pdyn*-positive neurons[18] **THC:** abolished in KO **Cocaine:** decreased or unchanged in KO	**Morphine:** unchanged in KO **THC:** unchanged in KO **Cocaine:** unchanged in KO	**Morphine:** reduced or Unchanged in KO **THC:** unchanged in KO **Nicotine:** reduced in KO

		Nicotine: abolished in KO **Alcohol:** abolished or unchanged in KO; abolished in DLX-MOR KO[19]		
Withdrawal or negative affect	Depressive- and anxiety-like responses	**Heroin:** decreased upon MOR KO in the DRN (AAV-Cre) [20]	KOR deletion in the BLA reduced anxiety [21]	–
	CPA	**THC:** decreased in KO	**THC:** abolished in KO **KOR agonists:** KOR deletion in DA neurons reduced CPA[22]	**THC:** unchanged in KO
	Increased motivation for self administration	**Heroin:** increased in DLX-MOR KO[17]	–	–
	Withdrawal	**Morphine:** abolished in KO **THC:** unchanged or decreased in KO **Nicotine:** decreased in KO **Alcohol:** earlier signs in KO	**Morphine:** abolished in KOR KO **THC:** unchanged in KOR KO	**Morphine:** unchanged in DOR KO **THC:** unchanged in DOR KO **Nicotine:** unchanged in DOR KO
Preoccupation or anticipation	Reinstatement	–	**Cocaine:** abolished in KOR KO (stress-induced)	–

AAV, adeno-associated virus; CPA, conditioned place aversion; CPP; conditioned place preference; DOR, delta opioid receptor; DLX, homeobox protein DLX; KO, knockout; KOR, kappa-opioid receptor; MOR, mu-opioid receptor; *Pdyn*, gene encoding preprodynoprhin; SA, self administration.

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