Usefulness of knockout mice to clarify the role of the opioid system in chronic pain

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Abstract

Several lines of knockout mice deficient in the genes encoding each component of the endogenous opioid system have been used for decades to clarify the specific role of the different opioid receptors and peptide precursors in multiple physiopathological conditions. The use of these genetically modified mice has improved our knowledge of the specific involvement of each endogenous opioid component in nociceptive transmission during acute and chronic pain conditions. The present review summarizes the recent advances obtained using these genetic tools in understanding the role of the opioid system in the pathophysiological mechanisms underlying chronic pain. Behavioural data obtained in these chronic pain models are discussed considering the peculiarities of the behavioural phenotype of each line of knockout mice. These studies have identified the crucial role of specific components of the opioid system in different chronic pain manifestations and have also opened new possible therapeutic approaches, such as the development of opioid compounds simultaneously targeting several opioid receptors. However, multiple questions still remain open and require further experimental effort to be clarified. The novel genetic tools now available to manipulate specific neuronal populations and precise genome editing in mice will facilitate in a near future the elucidation of the role of each component of the endoneous opioid system in chronic pain.

Abbreviations

MOP: Mu opioid receptor

DOP: Delta opioid receptor

KOP: Kappa opioid receptor

Oprm1: Gene coding for mu opioid receptor

Oprd1: Gene coding for delta opioid receptor

Oprk1: Gene coding for kappa opioid receptor

Pomc: Proopiomelanocortin gene

Pdyn: Prodynorphin gene

Penk: Proenkephalin gene

NOP: Nociceptin/orphanin receptor

N/OFQ: Nociceptin/orfanin FQ peptide

CFA: Complete Freund's adjuvant

PSNL: Partial Sciatic Nerve Ligation

DPDPE: [D-Pen2,5]-enkephalin

GABA: γ-Aminobutyric acid

DREADD: Designed Receptor Exclusively Activated by Designed Drugs

CNO: clozapine N-oxide

CRISPR: Clustered Regulatory Interspaced Short Palindromic Repeats

Cas9: CRISPR associated protein 9

Oprl1: Gene coding for Nociceptin/orphanin receptor

PKA: cAMP-dependent protein kinase

1. Introduction

The endogenous opioid system is composed of three opioid receptors, mu-(MOP), delta-(DOP) and kappa-(KOP) and their endogenous ligands derived from three precursors, proopiomelanocortin, proenkephalin and prodynorphin. Opioid precursor genes *Pomc*, *Penk* and *Pdyn*, respectively, were identified in the early 1980s, whereas the genes encoding for MOP (*Oprm1*), DOP (*Oprd1*) and KOP (*Oprk1*) were identified a decade later (Kieffer and Evans, 2009). Opioid peptides derived from *Penk*, mainly met and leu-enkephalin, are preferential ligands of DOP, although also show affinity for MOP. The opioid peptide derived from *Pomc*, β-endorphin, shows affinity for both MOP and DOP, whereas opioid peptides derived from *Pdyn*, dynorphins and neoendorphins, are preferential agonists of KOP (Kosterlitz, 1985).

The endogenous opioid system plays a crucial role in the control of nociceptive responses at peripheral, spinal and supra-spinal level (Figure 1). In physiological conditions, the neural process of encoding noxious stimuli starts with stimulation of Aδ and C nociceptors innervating peripheral tissues. These fibers synapse with projection neurons in the spinal cord dorsal horn, which send information to supra-spinal areas. At the peripheral level, opioid peptides released by immune cells during inflammation locally inhibit pain transmission (Rittner et al., 2008). At the spinal level, stimulation of presynaptic opioid receptors from nociceptors inhibits the release of neurotransmitters. Spinal cord dosal horn projection neurons convey inputs from peripheral and supra-spinal projections and post-synaptic opioid receptors inhibit their activity. However, activation of the endogenous opioid system at spinal level can also have pro-nociceptive effects in certain conditions, generally attributed to spinal dynorphins (Povdin et al., 2016).

Dorsal horn neurons project their axons to the thalamus and limbic areas of the brain, and thalamic neurons connect to the somatosensory cortex S1 and S2, allowing temporal and spatial discrimination of the noxious stimuli. Activation of limbic areas results in affective and motivational consequences of pain, which, in case of sustained activation, can evolve to pain-related anxiety, depression and cognitive impairment. Opioid receptors and peptides are expressed in these brain areas, which include amygdala, nucleus accumbens and medial prefrontal and anterior cingulate cortices. At the same time, emotional and cognitive factors are known to modulate pain perception (Bushnell et al., 2014). While a negative emotional state can increase the unpleasantness of pain regardless of the perceived intensity, a decreased attention to

the painful area can diminish the intensity of the pain sensation. This different pain modulation by cognitive and affective aspects is probably due to the different circuitry involved, being the attentional alterations associated with activity in the insula and the parietal and somatosensory cortices (Bushnell et al., 2014) and descending modulation by negative affect associated to activation of the periaqueductal gray (PAG) and the anterior cingulate and medial prefrontal cortices. Interestingly, these areas are also under control of the endogenous opioid system (Henriksen and Willock, 2008; Lutz and Kieffer, 2013).

The PAG receives inputs from amygdalar and cortical areas and sends projections to the rostral ventromedial medulla (RVM). In the RVM, On and Off neurons have axons that reach the dorsal horn of the spinal cord to facilitate (On cells) or inhibit (Off cells) nociceptive transmission (Ossipov et al., 2010). Opioid receptor activation in the PAG inhibits the cells that keep inhibited Off cells in the RVM, whereas On cells are the only cells directly inhibited by opioids in the RVM. The endogenous opioid system also modulates the activity of noradrenergic neurons in the locus coeruleus, which also inhibit synaptic transmission in the spinal cord. Thus, at this level opioids inhibit nociceptive transmission both by direct inhibition of On cells and disinhibition of Off cells, and by increasing noradrenergic activity in the spinal cord (reviewed in Nadal et al., 2013). The opioid system also participates in the control of other multiple physiological functions including emotional and rewarding responses, cognition, locomotion, feeding behavior, body temperature, endocrine, cardiovascular, respiratory and gastrointestinal functions, among others (Terenius, 2000; Polunina and Bryun, 2013; Bodnar, 2017).

Activation of nociceptive pathways during nociceptive and acute inflammatory pain avoids further damage to the organism and has an adaptive function. However, in some circumstances inflammatory pain perstists and becomes chronic, or chronic pain can be a result of abnormal functioning of the nervous system (pathological pain). Pathological maladaptive pain is not a symptom of a disorder, and can be associated to damage of the nervous system (neuropathic pain) or to conditions in which there is no neuronal damage or inflammation (dysfunctional pain) (Woolf, 2010). The modification of behavioural and physiological responses by the opioid system could influence the manifestations of acute and chronic pain.

Another receptor, the nociceptin/orphanin receptor (NOP), and its endogenous ligand, the nociceptin/orphanin FQ peptide (N/OFQ), were also proposed to integrate the endogenous opioid

system (Meunier, 1997). This receptor and endogenous peptide have been involved in nociceptive transmission, although its activation counteracts most of the classical opioid effects (Schröder et al., 2014; Kiguchi et al., 2016). Indeed, N/OFQ produces hyperalgesia in rodents when administered by intracerebroventricular route and analgesia when intrathecally administered, not suppressed by naloxone (Witkin et al., 2014). The actions of N/OFQ system are not restricted to nociceptive pathways. Indeed, NOP activation induces hypolocomotion, ataxia and loss of righting reflex, impairs spatial learning, increases feeding behaviour and regulates pituitary hormones release, among many others (Schröder et al., 2014; Toll et al., 2016).

Different lines of genetically modified mice have been generated with mutations targeting specific components of the opioid system. These genetic models have been invaluable tools to study the physiological role of the different opioid receptors and peptide precursors (Gavériaux-Ruff, 2013). Constitutive deletion of a targeted gene by homologous recombination in embryonic stem cells has allowed the generation of multiple lines of conventional knockout mice for nearly three decades. The phenotypic characterization of these conventional knockout mice has provided important advances in the knowledge of multiple physiological and pathological processes, including chronic pain. Genetic approaches targeting specific brain regions or neuronal populations are now providing additional advances in the knowledge of the specific mechanisms underlying chronic pain. Thus, the Cre-lox P system has been used to generate conditional deletions of opioid system components in specific tissues. The generation of conditional Cre/lox P knockouts requires the introduction of the sites for Cre recombinase (lox P sites) in direct orientation flanking the gene of interest. These “floxed” mice integrating the two loxP sites will delete the gene of interest upon delivery or expression of the Cre recombinase into the cells in which recombination is required (Morozov et al., 2003).

Knockout mice have been extensively used to evaluate the involvement of the opioid system in the control of nociceptive responses. Several reviews have recapitulated the advances provided by these genetic tools in pain control (Dierich and Kieffer 2004; Nadal et al., 2013). However, these reviews are mainly focused on the role of endogenous opioid components in acute pain and the antinociceptive responses induced by different opioid compounds. While moderate to severe acute pain can be efficiently treated with opioids, opioid treatment of chronic pain has multiple

deleterious consequences for the patients. Blunted efficacy of opioids, tolerance, hyperalgesia and addiction undermine the efficiency of these treatments (Trang et al., 2015). Understanding how the opioid system works during chronic inflammatory and neuropathic pain will be essential to optimize current treatments or to develop strategies for effective pain management. In the present review, we summarized the recent advances obtained using knockout mice in understanding the role of the different components of the opioid system in the pathophysiological mechanisms underlying chronic pain. The models used to evaluate and mimic inflammatory and neuropathic pain conditions in the opioid knockouts are recapitulated in Table 1. Behavioural data obtained in these models of chronic pain should be interpreted carefully considering the peculiarities of the behavioural phenotype of each line of knockout mice.

2. Studies on knockout mice deficient in MOP, DOP and their endogenous ligands

MOP constitutive knockout mice have been obtained by disruption of exon 1 (Sora et al., 1997; Tian et al., 1997; Schuller et al., 1999), exon 2 (Matthes et al., 1996), exon 2 and 3 (Loh et al., 1998; van Rijn and Whistler, 2009) or exon 11 (Pan et al., 2009) of the *oprm1* gene in mice with c57bl/6 or mixed c57bl/6-129S background (Figure 2). Additionally, conditional knockout with MOP exon 2 and 3 deleted in Nav1.8 (Weibel et al., 2013) and TRPV1 positive neurons (Corder et al., 2017) have also been published.

Due to the extended editing of the *Oprm1* gene (Pasternak and Pan, 2013) the different constructions of MOP knockouts resulted in the expression of different splice variants in the various strains generated. Exon 1 knockouts lack the canonical 7-transmembrane (7-TM) MOP, and hence are unsensitive to morphine. However, they keep the genomic sequence coding for 6 transmembrane (6-TM) domains, and were described to express 6-TM splice variants of the MOP (Schuller et al., 1999; Lu et al., 2015). This maintained their sensitivity to other opioids, including heroin, 6-acetylmorphine, morphine-6-glucuronide or fentanyl, which still could act through the 6-TM MOP (Schuller et al., 1999). However, exon 1 knockouts generated by other groups (Sora et al., 1997) showed decreased G-protein activation after exposure to these opioids, thus it is unclear whether these other exon 1 knockouts still express the 6-TM MOP. Interestingly, exon 11 knockouts maintain the 7-TM forms of the MOP but cannot produce the 6-

TM variants (Pan et al., 2009). Hence, these mice were sensitive to morphine but not to morphine-6-glucuronide or fentanyl. Moreover, exon 11 knockouts failed to show morphine-induced hyperalgesia, developed less tolerance and did not display hyperlocomotion after repeated morphine administration (Marrone et al., 2017), associating these negative consequences of opioid treatments to the 7-TM MOP splice variants. On the other hand, exon 2 or exon 2-3 knockouts are expected to lack both 6 and 7-TM variants (Pasternak and Pan, 2013) and showed loss of sensitivity to morphine and fentanyl (Matthes et al., 1996 ; Weibel et al., 2013). Thus, it is important to take into account the specific genetic modifications, since they may result in different behavioral outcomes depending on the phenotype being tested.

Exon 1, 2, and 2-3 knockouts showed increased sensitivity to heat, hypolocomotion and a disruption of the analgesic effects of MOP agonists (Ide et al., 2006, 2008; Pan et al., 2009; Kögel et al., 2011; Gavériaux-Ruff, 2013). Baseline mechanical sensitivity was generally unaltered when assessing light touch sensitivity with von Frey hairs, although nociceptive responses to stronger mechanical stimuli were increased (Fuchs et al., 1999; Martin et al., 2003). Indeed, abdominal contractions in response to colorrectal distension were also increased in constitutive exon 2-3 knockouts, but not in conditional Nav1.8 knockouts, suggesting a role of central MOP controlling basal colon mechanosensation (Gavériaux-Ruff et al., 2008).

A given cognitive or emotional trait in the rodent strains could impact the phenotypes observed in the chronic pain models. In the case of the MOP knockouts, basal anxiolytic and anti-depressive phenotype restricted to males were reported using classical behavioural paradigms (Filliol et al., 2000), in contrast to the increased anxiety in conflict and defensive conditions, aggressiveness and deficits in social behaviour independent of the gender of these mutants (Becker et al., 2014). Other features that may indirectly affect chronic pain manifestations include cognitive impairment not associated to attentional deficits (Jamot et al., 2003; Sanders et al., 2005), increased hematopoiesis or sexual dysfunction (Tian et al., 1997).

The literature exploring chronic pain in MOP knockouts have reported increased, no difference or decreased nociceptive behaviour depending on each specific study. Although this heterogeneity could seem a lack of reproductibility at the first glance, a closer inspection of the results may give some interesting conclusions. Divergent results may suggest a complex role for

MOP in the pathophysiology of chronic pain, could be explained by the multiplicity of MOP knockout models or also by methodological differences.

Partially conflicting results were obtained in different models of inflammatory pain. For instance, exon 1 knockout mice showed increased response in the late phase and no change in the early phase of the formalin test (Zhao et al., 2003) whereas exon 2 knockouts showed increased reflex responses in the early phase, and no change in the late phase (Martin et al., 2003). An effect of the different genetic deletions and certain methodological differences could have favored these divergent results. While the first study injected 2% formalin in the hindpaw and measured the number of flinches, the second one injected 5% formalin and measured the time licking, shaking or biting the paw. The significance of an increased response in each of the phases may have different interpretations (Table 1), although both studies showed antinociceptive effects of MOP.

Exon 1 MOP knockout mice showed divergent results in other models of inflammatory pain. Initial studies showed decreased number of writhings in the acetic acid model of abdominal pain (Sora et al., 1999), increased responsiveness to chemical stimulation in the formalin test (Qiu et al, 2000), and decreased heat sensitivity after the complete Freund's adjuvant (CFA) (Qiu et al., 2000), suggesting different MOP modulation depending on the pain model. However, later studies in exon 1 knockouts did not detect changes in heat sensitivity after CFA (Gendron et al., 2007), or in heat and mechanical sensitivity after carrageenan-induced inflammation (Mansikka et al., 2004). Another source of conflicting data was the DOP-mediated antinociception. Enhanced antinociceptive effects of the DOP agonists DPDPE and deltorphin-II were reported in exon 1 knockouts in c57bl/6-129/S background (Qiu et al., 2000), whereas reduced deltorphin-II and SNC80 antinociception was shown in a similar CFA model using c57bl/6 exon 1 knockouts (Gendron et al., 2007). In this case, different strain sensitivity to the DOP agonists may have favored this conflicting data, since previous studies already showed three-fold reduced morphine sensitivity in c57bl/6 vs. 129/S mice. Importantly, the reported differences in nociception between these strains could have contributed to the heterogeneity of the results in these opioid knockouts (Mogil and Wilson, 1997). Given the divergent results obtained in exon 1 knockouts, it should be clarified whether the different exon 1 strains express similar splice variants. While some groups (Schuller et al., 1999) characterized the expression of 6-TM MOP in these mice, this was not examined in exon 1 knockouts generated by other groups (Sora et al., 1997; Tian et

al., 1997). Future studies may help to clarify these apparently conflicting results and the specific participation of 6/7-TM splice variants in the chronic pain phenotypes.

Exon 11 knockouts (lacking 7-TM but preserving 6-TM variants) showed no change or marginal increases in mechanical sensitivity in the CFA model of inflammatory pain (Wieskopf et al., 2014) and unaltered mechanical sensitivity after spared nerve injury. However, these data were obtained with a dynamic plantar aesthesiomether (Table 1) and may not be directly comparable to mechanosensitivity data obtained with the other strains, generally evaluated using the von Frey filaments.

Using a similar model of inflammatory pain, exon 2 knockouts showed increased mechanical hypersensitivity after complete Freund's adjuvant (CFA) (Walwyn et al., 2016). However, other study showed unchanged responses to heat and mechanical stimuli after CFA using also exon 2 knockouts in similar c57bl/6 background (Gavériaux-Ruff et al., 2008). Methodological differences may have contributed to these divergent data. The former study (Walwyn et al., 2016) estimated the 50% paw withdrawal threshold, whereas the latter (Gavériaux-Ruff et al., 2008) used more restrictive conditions, considering the threshold as the thinest filament eliciting 60% of positive responses. Similarly, mice lacking MOP in Nav1.8 primary afferent fibers did not show altered sensitivity to inflammatory somatic or visceral pain, however MOP in these fibers participated in the analgesic effects of classical MOP agonists during chronic inflammation induced by CFA, but not in basal conditions (Weibel et al., 2013; Reiss et al.,2016). In the same line of results, the visceromotor response to colorrectal distension after the induction of experimental colitis was similar in exon 2-3 knockouts and control mice. Hence, exon 2 or 2-3 knockouts showed no effect or antinociceptive effect of MOP in inflammatory pain conditions, depending on the specific study.

Neuropathic pain models also showed divergent data in studies conducted with exon 1 or exon 2/2-3 MOP knockouts. Exon 1 knockouts exhibited enhanced mechanical, but not heat or cold hypersensitivity after unilateral nerve injury on L5 (Mansikka et al., 2004). Again, the interpretation of these results is difficult because the expression or absence of 6-TM MOP has not been characterized in these mice. Exon 2/2-3 knockouts showed no differences on mechanical sensitivity to von Frey hairs after cuffing the sciatic nerve (Bohren et al., 2010), whereas decreased nocifensive behaviour to thermal stimulation was observed in a model of

diabetic neuropathy (Kögel et al., 2011). In the same direction, exon 2 knockouts showed attenuated sensitivity to cold after partial ligation of the sciatic nerve (PSNL) (Maldonado, 2016), suggesting pronociceptive effects of MOP. This view may be in agreement with a recent study using conditional MOP exon 2-3 knockouts in TRPV1-positive neurons (Corder et al., 2017). The study revealed absence of morphine tolerance and morphine-induced hyperalgesia in these mice, attributing a role for peripheral MOP on these detrimental consequences of opioid treatments (Corder et al., 2017). Hence, according to the results obtained with exon 2/2-3 knockouts, this opioid receptor may play antinociceptive roles in certain conditions of chronic inflammatory pain, whereas the results obtained with neuropathic pain models suggest a maladaptive function of MOP in abnormal pain conditions. Thus, treatments with MOP agonists might be considered during chronic inflammatory pain, and these MOP agonists would act through peripheral sites. However, the application of MOP agonists during chronic neuropathic pain may be counterproductive.

Constitutive DOP knockouts were obtained by homologous recombination of *oprd1* exon 1 (Filliol et al., 2000) or 2 (Zhu et al., 1999; van Rijn and Whistler, 2009) in mice with mixed c57bl/6-129S or complete c57bl/6 background, and new conditional models have been obtained by deletion of exon 2 in primary afferents expressing Nav1.8 channels (Gaveriaux-Ruff et al., 2011) or in GABAergic neurons of the forebrain (van Rijn and Whistler, 2009; Chu Sin Chung et al., 2015). In these models, DOP appear critical to maintain affective stability and cognitive performance. Indeed, mice constitutively lacking DOP showed anxious and depressive-like phenotype (Filliol et al., 2000), and an impairment in learning and short-term memory (Le Merrer et al., 2013). It could be expected that a basal phenotype characterized by negative affect would favour nociceptive sensitivity (Bushnell et al., 2009). However, at least in baseline conditions, nociceptive thresholds to somatic thermal or mechanical stimuli were not modified (Filliol et al., 2000; Martin et al., 2003; Nadal et al., 2006; Gavériaux-Ruff et al., 2008). Interestingly, abdominal contractions in response to colorrectal distension were increased like in the MOP knockout animals, but this was not observed in conditional Nav1.8 knockouts, suggesting a role of central DOP on basal colon sensitivity.

The presence of DOP acquires more relevance in chronic pain conditions. Indeed, DOP knockouts developed increased thermal and mechanical sensitivity in models of chronic neuropathic (Nadal et al., 2006) and inflammatory pain (Gavériaux-Ruff et al., 2008), suggesting a protective role of DOP for the development of these chronic pain manifestations. This antinociceptive role of DOP was also observed in the late phase of the formalin test (Martin et al., 2003) and in pharmacological experiments with constitutive DOP knockouts revealing a crucial role of this receptor for the anti-allodynic effects of tryciclic antidepressants and DOP agonists (Benbouzid et al., 2008; Gavériaux-Ruff et al., 2008; Gavériaux-Ruff, 2013). On the contrary, the abdominal response to colorrectal distension after experimental colitis was similar in DOP knockout and wild-type mice (Reiss et al., 2016).

Conditional knockouts have been used to attribute the nociceptive phenotypes associated to DOP deletion to specific nervous system regions. Thus, DOP ablation in primary afferent fibers expressing Nav1.8 revealed the involvement of these receptors in chronic pain (Gaveriaux-Ruff et al., 2011). Indeed, these conditional mutants showed unchanged heat hyperalgesia but increased mechanical allodynia after CFA, and enhanced mechanical and cold hypersensitivity after PSNL (Gaveriaux-Ruff et al., 2011). Since constitutive DOP knockouts showed increased sensitivity to heat and this increase was absent in the conditional lines, this suggests that central DOP control heat hypersensitivity in chronic pain conditions. Another conditional knockout line targeting DOP in forebrain GABAergic neurons (Dlx5/6) was generated (Chu Sin Chung et al., 2015). Surprisingly, these mice showed lower levels of anxiety, in contrast to the anxious characteristics of constitutive DOP knockouts (Filliol et al., 2000). Although the consequences of this conditional DOP deletion in the development of chronic pain have not been yet investigated, it is yet unknown whether the heightened chronic pain sensitivity in constitutive DOP knockouts could be influenced by their inherent depressive and anxious phenotype. However, it seems clear that DOP have an overall protective function on nociceptive sensitivity during both chronic inflammatory and neuropathic pain conditions, partially mediated through their function on primary afferent neurons. While DOP from peripheral neurons control mechanical and cold sensitivity, DOP in central structures modulate heat hypernociception.

Knockout mice deficient in the precursors of the opioid peptides acting on MOP and DOP have also been generated. β-endorphin knockouts were obtained by a point mutation in exon 3 of the

Pomc gene in a mixed c57bl/6-129S background, which resulted in a premature stop codon not affecting the expression of other *Pomc*-derived peptides (Rubinstein et al., 1996). Baseline thermal and mechanical nociceptive thresholds were unaltered (Gendron et al., 2007; Petraschka et al., 2007; Fell et al., 2014; Walwyn et al., 2016) or slightly increased (Mogil et al., 2000; Trigo et al., 2009). These mutants showed normal anxiety-like behaviour (Trigo et al., 2009) and a reduced motivation for food (Hayward et al., 2002). In addition, these mice lacked stress-induced analgesia and showed a delayed thermal hyperalgesia after forced swimming (Parikh et al., 2011).

In chronic pain models, β-endorphin knockout mice showed normal nociceptive sensitization to thermal and mechanical stimuli in inflammatory (Gendron et al., 2007; Walwyn et al., 2016) and peripheral neuropathy models (Petraschka et al., 2007; Niikura et al., 2008a, 2008b; Labuz et al., 2016). However, these mice retained sensitivity to MOP agonists during neuropathic pain, whereas wild-type mice experienced the regular tolerance to MOP stimulation. These studies suggested that an enhanced release of β-endorphin during neuropathic pain produced stimulation of MOP and subsequent phoshorylation and desensitization, which was observed in wild-type but not in β-endorphin knockout mice (Petraschka et al., 2007; Niikura et al., 2008a, 2008b; Narita et al., 2013). Hence, β-endorphin activity would not be sufficient to modulate the nociceptive manifestations of chronic pain, although it could affect the functionality of opioid receptors.

Penk-deficient mice were generated in c57bl/6 and DBa2 backgrounds with deletions in exon 3 of the *Penk* gene (König et al., 1996; Ragnauth et al., 2001). It needs to be noted that *Penk* deletion not only supresses the production of enkephalins, but also of different peptides coded by the same gene (peptides B, E, I), and does not fully remove the production of enkephalins, since they are also codified by the *Pdyn* and *Pomc* genes (Höllt, 1986). In spite of this, *Penk* deficit increased supraspinal responses to nociceptive stimulation, but did not change reflex responses to heat or mechanical stimuli (Kingery et al., 2001; Gendron et al., 2007; Chen et al., 2008). *Penk* supression also enhanced aggressiveness and anxiety-like behaviour (König et al., 1996; Ragnauth et al., 2001; Bilkei-Gorzo et al., 2004), although the responses in specific tests may be strain-dependent (Bilkei-Gorzo et al., 2004). These mutants also showed a reduced motivation for food (Hayward et al., 2002). While enkephalin deficits are associated with increased

depressive-like behavior and enhancing enkephalin levels with enkephalinase inhibitors showed anti-depressant effects (Chu Sin Chung and Kieffer 2013), depressive-like behaviour in *Penk*-deficient mice was normal in basal conditions (Bilkei-Gorzo et al., 2007). Prominent increases of MOP and DOP in brain regions involved in emotional processing could have normalized this phenotype (Brady et al., 1999). However, acute stress provoked exacerbated anxiety and depressive-like behaviour in these mutants, whereas stress-induced analgesia was not modified (Ragnauth et al., 2001; Kung et al., 2010; Parikh et al., 2011). Thus, the anxious phenotype of the *Penk* knockout mice coexisted with increased supraspinal responses to noxious stimulation, but the magnitude of reflexive responses to nociceptive inputs was unaffected in physiological conditions.

Few studies have explored the consequences of *Penk* ablation in chronic pain (Labuz et al., 2016; Walwyn et al., 2016). Heat and mechanical sensitization was normal in models of inflammatory and neuropathic pain (Celik et al., 2016; Walwyn et al., 2016). Conversely, nerve-injured mice receiving T-lymphocytes from *Penk* knockout donors showed decreased anti-allodynic response compared to mice receiving wild-type lymphocytes (Basso et al., 2016), which could be equivalent to the reduced anti-allodynic effect of exogenous peptides in *Penk* knockouts with peripheral neuropathy (Celik et al., 2016). The absence of major modifications in the nociceptive manifestations of chronic inflammatory or neuropathic pain after the genetic suppression of the main precursors of the opioid peptides acting on MOP and DOP is in contrast to the changes on these chronic pain manifestations revealed in MOP and DOP knockouts. These controversial findings have suggested the possible relevance of ligand-independent opioid receptor constitutive activity in the control of nociceptive responses during chronic pain (Corder et al., 2013; Walwyn et al., 2016). Another plausible explanation is that endorphins and enkephalins can bind both MOP and DOP with very slight differences in affinity (Kosterlitz 1985). Thus, each peptide type may generate similar physiological responses in the absence of the other, and this may be furthered under pathological conditions that could modify their expression. According to the previous data, the aberrant affective behaviour of enkephalin knockouts did not have an impact on nociceptive sensitization, but the emotional-like manifestations of chronic pain have not been evaluated in these mice. Enkephalin supression may modify the affective consequences of chronic pain without altering the extent of the nociceptive sensitization, but this remains to be explored.

3. Studies on knockout mice deficient in KOP and their endogenous ligands

KOP knockout mice were generated by deleting exon 1 (Simonin et al., 1998) or 3 (Hough et al., 2000) of the *oprk1* gene in c57bl/6/129S or c57bl/6 background, respectively. These mice showed normal (Simonin et al., 1998; Martin et al., 2003; Xu et al., 2004; Negrete et al., 2016) or slightly enhanced (Martin et al., 2003; Gavériaux-Ruff et al., 2008) baseline sensitivity to mechanical and thermal stimuli. Inflammatory and nociceptive responses were unchanged in the formalin test, whereas visceral pain was increased in the writhing test (Simonin et al., 1998; Martin et al., 2003). In spite of the implication of KOP on dysphoria, anxiety and aversive effects of stress (Land et al., 2009), constitutive deletion of the receptor had no consequences on anxiety- and depressive-like behaviour (Simonin et al., 1998; Filliol et al., 2000), and spatial memory was unaffected (Jamot et al., 2003).

Removal of KOP favors a phenotype prone to nociceptive sensitization in chronic pain. These mutants showed increased heat sensitization after streptozotocin-induced diabetic neuropathy (Rutten et al., 2014), and enhanced heat and mechanical sensitivity as well as a contralateral mirror-image sensitization after PSNL (Xu et al., 2004). Surprisingly, the same authors showed that these knockouts displayed reduced spinal cord astrocytosis after PSNL (Xu et al., 2007). Increased and contralateral sensitization was also reported in the CFA model of inflammatory pain (Schepers et al., 2008), although another study did not show abnormal sensitivity in the same CFA model probably due to the high baseline sensitivity of the mice used (Gavériaux-Ruff et al., 2008). The model of monoiodoacetate-induced osteoarthritis pain also revealed enhanced mechanical sensitivity in these mutants, although anxiety-like behaviour and cognitive impairment associated to these chronic pain manifestations were attenuated (Negrete et al., 2016). Another model of osteoarthritis showed enhanced joint damage in KOR knockouts (Wu et al., 2017), and increased formation of cancellous bone was observed in these mice (Baldock et al., 2012), suggesting that the effects of KOP deletion on chronic osteoarthritis pain could also be local. However, no histological modifications were found on the articular cartilage in KOP knockouts after monoiodoacetate (Negrete et al., 2016). Taken all the results together, KOP seem

to have protective effects against nociceptive sensitization and detrimental effects promoting anxiety-like behaviour and cognitive impairment associated to chronic pain.

Pdyn-deficient mice were obtained by targeted disruption of exons 3 and 4 of the *Pdyn* gene in c57bl/6, 129S and Balb/c backgrounds (Sharifi et al., 2001; Zimmer et al., 2001; Loacker et al., 2007). *Pdyn* gene disruption results in the ablation of dynorphin, several enkephalin peptides and the α–neoendorphin. Baseline sensitivity to heat and mechanical stimuli was not modified (Wang et al., 2001; Zimmer et al., 2001; McLaughlin et al., 2003; Parikh et al., 2011), although certain experimental conditions have revealed increased sensitivity to mechanical (Walwyn et al., 2016) and heat stimuli (Wang et al., 2001). Furthermore, removal of the *Pdyn* gene decreased anxiety (Wittmann et al., 2009; Kastenberger et al., 2012), whereas depressive-like behaviour was generally unaffected, although punctual increases were also reported (Kastenberger et al., 2012). Dynorphin deficits suppressed stress induced-analgesia after forced-swimming or social defeat in the hot tail withdrawal test (McLaughlin et al., 2003), whereas the hot plate responses were preserved after different forced-swimming paradigms (Parikh et al., 2011).

Pdyn knockouts have been evaluated in different chronic pain models. Acute inflammatory pain was increased in the late phase of the formalin test (Wang et al., 2001). In contrast, mechanical hypersensitivity after chronic sciatic nerve constriction (Labuz et al., 2016), and mechanical and heat hypersensitivity after spinal nerve ligation or PSNL were decreased (Wang et al., 2001; Xu et al., 2004). In agreement, the latter study showed a reduction in KOP phosphorylation in the spinal cord accompanied by reduced astrocytosis in *Pdyn* knockouts after PSNL, suggesting a pronociceptive role of *Pdyn* gene products (Xu et al., 2007). Likewise, nerve-injured dynorphin-deficient mice did not develop tolerance to a KOP agonist, unlike wild-type mice (Xu et al., 2004). *Pdyn* knockouts showed enhanced mechanical nociception after CFA, however this was attributed to an increased baseline sensitivity (Walwyn et al., 2016). Another study failed to show differences in pain sensitization after CFA (Gendron et al., 2007). Conversely, mice lacking dynorphin showed increased sensitization to mechanical stimuli and developed less anxiety-like behaviour during osteoarthritis pain induced by monoiodoacetate (Negrete et al., 2016). Thus, dynorphins seem to play a complex role during chronic pain, dependent on the pain condition. Indeed, results in *Pdyn* knockouts suggest antinociceptive effects of *Pdyn* gene products in inflammatory pain conditions, however neuropathic pain models suggest

pronociceptive effects. While *Pdyn* antinociception matches with the pain-limiting effects of the KOP seen in *oprk1* knockouts, the enhanced pain phenotype in neuropathic conditions diverges from the antinociceptive role of the KOP observed in these models. Thus, dynorphins may facilitate pain sensitization through other opioid or non-opioid receptor/s during neuropathic pain. Additionally, *Pdyn* gene products could participate in the affective component of chronic pain, promoting anxiety-like behaviour.

4. Studies on knockout mice deficient in NOP and their endogenous ligand

Constitutive NOP knockout mice were generated by deletion of exon 2 and 3 (Clarke et al., 2001) and by deletion of exon 1 of the *oprl1* gene in a mixed c57bl/6-129S background (Nishi et al., 1997). NOP constitutive deletion did not modify acute thermal and mechanical nociception and morphine analgesia and withdrawal signs were unaltered suggesting that NOP was not essential in these responses (Nishi et al., 1997; Mamiya et al., 2001). Interestingly, these mutants showed a consistent reduction of tolerance to morphine analgesia revealing the involvement of NOP in the mechanisms underlying morphine tolerance (Ueda et al., 1997; Chung et al., 2006).

Knockouts deficient for the precursor of N/OFQ were generated by altering exon 2 in a C75BL6 background (Köster et al., 1999). As a consequence, these animals were lacking OFQ, OFQ2 and nocistatin. These mutants showed an elevated basal pain threshold in the tail-flick, but developed normal stress-induced analgesia and spatial learning performance. The mutants showed impaired adaptation to basal stress suggesting a role of N/OFQ in these responses to stress. In contrast, other authors have revealed an increased sensitivity in the tail-flick test in N/OFQ knockouts (Chen et al., 1999; Mogil and Pasternak, 2001).

The use of both knockout lines has also provided evidence on the participation of the N/OFQ system in other physiological processes, such as locomotion (Marti et al., 2004) and emotional-like responses (Redrobe et al., 2002; Gavioli et al., 2003). More recently, NOP knockout rats have been obtained by means of the premature stop codon using N-ethyl-N-nitrosourea-driven mutagenesis (Homberg et al., 2009). Mutant rats are more sensitive to morphine reward (Rutten et al., 2011) and showed differences in anxiety and mood-related behaviours, locomotion and nociception, mainly in the formalin test (Rizzi et al., 2011).

The role of the N/OFQ system in chronic pain was also studied using NOP and N/OFQ knockout mice (Depner et al., 2003). These mutants showed stronger nociceptive responses in late phase of the formalin test and increased thermal pain in the zymosan inflammatory model suggesting a protective role of the N/OFQ system during prolonged inflammatory pain. In contrast, NOP deletion prevented the appearance of postherpetic allodynia, although did not affect the development of skin lesions and allodynia during the presence of these lesions (Sasaki et al., 2008). Therefore, the N/OFQ system may be involved in the transition from herpetic to postherpetic allodynia.

NOP deficient mice were also used to study the interdependency between NOP and the different opioid receptors in streptozotocin-induced diabetic polineuropathy (Rutten et al., 2014). Selective agonists for each opioid receptor induced smaller analgesic responses in the hot plate in MOP, DOP, KOP and NOP knockouts compared with wild-type littermates. No evidence was shown on the interdependency of NOP and MOP suggesting the interest of its concurrent activation in chronic pain. In contrast, the antinociceptive (Lutfy et al., 2003), motor stimulatory and rewarding actions (Marquez et al., 2008) of buprenorphine were enhanced in NOP knockouts. The interaction between NOP and opioid receptors may work at circuit level as they share common signalling pathways in different anatomical regions, such as spinal cord, PAG and RVM (Günther et al., 2017). The effect of mixed NOP/MOP agonists inhibiting tolerance and dependence compared with pure MOP agonists may be due to a chronic desensitization of NOP signalling in reward and tolerance circuits (Lufty et al., 2001), as similar results were seen after a co-treatment of MOR receptor agonists with NOP antagonists (Chung et al., 2006).

NOP mediated effects on pain may be more complex than those from other opioid receptors. Its activation is followed either by pronociceptive or antinociceptive effects depending on the administration route and pain modalities in rodents (Schröder et al., 2014). Activation of spinal NOP in models of chronic pain caused potent antihyperalgesic and antiallodynic effects that can be explained at least in part by an up-regulation of those receptors in the spinal cord (Kiguchi et al., 2016). However, the supraspinal actions of NOP activation gave conflicting results depending of the model. While it seemed to elicit pronociceptive effects in inflammatory pain models, antinociceptive effects were observed in neuropathic pain models, whereas antagonists produced similar attenuation in both types of pain (Kiguchi et al., 2016). Additionally, it has

been observed that preproN/OFQ and NOP receptor knockout mice showed increased inflammatory hyperalgesia in the formalin assay, but not in an acute pain assay (Depner et al., 2003). These results were similar to those obtained in NOP receptor knockout rats (Rizzi et al., 2011). In summary, these studies indicate the possibility that the NOP system behaved differently in the different pain models and that its plasticity may participate in some degree in the nociceptive responses induced by chronic pain models (Toll et al., 2016). Heterogeneous outcomes may be explained by various levels of spinal vs. supraspinal participation of the NOP/NOFQ system under different pain states (Kiguchi et al., 2016).

5. Clinical interest of the multitarget approach

Findings obtained with these N/OFQ system knockouts and results of pharmacological studies have suggested a new therapeutic approach based on the development of opioid compounds targeting several opioid receptors in contrast to the traditional high selectivity of agonists (Bird and Lambert, 2015). Early pharmacological studies already demonstrated beneficial effects of coadministering several drugs acting in different opioid receptors. Thus, a reduction in the development of morphine tolerance was revealed when concomitantly administered with the DOP antagonist naltrindole (Abdelhamid et al., 1991). Similarly, morphine tolerance did not appear in *Penk* knockouts (Nitsche et al., 2002) and several studies have revealed the blockade of morphine tolerance in DOP and NOP knockouts (Ueda et al., 1997; Zhu et al., 1999; Chung et al., 2006). In agreement, various mixed MOP/NOP ligands have demonstrated potent analgesic effects in several rodent models together with attenuation in the development of tolerance or physical dependence (Suktankar et al., 2013; Sobczak et al., 2014; Zielińska et al., 2015), suggesting the potential interest of using drugs simultaneously targeting NOP and MOP (Blair Journigan et al., 2014). Recently, Toll et al. (2016) have reviewed the influence of the activation or blockade of N/OFQ system on the development of opioid tolerance. Morphine tolerance is significantly reduced in NOP or ppN/N/OFQ KO mice (Ueda et al., 1997; Chung et al., 2006). Additionally, N/OFQ antibody partially reversed the tolerance associated to chronic morphine administration (Tian and Han, 2000). These results agree with the report that the co-administration of J-113397, an antagonist of the NOP, blocks tolerance development in mice (Ueda et al., 1997, Chung et al, 2006). The application of such antagonist in ventrolateral

periaqueductal gray blocks the analgesic action of morphine (Scoto et al., 2010) and DAMGO (Parenti and Scoto, 2010). Although i.c.v. injection of N/OFQ after a daily systemic administration of morphine also blocked morphine tolerance (Lufty et al., 2001), NOP antagonists also blocked the expression of tolerance to a daily injection of morphine (Zaratin et al., 2004; Chung et al., 2006). Toll et al. (2016) concluded that chronic morphine leads to up-regulation of brain NOP system, which attenuates morphine analgesia and can be blocked with a NOP antagonist. They suggested the need of new studies to better understand the implication of NOP system, given the conflicting results with agonists and antagonists (Toll et al., 2016). In this sense, a novel full MOP agonist and NOP partial agonist displaying subnanomolar affinity for both receptors, cebranopadol, has been extensively studied at preclinical level (Bird and Lambert, 2015; Sałat et al., 2015). This drug has long-term analgesic effects in acute and chronic pain models, and its side effects are less severe than those of standard opioid drugs. Indeed, motor coordination and respiratory function remains unaltered at high doses and tolerance is delayed compared with morphine (Sukhtankar et al., 2013; Linz et al., 2014; Schunk et al., 2014; Raffa et al., 2017). According to ClinicalTrials.gov (National Institutes of Health, 2017), seven Phase II clinical trials have been completed with cebranopadol. These studies analysed its analgesic effects in painful diabetic polyneuropathy, postoperative pain, severe chronic low back pain, and chronic pain due to osteoarthritis of the knee. Two Phase III clinical trials were also carried out in cancer pain. Some of these clinical studies were finished several years ago, but the results have not been yet published. This information would be necessary to understand the real clinical interest of targeting with a same ligand MOP and NOP for the treatment of acute or chronic pain in patients.

6. Current and future directions

Novel genetic approaches have been recently developed to manipulate specific neuronal populations in live animals as well as new techniques that facilitate the simplicity of making gene mutations in rodents. The application of these new techniques can provide important advances in the near future for understanding multiple neurophysiological processes. The novel genetic techniques most commonly employed to selectively manipulate specific neuronal populations are optogenetics using channels activated by light, and chemogenetics using

engineered G-protein coupled receptors activated by otherwise inert drugs (Whissell et al., 2016).

Optogenetic approaches have been applied to manipulate specific neuronal populations and have been recently used to study specific components of the pain circuitry (Copits et al., 2016). In these approaches, light-sensitive proteins, called opsins, are expressed in genetically defined neuronal populations. The majority of these opsins are light-gated ion channels and pumps isolated from diverse microorganisms (Fenno et al., 2011). Excitatory opsins are cation selective and gate inward photocurrents that depolarize neurons, whereas inhibitory opsins are most frequently pumps that mediate chloride influx or proton efflux to silence neuronal firing (Copits et al., 2016). These optical approaches have also been developed for manipulating intracellular signaling cascades associated to G-protein coupled receptors, including MOP (Siuda et al., 2015). Opsins are delivered into the nervous system through viral vectors or transgenesis (Copits et al., 2016). Cre-dependent adeno-associated viruses containing opsin genes are usually injected into transgenic mice where Cre-recombinase expression is restricted to genetically defined cell types. On the other hand, crossing Cre driver transgenic mice with genetically encoded opsin mouse lines can enable specific photo-manipulation of defined neuronal populations (Wang et al., 2016). These manipulations have already been employed to clarify the neuronal pathways involved in chronic pain control. Thus, optogenetic manipulation of the neuronal activity in the medial prefrontal cortex (Ji and Neugebauer, 2012; Zhang et al., 2015) and basolateral amygdala (Kiritosi et al., 2016) has identified the specific contribution of these circuits in the sensory and emotional components of chronic pain. Optogenetic stimulation of cortical projections to the nucleus accumbens decreased chronic pain manifestations (Sugimura et al., 2016), whereas optogenetic stimulation of the central amygdala induced visceral pain (Crock et al., 2012). These approaches have also allowed the dissection of specific roles of serotonergic neurons of the rostral ventral medulla (Cai et al., 2014) and subpopulations of locus coeruleus noradrenergic neurons (Hickey et al., 2014) in pain control. An optogenetic approach has also been used to dissect the specific contribution of opioid and GABA receptors in the pre-synaptic modulation of the nociceptive information transmitted to spinal neurons (Honsek et al., 2015). Interestingly, MOP pre-synaptic activation mainly inhibited C-fibers innervating lamina I spinal cord neurons whereas DOP pre-synaptic activation did not modify these responses. In a similar location (spinal cord laminae I/II) another study found that a MOP agonist inhibited excitatory

postsynaptic currents elicited by TRPV1 nociceptors optogenetically activated. The inhibition disappeared after removal of the MOP agonist, and was followed by long-term potentiation indicative of excitatory opioid effects. Since these effects were absent in conditional knockouts lacking MOP in TRPV1 nociceptors, and correlated with suppression of opioid-induced hyperalgesia in these mice, a fundamental role of presynaptic MOP could be defined in these processes (Corder et al., 2017). Hence, these interesting approaches are already helping to understand how opioid receptors function in specific locations of the nociceptive pathways.

The chemogenetic approach most widely used in neuroscience to selectively manipulate specific brain circuits by modifying the activity of G-protein coupled receptors is the Designed Receptor Exclusively Activated by Designed Drugs (DREADD) technique (Whissell et al., 2016). The engineered designer receptor must first be selectively expressed in the targeted neuronal population. DREADDs are usually modified muscarinic receptors coupled to an inhibitory (Gi) or excitatory (Gq, Gs) signaling cascade with low affinity for endogenous ligands and activated by synthetic compounds, such as clozapine N-oxide (CNO), an inert and orally available drug. The genes encoding these designer receptors are usually introduced in cells by Cre-dependent adeno-associated viruses restricting DREADD expression to cells that selectively express Cre. The administration of CNO will decrease or increase the activity of this specific neuronal circuit depending on the signaling cascade coupled to the designer receptor expressed (Whissell et al., 2016). These techniques have already been used to clarify the specific involvement of different spinal cord neuronal populations in itch (Bourane et al., 2015), acute (Saloman et al., 2015; Peirs et al., 2015) and chronic pain (Peirs et al., 2015), and supraspinal circuits of visceral pain (Jurik et al., 2015). However, none of these studies has yet coped with the function of the endogenous opioid system in chronic pain conditions.

The use of Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR)/Cas9 technology is revolutionizing genetics in multiple organisms including laboratory mice. The CRISPR system is a versatile antiviral defense mechanism that provides immunity for a host bacterium against extrachromosomal genetic material (Mojica and Montoliu, 2016). After the acquisition of foreign DNA by the bacterium, this system involves the synthesis and maturation of CRISPR RNA followed by the formation of RNA-Cas nuclease protein complexes that will selectively recognize and destroy the targeted foreign DNA by Cas nuclease cleavage. The

CRISPR/Cas9 is a versatile system able to recognize virtually any sequence in the genome, including the mammalian genome, and introduce a controlled break in the DNA (Singh et al., 2015). This novel technology has opened a plethora of possibilities for precise genome editing in mice allowing nearly any possible change mimicking human coding variants to be introduced in the mouse genome. An important progress has been already made in targeting specificity, simplicity of making gene-editing mice and bioinformatics design (Pulido-Quetglas et al., 2017). Although improvements may still be needed to increase precise editing efficiency and decrease mosaicism (Singh et al., 2015), this technology provides entirely new tools extremely useful to conduct genetic mouse research faster and better. A recent study on a model of intervertebral disc degeneration (Stover et al., 2017) detected specific changes in the activity of dorsal root ganglion neurons, associated to the A kinase anchor protein AKAP150, a protein that recruits the cAMP-dependent protein kinase (PKA) to the dendrites. These authors utilized CRISPR epigenome editing to modulate endogenous expression of AKAP150 and could erase the pathologic neuronal activity while preserving normal activity. This highlights the potential use of CRISPR as a pain neuromodulatory strategy. Due to the close relationship of PKA with the processes associated to opioid receptor stimulation, it would be interesting to evaluate opioid function in these experimental conditions, however the possibilities offered by CRISPR/Cas9 are immense.

All these novel genetic techniques will certainly provide in the near future important advances to our understanding of the neurobiological mechanisms underlying chronic pain and the specific involvement of the different components of the endogenous opioid system in these pathophysiological processes. Optogenetic or chemogenetic control of MOP expressed in genetically defined circuits could clarify their role in the nociceptive and affective manifestations of chronic pain, whereas an optimized genomic editing could avoid compensatory changes and disrupt the expression of opioid peptides with common receptor affinities to clarify their participation in chronic pain phenotypes.

7. Concluding remarks

Studies using knockout mice have allowed identifying the crucial role of DOP and KOP on chronic inflammatory and neuropathic pain (Figure 4). However, these approaches have not yet clarified the precise role of MOP in the development of different chronic pain conditions. Data suggest that stimulation of peripheral and central MOP could exert antinociceptive effects during certain chronic inflammatory pain conditions, but it may be counterproductive during pathological neuropathic pain or after repeated opioid treatments. The clarification of the role of the different MOP splice variants in the development of chronic pain may as well open new therapeutic avenues. MOP is the main opioid receptor for acute nociceptive control and the elucidation of its specific roles in chronic pain merits further studies. In contrast to these findings in opioid receptor knockouts, the suppression of the main precursors of the opioid peptides acting on MOP and DOP had not major consequences on chronic pain manifestations. This suggests either a possible role of ligand-independent opioid receptor constitutive activity or a redundancy in the opioid system in which the presence of one opioid peptide can supplement the abscence of other with similar receptor affinity. Interestingly, β-endorphin activity was revealed necessary for the development of morphine tolerance, whereas the effects of dynorphins could be different depending on the chronic pain condition. Another important open question is to understand the possible influence of the emotional changes revealed in these knockouts in chronic pain manifestations, since the affective aspects of chronic pain may be independent of strictly nociceptive manifestations. The N/OFQ system also seems to play an important role in specific chronic pain conditions, and seems essential for the development of opioid analgesic tolerance. These findings has opened a new possible therapeutic approach consisting in mixed MOP/NOP ligands that showed beneficial effects in preclinical studies, but the results of the already finished clinical trials have not been yet published. The novel genetic tools now available to manipulate specific neuronal populations are already providing answers on the functioning of the opioid system, and a quick and precise genome editing in mice will facilitate the elucidation of most of these still open questions in the pathophysiology and treatment of chronic pain.

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Competing Interests' Statement

The authors have no competing interests to declare

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