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Disruption of the κ-opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective κ-agonist U-50,488H and attenuates morphine withdrawal

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μ-, δ- and κ-opioid receptors are widely expressed in the central nervous system where they mediate the strong analgesic and mood-altering actions of opioids, and modulate numerous endogenous functions. To investigate the contribution of the κ-opioid receptor (KOR) to opioid function *in vivo*, we have generated KOR-deficient mice by gene targeting. We show that absence of KOR does not modify expression of the other components of the opioid system, and behavioural tests indicate that spontaneous activity is not altered in mutant mice. The analysis of responses to various nociceptive stimuli suggests that the *KOR* gene product is implicated in the perception of visceral chemical pain. We further demonstrate that KOR is critical to mediate the hypolocomotor, analgesic and aversive actions of the prototypic κ-agonist U-50,488H. Finally, our results indicate that this receptor does not contribute to morphine analgesia and reward, but participates in the expression of morphine abstinence. Together, our data demonstrate that the *KOR*-encoded receptor plays a modulatory role in specific aspects of opioid function.

Keywords: nociception/opioid pharmacology/κ-opioid receptor knock-out/spontaneous behaviour

Introduction

Opium has been used for medical purposes for more than 4000 years. Morphine, the major active alkaloid of opium, remains the most important compound used in medicine for the treatment of severe pain. However, opioid administration is associated with severe side effects (see Schug *et al.*, 1992), and the illegal abuse of opioids, particularly heroin, represents a major public health problem. Three different receptor classes, μ-, δ- and κ-, mediate the pharmacological actions of exogenous opioids (see Browstein, 1993) and interact with endogenous peptides under normal physiological conditions. Opioid peptides derive from precursor proteins known as proopiomelanocortin (POMC), proenkephalin (PENK) and prodynorphin (PDYN) (see Rossier, 1982). In addition, novel peptides recently have been isolated from brain that may represent a class of highly μ-selective endogenous opioids (Zadina *et al.*, 1997). The endogenous peptides and their receptors are highly expressed throughout the central nervous system and play a major role in the control of pain (see Dickenson, 1991; Roques *et al.*, 1993) and stress responses (see Akil *et al.*, 1984). The opioid system also regulates some aspects of behaviour, including locomotion, mood, learning and memory, and influences autonomic, neuroendocrine and immune responses (see Olson *et al.*, 1996).

There is good evidence that each receptor plays a specific role in the physiological and pharmacological responses to opioids. Binding site and mRNA mapping shows distinct, while partially overlapping, distribution patterns (Waksman *et al.*, 1986; see Mansour *et al.*, 1995). Pharmacological studies suggest that κ-opioid receptors (KORs) participate in the control of chemical, mechanical and thermal pain, mainly at the spinal level (see Millan, 1990) where all three receptor classes are expressed abundantly and mediate opioid analgesia (see Dickenson, 1991). In the limbic system, KORs modulate mood and locomotion in opposition to μ-opioid receptors (MORs) and δ-opioid receptors (DORs) (see Millan, 1990). Indeed μ- and δ-agonists both display reinforcing properties (Di Chiara and North, 1992; Koob, 1992; Spanagel *et al.*, 1992) and increase locomotor activity in most behavioural paradigms used in mice (Murray and Cowan 1990; Di Chiara and North, 1992), while κ-agonists have strong dysphoric and sedative effects (Pfeiffer *et al.*, 1986). These opposite actions are probably due to the fact that selective opioid agonists may enhance (μ, δ) or reduce (κ) the activity of mesolimbic dopaminergic neurons involved in the reward responses (Di Chiara and Imperato, 1988). Another major aspect of opioid action is the development of long-term adaptative changes following chronic exposure to opioid agonists leading to dependence. The MOR has been proposed as a major factor in the development and expression of physical dependence, although δ- and κ-agonists have also been shown to exhibit dependence liability (Cowan *et al.*, 1988; Maldonado *et al.*, 1992).

In many instances, it has been difficult to separate clearly the specific pharmacological responses mediated by each opioid receptor. Conclusions from pharmacological studies rely on the use of μ-, δ- and κ-selective compounds whose specific action may be questioned, particularly in the case of *in vivo* experiments where the route of administration and the pharmacokinetic properties of the drug are critical. The recent molecular cloning of opioid

receptors and the availability of gene targeting technology now provide the opportunity to characterize unambiguously the physiological role of each receptor type and to re-evaluate the specific mode of action of opioid drugs (see Valverde *et al.*, 1997). Three genes encoding a MOR, a DOR and a KOR have been identified (see Kieffer, 1995). Previously, we have disrupted the *MOR* gene in mice by homologous recombination and demonstrated that the encoded MOR is a mandatory component of the opioid system for the main biological actions of morphine (Matthes *et al.*, 1996).

We have now applied a similar strategy to inactivate the *KOR* gene in mice, and we report here the first characterization of KOR-deficient mutant mice. We show that the absence of *KOR*-encoded receptors does not markedly modify the spontaneous behaviour of mutant mice. Our data indicate no alteration in the perception of thermal, mechanical and inflammatory noxious stimuli, whereas the nociceptive threshold is modified in response to visceral chemical pain. We demonstrate that locomotor, analgesic and dysphoric effects of a prototypic κ-agonist, U-50,488H, is essentially mediated by the *KOR* gene product. Finally our results suggest that morphine analgesia and reward are unchanged, while physical dependence on morphine is attenuated in KOR-deficient mice.

Results

Generation of KOR-deficient mice

We used a PCR-generated probe encoding the N-terminal part of the mouse KOR to screen a mouse 129 genomic library and obtained a fragment of the *mKOR* gene. To create a null mutation in the *KOR* gene by homologous recombination (Capecchi, 1989), we generated a targeting construct by replacing the initiation codon and the N-terminal coding region of mKOR (amino acids 1–79) by a neomycin resistance cassette (Figure 1A). One embryonic stem (ES) cell clone harbouring the desired homologous recombination was identified and injected into C57BL/6 host blastocysts to generate chimeric mice. Male chimeras were mated to C57BL/6 females to produce F_1 offspring carrying germline transmission of the mutated *mKOR* allele. Male and female F_1 *mKOR* heterozygotes (+/–) were inter-crossed to generate F_2 homozygous (–/–) mutants, as demonstrated by Southern blotting (Figure 1B). Mice homozygous for the mutation were born in the expected Mendelian proportion, indicating that there was no *in utero* or post-natal mortality.

The KOR-deficient mice appeared healthy and had no gross abnormalities. The body weight of 6-week-old mutant mice was not significantly different from that of their wild-type littermates ($n = 25$). We observed an increase in the size of litters obtained from heterozygous (7.9 ± 0.40, $n = 55$) and homozygous (7.7 ± 0.6, $n = 16$) founder animals compared with wild-type (6 ± 0.4, $n = 72$) mice. These differences were statistically significant when compared with the wild-type in both cases (heterozygous, $U = 1302$, $P < 0.01$; homozygous, $U = 382.5$, $P < 0.05$). There was no obvious difference in numbers of males and females in the offspring of mutant animals.

Expression of opioid receptor-binding sites

We have investigated the absence of KOR protein in mutant mouse brain by binding studies using [^{3}H]CI-977, a high affinity ligand with very good κ-selectivity and low non-specific binding (Boyle *et al.*, 1990; Hunter *et al.*, 1990). Saturation analysis of [^{3}H]CI-977 binding on total brain homogenates showed a 50% reduction of KOR-binding sites in heterozygous animals and a total loss of KOR-binding sites in homozygous mutants (Table I). We performed autoradiographic mapping of KORs throughout the brain using the same radioligand (Figure 2). In brains from heterozygous mice, there was a loss of about half the number of sites throughout all brain structures where KORs are expressed. In homozygous brains, levels of binding were not different from that of non-specific binding images. These data confirmed that our gene targeting strategy led to a complete loss of KOR-binding sites.

To assess the influence of the *KOR* gene deletion on the remaining opioid receptor sites, we have quantified the number of μ- and δ-sites on whole brain membranes using specific μ- (DAMGO) and δ- (DELT I) radioligands. For the two receptors, we found similar K_d and B_{max} values (Table I) across the three genotypes. Autoradiographic mapping confirmed that the distribution of binding sites forMORs and DORs was unaffected throughout the brain in both heterozygous and homozygous animals. These results indicate the absence of compensatory changes at the level of MORs and DORs in the brain of KOR-deficient animals.

Expression of endogenous opioid peptide genes

Three opioid peptide precursor genes have been described, POMC whose synthesis is restricted to the arcuate nucleus of the hypothalamus and to intermediate and anterior lobes of the pituitary, PENK and PDYN, which both display a similar widespread expression pattern in the central nervous system (Khachaturian *et al.*, 1985). The effect of the absence of the KOR on the transcription pattern of these genes was investigated by *in situ* hybridization analysis. There was no modification in the distribution of the PENK mRNA in olfactory tubercle, piriform cortex, nucleus accumbens and caudate putamen across mouse genotypes (Figure 3). The PDYN mRNA distribution was also unaffected by the lack of KORs, with a signal high in the nucleus accumbens and weak in the caudate putamen for all three genotypes (+/+, +/– and –/–) (Figure 3). In addition, labelling of paraventricular hypothalamic and supraoptic nuclei by the PDYN probe was unchanged in mutant mice (not shown). POMC expression was also similar in wild-type and mutant mice, in arcuate neurons (Figure 3, upper panels) and in the pituitary gland (Figure 3, lower panels), where strong labelling of the entire intermediate lobe and moderate labelling of the anterior lobe were observed. Therefore labelling patterns appeared indistinguishable across genotypes, suggesting that the distribution and expression levels of opioid peptides were not modified in KOR-deficient mice.

Spontaneous behavioural responses in KOR-deficient mice

To investigate whether the absence of κ-sites modifies spontaneous behaviour, we have compared the responses

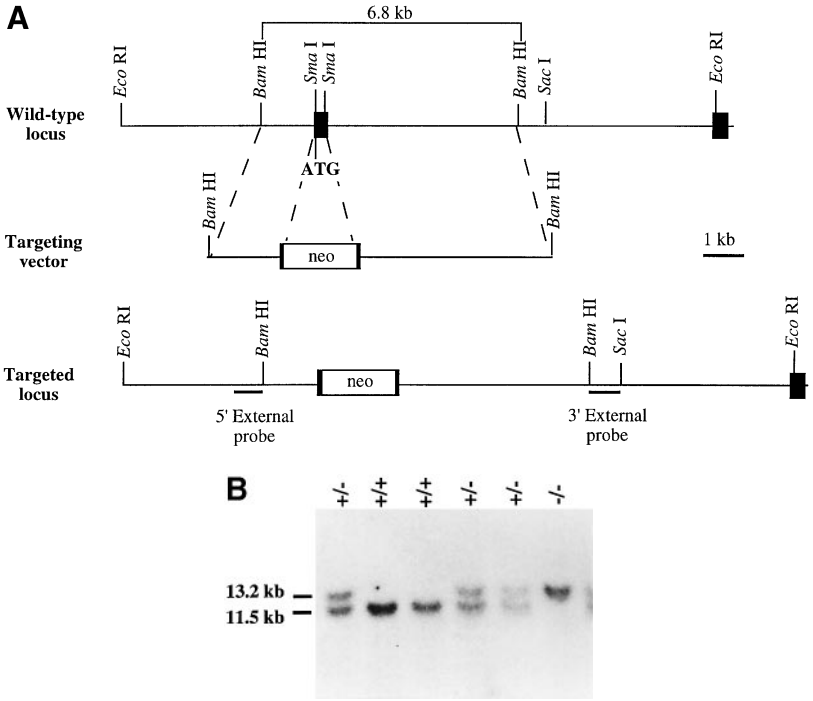

Fig. 1. Generation of KOR-deficient mice. (**A**) Representation of the wild-type *mKOR* locus (top), the targeting vector (middle) and the recombinant allele (bottom). The black boxes indicate the first and second coding exons. The positions of the 5′ and 3′ external probes used for Southern blot analysis are indicated by thick bars. (**B**) Genomic Southern blot analysis of mouse tail DNA digested with *Sac*I and *Eco*RI and hybridized with the 5′ probe. The expected sizes are 11.5 kb for the wild-type DNA and 13.2 kb for the mutant DNA. The genotype of each mouse is indicated on top of each lane.

Table I. Saturation analysis of κ-, μ- and δ-opioid receptor sites in brain membranes of wild-type (+/+), heterozygous (+/–) and homozygous (–/–) mice

	[^{3}H]CI-977		[^{3}H]DAMGO		[^{3}H]DELT I	
	K_d[a]	B_{max}	K_d	B_{max}	K_d	B_{max}
+/+	0.137 ± 0.001	0.040 ± 0.002	1.26 ± 0.24	0.153 ± 0.024	0.42 ± 0.02	0.089 ± 0.001
+/–	0.173 ± 0.004	0.020 ± 0.002	1.36 ± 0.11	0.146 ± 0.003	0.380 ± 0.004	0.099 ± 0.01
–/–	und[b]	und	1.39 ± 0.12	0.139 ± 0.014	0.319 ± 0.07	0.090 ± 0.008

[a]K_d (nM) and B_{max} (pmol per mg protein) values are the mean ± SEM of at least two experiments performed in triplicate.
[b]und: undetectable.

of homozygous mutant mice and their wild-type littermates in different behavioural paradigms during three consecutive days for each test. The results are summarized in Table II. First, spontaneous locomotor responses were evaluated under non-stressful conditions in locomotor activity boxes (n = 14). Both groups of mice showed a significant habituation to the new environment as revealed by the decrease in the activity counts on the third day when compared with the first day (P <0.05), but no difference was observed between the two genotypes. We have also investigated the circadian changes in locomotor activity. Both groups of animals showed a similar increase in the spontaneous locomotor activity during the dark period. When the locomotor activity was recorded at 2 h, wild-type and the mutant mice showed an increase of 26 and 19% respectively, in comparison with the previous values obtained at 14 h (data not shown). Behaviour was also studied under stressful conditions by using the open-field test. Both groups of mice showed a rapid habituation to the open-field that was evident from the second day (latency time to move out, P <0.01). Although habituation to the number of squares crossed was stronger in the group of mutant mice (P <0.05 on the third day) than in the wild-type group (n.s. on the third day), there were no significant differences between *KOR* –/– and *KOR* +/+ mice (n = 15) in any of the behavioural events evaluated. Emotional-related responses of KOR-deficient animals were investigated using the elevated O-maze and elevated plus-maze. Both groups of mice showed a similar habituation to the tests, and no significant differences were observed between *KOR* –/– and *KOR* +/+ mice. Finally, spontaneous alternation was evaluated in the Y maze in a single observation period in mutant mice and their wild-type littermates (n = 15). No significant differences between the two groups of mice were observed in the behavioural responses evaluated in this test (alternation and global activity). The scores for spontaneous alternation were respectively 63.0 ± 2.1% and 65.7 ± 1.2% for the

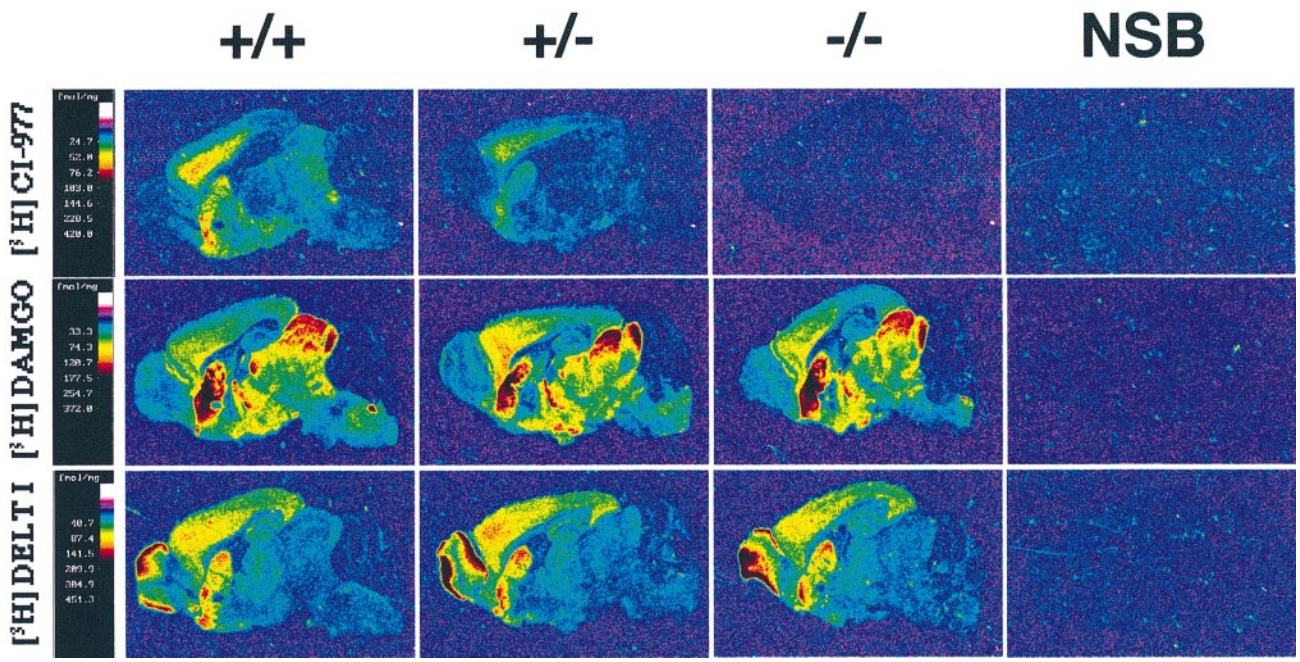

Fig. 2. Computer-enhanced colour autoradiograms of sagittal sections from wild-type (+/+), heterozygous (+/–) and homozygous (–/–) mice showing κ- (upper row), μ- (middle row) and δ- (bottom row) opioid receptor binding. μ-Receptors were labelled with [^{3}H]DAMGO, δ-receptors were labelled with [^{3}H]DELT I and κ-receptors were labelled with [^{3}H]CI-977 in adjacent sections from each brain. The colour bar shows a pseudo-colour interpretation of the relative density of black and white film image calibrated in fmol/mg tissue. Non-specific binding (NSB), determined in the presence of excess naloxone, was at background levels and is shown in the far right column for each ligand. Wild-type, heterozygous and homozygous sections were processed in parallel throughout binding and for development of autoradiograms.

wild-type and mutant mice (data not shown). In conclusion, no marked differences between homozygous mutant mice and their wild-type littermates were found in any of these observations, suggesting the absence of major impairment of spontaneous behaviour in mutant mice.

To examine whether mice deficient in the *KOR* gene exhibit altered pain perception, we have exposed mutant and wild-type animals to a number of nociceptive stimuli, including inflammatory (time spent in licking the right forepaw after formalin injection, see Figure 4A), mechanical (threshold in the tail pressure test, see Figure 4B), chemical (number of writhes after i.p. acetic acid injection, see Figure 4C) and thermal pain (tail-withdrawal latency in the tail immersion test, see Figure 4D, paw lick latency in the hot plate test, see Figure 4E). Both groups of mice (n = 15–20) exhibited similar nociceptive responses in all the nociceptive thresholds evaluated, except for the abdominal constriction test. In this assay, the group of mutant mice showed a significant increase (P <0.01) in the number of writhes induced by acetic acid as compared with their wild-type littermates (Figure 4C), indicating an involvement of the *KOR* gene product in the perception of this noxious stimulus. We have further compared responses of heterozygous mutant mice with those of homozygous and wild-type animals in this test. The number of abdominal constrictions in KOR +/– mice (12.67 ± 3.29, n = 12) was similar to that of wild-type animals (13.12 ± 2.16, n = 12), again significantly lower than in KOR –/– mice (19.66 ± 4.18, n = 5) (not shown). The absence of a gene dosage effect in this response suggests the existence of spare receptors in neurons mediating chemical visceral pain.

Pharmacological action of the κ-selective agonist U-50,488H in KOR-deficient mice

The arylacetamide compound U-50,488H was the first highly κ-selective agonist to be synthesized (Lahti *et al.*, 1982; Vonvoigtlander *et al.*, 1983). U-50,488H is therefore considered as one of the standard pharmacological agents to study KOR-mediated biological responses. We have used two different nociceptive models, the hot plate (jumping and licking responses) and the tail immersion tests, to assess U-50,488H analgesic action in *KOR* +/+ and *KOR* –/– mice. The compound was administered by the s.c. route at doses of 6.66 and 20 mg/kg, conditions that we have shown to induce a dose-dependent antinociception in 129/sv/C57Bl/6 hybrid mice (R.Maldonado, unpublished). U-50,488H induced dose-related antinociception in wild-type mice, evidenced by increases in tail withdrawal and licking latencies (n = 8–10) (Figure 5A and B). However, U-50,488H failed to induce any antinociceptive response in KOR-deficient mice (n = 8–10) (Figure 5A and B). A similar result was obtained for jumping latencies in the hot plate test (not shown).

Besides analgesia, U-50,488H induces other pharmacological responses *in vivo* that we also examined in this study. In contrast to μ- and δ-agonists, U-50,488H is known to reduce locomotor activity in mice. The effect of U-50,488H (6.66 and 20 mg/kg, s.c.) upon spontaneous horizontal locomotor activity was evaluated. A strong and dose-dependent decrease in the number of squares crossed was observed in wild-type mice (n = 8–10), as previously reported (Vonvoigtlander *et al.*, 1983). In mutant animals (n = 8–10), only the highest dose of U-50,488H (20 mg/kg) induced a slight decrease in the locomotor activity,

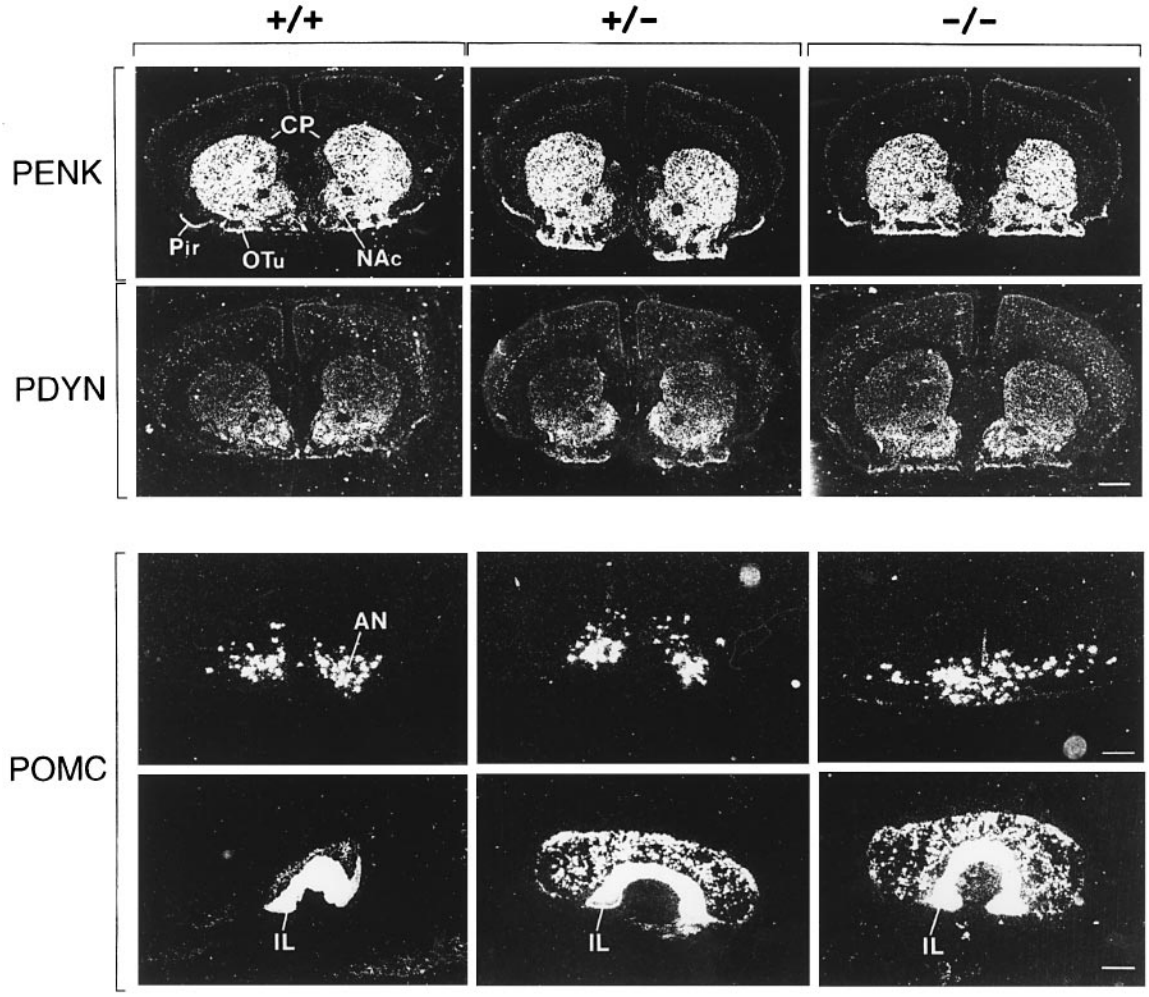

Fig. 3. Expression of endogenous opioid peptide genes in wild-type (+/+), heterozygote (+/–) and homozygote (–/–) brains. *In situ* hybridization analysis of PENK and PDYN transcripts on coronal brain sections at the level of the caudate putamen (scale bar = 900 μm) and of POMC transcripts at the levels of hypothalamus (upper POMC panel, scale bar = 250 μm) and pituitary gland (lower POMC panel, scale bar = 330 μm). All the sections are shown under dark-field illumination, with the signal grain appearing in white. Antisense probes are indicated on the left. PENK, proenkephalin; PDYN, prodynorphin; POMC, proopiomelanocortin; NAc, nucleus accumbens; CP, caudate putamen; OTu, olfactory tubercle; Pir, piriform cortex; AN, arcuate nucleus; IL, intermediate lobe of the pituitary gland.

Table II. Evaluation of the behavioural responses of the mice lacking κ-opioid receptors and their wild-type littermates

Behavioural test	Day 1		Day 2		Day 3	
	Wild-type	Mutant	Wild-type	Mutant	Wild-type	Mutant
Locomotor activity box						
Counts	146.2 ± 14.1	147.6 ± 16.5	125.8 ± 12.8	110.7 ± 11.6	99.2 ± 11.1*	96.5 ± 11.2*
Open field						
Squares crossed	259.4 ± 24.0	270.0 ± 20.0	228.0 ± 26.8	209.0 ± 28.0	203.0 ± 29.5	165.0 ± 28.0*
Rearing	23.8 ± 4.0	16.0 ± 5.0	18.4 ± 4.4	17.0 ± 3.9	19.7 ± 4.5	12.6 ± 2.8
Latency	18.3 ± 5.50	12.7 ± 2.83	1.80 ± 0.50**	2.57 ± 0.98**	2.70 ± 0.96**	2.50 ± 1.63**
Elevated O-maze						
Total No. of visits	17.8 ± 2.92	14.4 ± 2.86	9.62 ± 2.01*	9.75 ± 2.07	11.8 ± 2.32	7.37 ± 1.42*
Time in open arms (%)	10.2 ± 1.97	9.25 ± 2.18	5.23 ± 1.05	6.68 ± 1.96	5.25 ± 0.97	3.20 ± 0.734
Elevated plus-maze						
Total No. of visits	20.8 ± 2.45	18.2 ± 1.94	8.70 ± 1.42**	9.00 ± 1.13**	7.85 ± 1.50**	4.61 ± 0.76**
Time in open arms (%)	15.9 ± 2.90	14.7 ± 2.11	6.16 ± 1.60**	6.90 ± 1.34**	4.73 ± 1.52**	3.53 ± 1.52**
Visits to open arms (%)	38.5 ± 1.92	38.3 ± 1.90	32.0 ± 3.22	29.7 ± 4.07	23.5 ± 3.80**	19.3 ± 5.05**

*P <0.05, **P <0.01 as compared with the first day observation in the same group of animals (Dunnett's test).

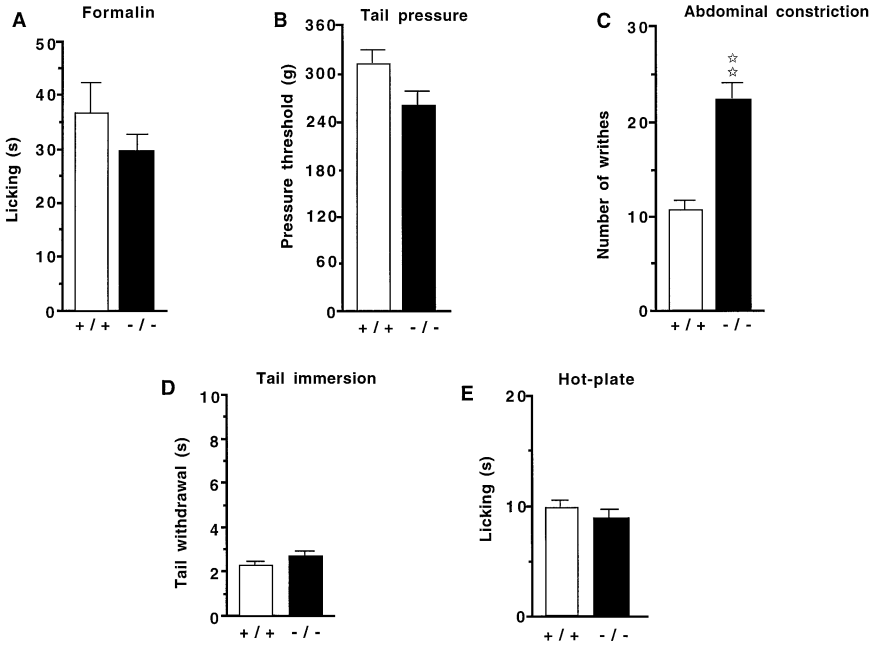

Fig. 4. Spontaneous nociceptive thresholds of KOR-deficient mice (–/–; filled columns) and their wild-type littermates (+/+; open columns). (**A**) Formalin test. Values on the *y*-axis represent time (seconds) spent in licking the right forepaw. (**B**) Tail pressure test (pressure threshold in grams). (**C**) Abdominal constriction test (number of writhes). (**D**) Tail immersion test (tail withdrawal latency in seconds). (**E**) Hot plate test (paw lick latency in seconds). The number of animals in each test was between 10 and 14. ☆☆$p < 0.01$; comparison between wild-type and mutant groups (two-tailed Student *t*-test).

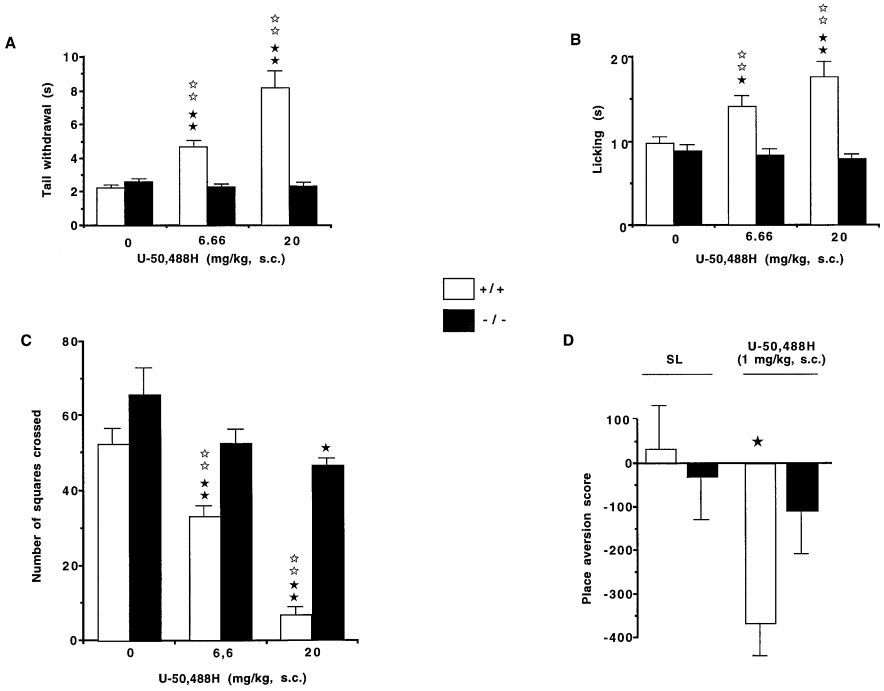

Fig. 5. Pharmacological responses induced by the administration of the selective κ-agonist U-50,488H in KOR-deficient mice (–/–; filled columns) and their wild-type littermates (+/+; open columns). (**A**) Tail immersion test. (**B**) Hot plate test. Values on the *y*-axis for nociceptive tests represent the latencies in seconds. (**C**) Spontaneous horizontal locomotor activity. Values on the *y*-axis represent the number of squares crossed during 5 min. (**D**) Conditioned place aversion. Values on the *y*-axis represent scores in seconds, as previously described (Valverde *et al.*, 1996). The number of animals was between eight and 10 in all the experimental groups. Data are expressed as mean ± SEM. ★$p < 0.05$; ★★$p < 0.01$ comparison between U-50,488H- and saline-treated animals (Dunnett's test). ☆$p < 0.05$; ☆☆$P < 0.01$ comparison between wild-type and mutant groups receiving the same treatment (Dunnett's test).

but this response was significantly lower than in wild-type mice (Figure 5C).

The activation of KORs has been reported to increase secretions (Ashton *et al.*, 1989) and we investigated the effects of the κ-agonist U-50,488H on salivation. U-50,488H at the dose of 6.6 mg/kg, s.c. produced salivation in nine out of 10 wild-type mice during a 5 min observation period, whereas this effect was seen in two out of 10

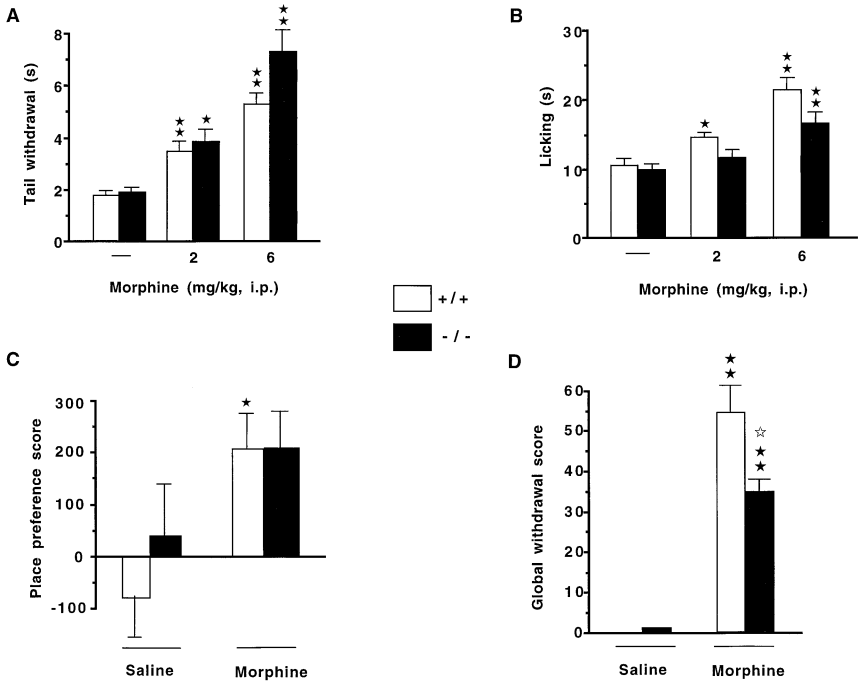

Fig. 6. Pharmacological responses induced by morphine administration in KOR-deficient mice (–/–; filled columns) and their wild-type littermates (+/+; open columns). (**A**) Tail immersion test. (**B**) Hot plate test. Values on the *y*-axis for nociceptive tests represent the latencies in seconds (n = 8–10). (**C**) Conditioned place preference paradigm. Values on the *y*-axis represent scores in seconds as previously described (Valverde *et al.*, 1996) (n = 10). (**D**) Naloxone-precipitated morphine withdrawal syndrome. Results are expressed as a global withdrawal score, as previously reported (Maldonado *et al.*, 1992) (n = 15–17). Data are expressed as mean ± SEM. ★p <0.05; ★★p <0.01 comparison between morphine- and saline-treated animals. ☆p <0.05; ☆☆P <0.01 comparison between wild-type and mutant groups receiving the same treatment (Dunnett's test).

mutant mice only (χ^2: 9.899, p <0.01). When U-50,488H was administered at the dose of 20 mg/kg, all the wild-type mice (n = 10) showed an increased salivation, but none of the mutant mice (n = 10) exhibited this response (χ^2: 20, p <0.001) (data not shown).

Previous studies have demonstrated that the stimulation of KORs produces dysphoric properties, clearly revealed by using the conditioned place aversion paradigm in mice (Mucha and Herz, 1985). In a pilot study, we evaluated the ability of U-50,488H to induce aversive effects in wild-type mice with the same genetic background in order to choose an effective dose to be tested in mutant mice. The κ-selective agonist, administered s.c., produced a significant place aversion (P <0.05; n = 8–10) at the two doses used, 1 mg/kg (score: –218.3 ± 40) and 3 mg/kg (score: –208.0 ± 45) (data not shown), as previously reported (Funada *et al.*, 1993). The dose of 1 mg/kg, s.c., was used in mutant animals in the conditioned place aversion paradigm. As shown in Figure 5D, this dose of U-50,488H induced a strong place aversion in wild-type mice, without exhibiting a significant effect in mutant animals (n = 10). Altogether, these results suggest that the *KOR* gene product plays a major role in all the pharmacological responses to U-50,488H that we have examined.

Morphine action in KOR-deficient mice

In order to assess the possible contribution of KORs to morphine action, we have evaluated the responses to morphine in *KOR* –/– mice and their wild-type littermates. The antinociceptive effects of morphine in the tail immersion and the hot plate tests are shown in Figure 6A and B (n = 8–10). Morphine, administered at doses of 2 and 6 mg/kg (i.p.), induced a strong and dose-dependent antinociceptive effect in both wild-type and mutant animals in the two tests. There were no significant differences between the two genotypes in any of the nociceptive thresholds evaluated, suggesting that morphine analgesia is not modified markedly in KOR-deficient mice.

The rewarding properties of morphine were investigated in wild-type and mutant mice using the place preference paradigm (Valverde *et al.*, 1996). In this experiment, we used a low dose of morphine (1 mg/kg, s.c.) to produce a submaximal rewarding response, in order to be able to evaluate any facilitation or inhibition that may result from the absence of KORs. Under these experimental conditions, morphine administration produced a similar conditioned place preference in both mice genotypes.

The behavioural expression of naloxone-precipitated withdrawal syndrome was investigated in morphine-dependent wild-type and mutant mice (n = 15–17). Several signs of withdrawal were slightly attenuated in mutant animals, such as body tremor (wild-type: 5.4 ± 0.2; mutant: 4.1 ± 0.4, P <0.01), ptosis (wild-type: 5.3 ± 0.2; mutant: 3.5 ± 0.3, P <0.01), jumping (wild-type: 18.8 ± 6.0; mutant: 5.5 ± 2.3, n.s.), sniffing (wild-type: 10.5 ± 2.1; mutant: 5.6 ± 1.3, n.s.) and diarrhoea (wild-type: 1.0 ± 0.2; mutant: 0.5 ± 0.2, n.s.), whereas other signs such as wet dog shakes (wild-type: 14.5 ± 3.5; mutant: 11.7 ± 2.2, n.s.), teeth chattering (wild-type: 5.6 ± 0.1; mutant: 4.9 ± 0.3, n.s.) and paw tremor (wild-type: 14.5 ± 3.5; mutant: 11.7 ± 2.2, n.s.) were not modified. A global withdrawal score was calculated for each animal by giving a proportional weighting to each individual sign and using a range of possible scores from 0 to 100, as previously reported (Maldonado *et al.*, 1992).

As shown in Figure 6D, the global severity of morphine withdrawal syndrome was significantly decreased in mutant mice ($P < 0.05$). Thus, the chronic administration of increasing morphine doses (see Materials and methods) followed by naloxone-precipitated withdrawal attenuates abstinence signs in KOR-deficient mice as compared with wild-type mice.

Discussion

In the last 15 years, KORs have attracted great attention (Millan, 1990) particularly because κ-agonists are potent analgesics which cause less respiratory depression, nausea and constipation, and have reduced abuse potential compared with morphine. KORs have been studied extensively using pharmacological approaches, but their precise role in several functions remain unclear. In the present study, we have used a genetic approach to generate knock-out mice for the *KOR* gene. KOR-deficient mice are fertile, and adult animals do not present any obvious anatomical abnormalities. The absence of KORs has little influence on mouse spontaneous behaviour under non-stressful (locomotor activity boxes) or stressful (open-field) conditions nor on circadian rhythm locomotor activity. Emotional responses are similar in mutant and wild-type mice when evaluated in two animal models of anxiety (the elevated plus-maze and the elevated O-maze). Mutant mice also show a similar alternation to wild-type animals in the Y maze, a paradigm which is sensitive to changes in arousal and memory function (Anisman and Kokkinidis, 1975). Altogether, these data suggest that the activity of the *KOR* gene is not essential to maintain normal spontaneous responses under home cage conditions or novel environmental situations. This is in accordance with pharmacological studies indicating that the acute administration of a κ-antagonist has no apparent effect on overt behaviour (Tortella *et al.*, 1989). Alternatively, we cannot exclude that the absence of a *KOR*-encoded receptor has been compensated for by other mechanisms.

Knock-out mice for several genes of the opioid system have now been constructed. Homozygous mutant mice which lack β-endorphin (Rubinstein *et al.*, 1996), pre-proenkephalin (König *et al.*, 1996), the MOR (Matthes *et al.*, 1996; Sora *et al.*, 1997; Tian *et al.*, 1997), the DOR (Zhu *et al.*, 1997) or the KOR (this study) reach adult age without apparent anatomical deficits. This suggests that the absence of a single component of the opioid system does not markedly alter development, although all these genes are known to be transcribed during ontogenesis (Pintar and Scott, 1993). In adult animals, changes in locomotor activity were described for MOR- (Matthes *et al.*, 1996; Tian *et al.*, 1997) and pre-proenkephalin-deficient mice (König *et al.*, 1996), but this was not observed in KOR-deficient mice. Also, a modification in emotional-related responses was revealed in pre-proenkephalin knock-out mice using two animal models of anxiety (the open-field and O-maze tests, see König *et al.*, 1996), while no change was found in mice lacking the KOR. Together, the data suggest a minimal implication of the *KOR* gene in spontaneous behaviour as compared with other components of the opioid system. In the future, it would be most interesting to know whether the apparent low endogenous κ-tone can be confirmed using mice lacking the prodynorphin gene, which was suggested to encode the preferred endogenous ligand for the KOR (Goldstein and Naidu, 1989). Also the observation that spontaneous behaviour of mice lacking the KOR appears normal under basal conditions does not exclude a role for the *KOR*-encoded receptor in marked stressful situations. Future studies involving various stressors or chronic pain may reveal altered behavioural responses in *KOR* –/– mice.

We have analyzed the effects of the *KOR* null mutation on pain perception using different types of acute noxious stimuli. The results indicate no change in the nociceptive threshold after the application of thermal stimuli (tail immersion and hot plate tests), suggesting that the *KOR*-encoded protein does not participate to the perception of thermal pain. This result differs from data obtained with mice lacking the MOR (Sora *et al.*, 1997) or pre-proenkephalin (König *et al.*, 1996), and underscores a differential implication of *MOR*- and *KOR*-encoded receptors in pain control. The response of KOR-deficient mice was also found to be unchanged after the application of a mechanical (tail pressure test) or chemical stimulus producing local tissue damage (formalin test), suggesting the absence of a tonic implication of the *KOR*-encoded receptor in these responses. Interestingly, when acetic acid was injected to produce peritoneal irritation (writhing test), mutant mice showed a dramatic decrease in their nociceptive threshold, as revealed by a 2-fold elevated number of writhes. In line with previous pharmacological studies (Tyers, 1980; Schmauss *et al.*, 1983; Wards and Takemori, 1983), this finding provides the first genetic evidence for an implication of the *KOR*-encoded receptor in the control of chemical visceral pain. This result differs from data obtained with MOR knock-out mice, who did not exhibit an increased number of writhes using the same test (unpublished results). Again, this supports the notion that KOR plays a role different from MOR in the processing of nociceptive information.

The arylacetamide compound U-50,488H has been widely used as a classical κ-agonist. It was therefore important to correlate the pharmacological actions of this compound to the *KOR*-encoded receptor, by measuring U-50,488H responses *in vivo* in KOR-deficient mice. The analgesic response induced by U-50,488H, as measured by the hot plate and tail flick tests, is abolished in animals lacking KORs. This indicates that the *KOR* gene product represents a major target for the action of U-50,488H on nociception. Of note is the observation that U-50,488H seems to induce weak responses in mutant mice when considering behavioural responses. First, a slight reduction of locomotor activity in mutant mice was observed when U-50,488H was administered at the highest dose. Second, U-50,488H failed to produce a significant place aversion in mutant mice, but a trend to avoid the drug-associated compartment seemed to remain in this group. One possible explanation for the residual locomotor and aversive responses in mutant mice could be a cross-reactivity of U-50,488H with other opioid receptors. However, this hypothesis seems unlikely since the activation of MORs and DORs produces opposite effects, i.e. an increase in locomotor activity (Locke and Holtzmann, 1986; Murray and Cowan, 1990; Di Chiara and North, 1992) and place preference (Shippenberg *et al.*, 1987; Matthes *et al.*, 1996). Besides, the doses of U-50,488H used, mainly in the place

aversion paradigm (1 mg/kg), have been reported to be highly selective for KORs (Vonvoigtlander *et al.*, 1983). A more likely hypothesis is the existence of other receptor sites that would respond to U-50,488H. These sites may be other κ-receptor subtypes, as previously suggested (see Traynor, 1989; see Pasternak, 1993; see Mansour and Watson, 1993; see also discussion below), or alternatively non-opioid receptors. We conclude that U-50,488H mainly activates the *KOR*-encoded receptor, as revealed by the suppression of U-50,488H antinociception and most of the behavioural responses in mutant animals. In addition, U-50,488H may stimulate other receptor sites that would account for the observed residual behavioural responses. These sites would not be involved in the analgesic responses produced by U-50,488H and remain to be characterized.

We have demonstrated previously that the main biological actions of morphine are abolished in mutant mice lacking the MOR, including analgesia, reward and physical dependence (Matthes *et al.*, 1996). This demonstrates that the *MOR* gene product is necessary for these effects of morphine, but does not exclude a possible role for other opioid receptors in modulating morphine effects. There is evidence that morphine may act at non-μ receptors when administered at high doses (Takemori and Porthoghese, 1987). Indirect evidence has suggested the possibility of functional interactions between opioid receptors (reviewed in Rothman *et al.*, 1993), and KORs have been reported to modulate some responses produced by the activation of other opioid receptors (Funada *et al.*, 1993; Tao *et al.*, 1994). In order to investigate this hypothesis further, we have evaluated several pharmacological responses induced by morphine administration in *KOR* –/– mice. Our results show that the analgesic responses produced by morphine are not significantly modified in mutant mice, suggesting that KORs do not play an important role in morphine-induced antinociception, at least after the application of thermal stimuli. We anticipated that the absence of KORs may allow a potentiation of morphine reward in *KOR* –/– mice, because of the reported opposing actions of μ- and κ-agonists in modulating the endogenous tone of mesolimbic dopaminergic neurons (Shippenberg *et al.*, 1987) and the ability of κ-agonists to block morphine reward (Funada *et al.*, 1993). Our data, however, indicate that morphine-conditioned place preference is not altered in KOR-deficient mice, suggesting that the absence of κ-sites has little influence on the neuronal mechanisms involved in the rewarding properties of morphine. In contrast, morphine physical dependence was markedly modified in KOR-deficient mice, as revealed by a reduced behavioural expression of naloxone-precipitated withdrawal. Therefore, the *KOR* gene product, although not essential, also participates in the expression of morphine abstinence. Receptors arising from the *KOR* gene may functionally modulate this MOR-mediated response similarly to that of other neurotransmitter systems, including noradrenergic, serotoninergic, glutamatergic or GABA-ergic systems (see Bhargava, 1994).

Pharmacological studies performed in several laboratories have suggested the existence of multiple KORs (see Traynor, 1989; Mansour and Watson, 1993; Pasternak, 1993). The best characterized subtype is referred to as a κ_1-receptor due to its capacity to bind arylacetamide compounds like U-50,488H, U-69,593, PD117302 and CI-977 (see Dhawan *et al.*, 1996). The KOR which stems from the *KOR* gene has been shown to bind U-50,488H and U-69,593 with nanomolar affinity and has therefore been proposed to be a κ_1-receptor subtype (see Kieffer, 1995). Other putative KOR sites have been suggested based on the observation that the benzomorphan opioid ligand, bremazocine, labels a greater number of κ-sites than arylacetamide compounds under conditions where μ- and δ-binding are suppressed (Robson *et al.*, 1985; Smith *et al.*, 1989; Unterwald *et al.*, 1991; Kitchen *et al.*, 1997). These sites, however, have been difficult to characterize due to the lack of selective ligands, and the existence of distinct KOR proteins remains a matter of controversy (see Traynor, 1989; Richardson *et al.*, 1992). Our analysis of KOR-deficient mice clearly indicates that inactivation of the *KOR* gene leads to the suppression of κ_1 sites, as shown by the lack of [^{3}H]CI-977 binding on brain and the strong reduction or abolition of *in vivo* responses to U-50,488H administration. Detailed radioligand-binding studies using less selective κ-compounds combined with μ- and δ-suppressing ligands are currently underway in KOR-deficient mice to determine if there are κ-subtypes derived from another gene. In the future, the concomitant disruption of *KOR*, *MOR* and *DOR* genes should resolve definitively the issue of the existence of as yet uncloned opioid receptor genes.

Materials and methods

Production of KOR–/– mice

Mouse *KOR* genomic clones were isolated from a λFIX II strain SVJ129 library (Stratagene) by hybridization with a PCR product of 230 bp encoding the N-terminal part (amino acids 6–77) of mKOR and the position of the first coding exon was mapped by standard procedures. A 6.8 kb *Bam*HI fragment containing the first exon was cloned into pBluescript SK (Stratagene) in which the *Sma*I site was killed. Two *Sma*I sites were then created by site-directed mutagenesis, allowing deletion of a 234 bp fragment containing the start codon. This fragment was replaced by a neo cassette of 1.9 kb which contains a neo^r gene with a PGK promoter and a PGK polyadenylation signal at its 5′ and 3′ ends, respectively. The resulting targeting vector contained 1.3 kb of upstream sequences as the 5′ arm and 5.2 kb of intron sequences as the 3′ arm. The targeting vector was linearized and electroporated in P1 ES cells (Lufkin *et al.*, 1991). Neomycin-resistant clones were screened by Southern blot analysis using random primed ^{32}P-labelled 5′ and 3′ external probes (see Figure 1), and a Neo probe. The 3′ probe is a 700 bp *Bam*HI–*Sac*I fragment and the 5′ probe is a 600 bp *Sac*I–*Bam*H1 fragment. The *Sac*I site used to generate the 5′ probe is absent in the genomic sequence but originates from the library cloning vector. A positive clone was microinjected into C57BL/6 blastocysts (Lufkin *et al.*, 1991) and gave rise to chimeric offspring which in turn were mated with C57BL/6 mice. Agouti-coat pups were genotyped by Southern blot analysis of tail DNA for germline transmission.

Binding on brain homogenates

Binding was performed as described (Kieffer *et al.*, 1992) using 100 μg of total brain membrane proteins (Ilien *et al.*, 1988) incubated in 50 mM Tris–HCl pH 7.4, 1 mM EDTA at 25°C for 1 h with [^{3}H]D-Ala^2-MePhe^4-Gly-ol^5 enkephalin (DAMGO, Amersham, 54 Ci/mmol), [^{3}H]D-Ala^2 deltorphin I (DELT I, Zeneca custom synthesis, 47 Ci/mmol) or [^{3}H]-[5R-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro [4.5]dec-8-yl] benzo [*b*] furan-4-acetamide (CI-977, Amersham, 47 Ci/mmol) at concentrations of 0.05–6.4, 0.05–6.4 and 0.01–1.28 nM, respectively. Naloxone (Sigma) was used at a 2 μM concentration to determine non-specific binding. Experiments were performed in triplicate using at least two distinct membrane preparations, each made from three brains. Binding data were analysed using the EBDA-LIGAND program (Biosoft).

Autoradiographic procedures

Mice were killed by decapitation, and intact brains were removed and immediately frozen in isopentane at –20°C. Brains were stored at –70°C for a maximum period of 1 month until sectioning. For autoradiographic mapping, 20 μm frozen coronal sections were cut (300 μm apart) throughout fore- and midbrain structures in a cryostat (Zeiss Microm 505E), thaw-mounted onto pre-cleaned and gelatin-subbed ice-cold microscope slides and processed for autoradiography as described previously (Kitchen *et al.*, 1995). In addition, saggital sections were cut to assess levels of binding from fore- to hindbrain regions. Adjacent sections were cut for determination of total binding for μ-, δ-, κ-binding with [^{3}H]DAMGO, [^{3}H]DELT I and [^{3}H]CI-977. An additional set of sections were cut adjacent to these for determination of non-specific binding, which was determined for all ligands with naloxone (1 μM for DAMGO and CI-977, and 10 μM for DELT I). For autoradiographic visualization of binding sites, incubations were carried out as described previously (Kitchen *et al.*, 1997), with the exception that film exposure time for [^{3}H]CI-977 was 6 weeks.

In situ hybridization

Cryostat sections (10 μm) were prepared from frozen brains and hybridized with ^{35}S-labelled antisense riboprobes as described (Décimo *et al.*, 1995). The PENK probe was synthesized from a 800 bp *Pvu*II–*Xba*I cDNA fragment cloned into pBluescript, the PDYN probe from a 1700 bp *Pst*I–*Eco*RI fragment subcloned into pSP64 and the POMC probe from a 400 bp *Not*I–*Nco*I fragment cloned into pBluescript. Brains from the three genotypes were treated and hybridized in the same experimental series, with the same riboprobes, and exposed under Kodak NTB-2 emulsion for 4 days (POMC, hypophysis), 1 week (PENK) and 2 weeks (PDYN and POMC, arcuate nucleus region). No signal was obtained using control antisense probes.

Behavioural experiments

Animals were housed in a temperature-controlled environment (21 ± 1°C) with free access to food and water. Mice were allowed to become acclimatized to the experimental room and were handled during 1 week before starting the experimental sequence. All the experiments were performed in a soundproof room by an observer who did not know the genetic constitution of each animal nor their treatment. Behavioural tests and care of the animals were in accordance with standard ethical guidelines (NIH, 1995), and were approved by the local ethical committee. Mice were divided into two different groups. Each group contained 30 wild-type and 30 mutant mice. The first group of animals was used to evaluate the pharmacological effects of U-50,488H. Before receiving any treatment, several spontaneous behavioural responses were evaluated in these mice (locomotor response, $n = 15$, and elevated plus maze, $n = 15$). The spontaneous nociceptive threshold was then evaluated after inflammatory ($n = 10$), mechanical ($n = 10$) and chemical stimuli ($n = 10$). After 1 week, a single dose of U-50,488H was administered to each animal and its effects on locomotor activity, salivation and antinociception (thermal stimuli) were evaluated. One week later, the place conditioning paradigm (pre-conditioning phase) was started in order to evaluate the conditioned place aversion produced by U-50,488H. In a second group of mice, the pharmacological responses produced by morphine were investigated. First, several spontaneous behavioural responses were evaluated in these mice (elevated O-maze, $n = 15$, and spontaneous alternation, $n = 15$). The antinociceptive responses were then evaluated after an acute administration of morphine. One week later, place conditioning experiments were started in this group. One week after the testing phase of such behavioural paradigms, the first injection corresponding to the chronic morphine treatment schedule was administered. The reason for this specific order was (i) to evaluate spontaneous behaviour in animals free of any pharmacological treatment, (ii) to administer one single compound to each animal, (iii) to give the acute administration before the repeated treatments and (iv) to induce dependence at the end of the experimental sequence taking into account the high doses required. Animals were always counterbalanced after each individual experiment.

Locomotor activity boxes. Mice were placed individually in locomotor activity boxes consisting of a plastic rectangular area (255 cm×205 cm) with two crossed photocells, isolated in soundproof furniture and in almost complete darkness (<5 lux). The number of activity counts was evaluated at 14.00 h, for a period of 10 min, for three consecutive days (1st, 2nd and 3rd days). Locomotor activity was recorded at 14.00 h on day 4, and at 2.00 h on day 5, in order to evaluate the circadian changes.

Open-field test. The open-field was a rectangular area (70 cm wide, 90 cm long and 60 cm high) brightly illuminated from the top (500 lux). A total of 63 squares (10 cm×10 cm) were drawn with black lines on the white floor of the field. Six events were recorded during an observation period of 5 min: the number of squares crossed, the number of rears, the latency time to move out and to cross two squares, the number of grooming bouts, the number of defecation boli left in the field, and the number of urination events. Mice were exposed to the test at 14.00 h for three consecutive days.

Elevated plus-maze. The elevated plus-maze consisted of a wooden apparatus with two open arms (16 cm long×5 cm wide) and two closed arms (16 cm long×5 cm wide) with open tops arranged such that the two open arms were opposite each other. The maze was elevated 30 cm above the ground and illuminated from the top (100 lux). At the beginning of the experiments, mice were placed in the middle of the maze facing one of the open arms. The total number of visits to the open arms, the total number of visits to the closed arms, the cumulative time spent in the open arms and the cumulative time spent in the closed arms were then measured for 5 min. An arm visit was recorded when a mouse moved all four paws into the arm. The position of the mouse was recorded by a videocamera, and the time spent in each arm was analysed by a program provided with the Videotrack II, 2.12 version computer (Viewpoint, France). Mice were exposed to the test at 14.00 h for three consecutive days.

Elevated O-maze. The O-maze (diameter, 46 cm; runway width, 5.5 cm) consisted of a Plexiglas apparatus with two open and two wall-enclosed sectors of equal size. The maze was elevated 50 cm above the ground and illuminated from the top (100 lux). Animals were placed on one open sector in front of an enclosed sector. The position of the mouse was recorded by a videocamera, and the time spent in each arm was measured for 5 min by a program provided with the Videotrack II, 2.12 version computer. Time in a new sector was measured as soon as the animal entered with all four paws. General activity was assessed by counting all entries with at least two paws into a new sector. Mice were exposed to the test at 14.00 h for three consecutive days.

Spontaneous alternation. The apparatus consisted of a symmetrical wooden Y-maze with arms 25.0 cm long, 8.0 cm wide and 15.0 cm high. The apparatus was illuminated from the top (100 lux), and the floor was covered with a small amount of sawdust. The proportion of alternation and the global activity were calculated as previously reported (Anisman and Kokkinidis, 1975). Mice were tested only once in this paradigm at 14.00h.

Tail immersion test. The antinociceptive responses were determined using water at 50 ± 0.5°C as the nociceptive stimulus. The mice were maintained in a cylinder and their tails were immersed in the heated water. The latency to a rapid flick of the tail was taken as the endpoint, and the maximum latency allowed was 10 s.

Hot plate test. The test was based on that described by Eddy and Leimbach (1953). A glass cylinder (16 cm high, 16 cm diameter) was used to keep the mice on the heated surface of the plate, which was kept at a temperature of 50 ± 0.5°C using a thermo-regulated water circulating pump. Two nociceptive thresholds were evaluated: licking of the paws and jumping. The cut-off was 30 s and 240 s respectively for licking and jumping responses.

Tail pressure test. The local pressure required to elicit tail withdrawal was determined. Nociceptive thresholds were determined by a modification of the method reported by Randall and Selitto (1957). Increasing pressure was applied locally to the tail by using the Basile analgesia meter (tip diameter: 1 mm) (Apelex, France) until a tail withdrawal response was elicited. The cut-off was established at 600 g. Three consecutive determinations, separated by 15 s, were made, and the average of the three responses was determined.

Writhing test. Mice received 0.1 ml/10 g of body weight of a solution of 0.6% acetic acid by the i.p. route. The injection produced the typical reaction, which is characterized by contractions of the abdominal musculature followed by extension of the hind limbs (Koster *et al.*, 1959). The mice were placed in individual transparent containers and the number of writhes per animal during the 10 min period between 5 and 15 min after acetic acid injection was counted.

Formalin test. The test, adapted from Hunskaar *et al.* (1985), was carried out in a glass cylinder chamber (16 cm high×16 cm diameter). The

mice were placed in the test chambers for 30 min. After this adaptation period, 20 µl of 5% formalin was injected s.c. into the dorsal surface of the right hindpaw of the mouse, using a 26-gauge needle connected to a microsyringe. Each mouse was returned immediately to the observation chamber after injection, and its nociceptive response was recorded immediately after formalin injection over a 5 min period. Only licking or biting of the injected hindpaw was defined as a nociceptive response, and its total duration was registered by means of a stopwatch.

Measurement of locomotor activity after drug administration. Mice were placed in a transparent rectangular cage (30 cm×26 cm×30 cm), 5 min after drug injection. The apparatus was located in a soundproof room, and the illumination at the floor level was 100 lux. Mice displacements were measured by drawing 12 squares on the floor, and counting the number of crosses. This direct observation allows assessment of abnormal mouse behaviour.

Place conditioning paradigm. Tests were performed as previously reported (Valverde *et al.*, 1996), except for the conditioning time (18 min) and the size of compartments (15 cm×15 cm×15 cm). Data are expressed in scores calculated as the difference between the time spent in the drug-associated compartment during the testing and the pre-conditioning phases.

Morphine withdrawal syndrome. Opiate dependence was induced by repeated i.p. injection of morphine-HCl, at an interval of 12 h, over 6 days. The morphine-HCl dose was increased progressively as follows: 1st day, 20 mg/kg; 2nd day, 40 mg/kg; 3rd day, 60 mg/kg; 4th day, 80 mg/kg; 5th day, 100 mg/kg; 6th day (only one injection in the morning), 100 mg/kg. Control mice were treated with saline under the same conditions. Withdrawal was precipitated only once in each animal by injecting naloxone-HCl (1 mg/kg, s.c.) 2 h after the last morphine administration and was evaluated as previously reported (Maldonado *et al.*, 1996). Taking into account all the individual signs, a global withdrawal score was calculated for each animal by using a range of possible scores from 0 to 100, as previously reported (Maldonado *et al.*, 1992).

Statistical analysis

Individual group comparisons were made using a two-way ANOVA between-subjects analysis (factors of variation: mutation and shocks). Following a significant main effect by one-way ANOVA, individual dose effects were analysed using Dunnett's test comparisons. The Mann–Whitney U test was used for the statistical analysis of the litter sizes, the χ^2 test for the salivation and the unpaired two-tailed Student's *t*-test for the spontaneous nociceptive thresholds. The level of significance was $p < 0.05$.

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