Activity of the δ-Opioid Receptor Is Partially Reduced, Whereas Activity of the κ-Receptor Is Maintained in Mice Lacking the μ-Receptor

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Previous pharmacological studies have indicated the possible existence of functional interactions between μ-, δ- and κ-opioid receptors in the CNS. We have investigated this issue using a genetic approach. Here we describe *in vitro* and *in vivo* functional activity of δ- and κ-opioid receptors in mice lacking the μ-opioid receptor (MOR). Measurements of agonist-induced [^{35}S]GTPγS binding and adenylyl cyclase inhibition showed that functional coupling of δ- and κ-receptors to G-proteins is preserved in the brain of mutant mice. In the mouse vas deferens bioassay, deltorphin II and cyclic[D-penicillamine^2, D-penicillamine^5] enkephalin exhibited similar potency to inhibit smooth muscle contraction in both wild-type and MOR −/− mice. δ-Analgesia induced by deltorphin II was slightly diminished in mutant mice, when the tail flick test was used. Deltorphin II strongly reduced the respiratory frequency in wild-type mice but not in MOR −/− mice. Analgesic and respiratory responses produced by the selective κ-agonist U-50,488H were unchanged in MOR-deficient mice. In conclusion, the preservation of δ- and κ-receptor signaling properties in mice lacking μ-receptors provides no evidence for opioid receptor cross-talk at the cellular level. Intact antinociceptive and respiratory responses to the κ-agonist further suggest that the κ-receptor mainly acts independently from the μ-receptor *in vivo*. Reduced δ-analgesia and the absence of δ-respiratory depression in MOR-deficient mice together indicate that functional interactions may take place between μ-receptors and central δ-receptors in specific neuronal pathways.

Key words: μ-opioid receptor knock-out; μ-δ-κ-opioid receptor interactions; G-protein coupling; δ-κ analgesia; respiration; vas deferens

Opiates and endogenous opioid peptides act through multiple receptors, classified as μ-, δ- and κ-opioid receptors. Each receptor class displays a unique tissue distribution pattern (Mansour and Watson, 1993) and a distinct pharmacological profile (Goldstein and Naidu, 1989). The three opioid receptors participate in mediating the biological actions of opioids, with a distinct contribution of each receptor type (Millan, 1990). All three receptors mediate opioid-induced analgesia, with μ-receptors essentially responsible for supraspinal analgesia, whereas μ-, δ- and κ-receptors participate in the control of pain at the spinal level (Dickenson, 1991). The three receptors also mediate the mood-altering properties of opioid compounds, and it has been shown that μ- and δ-ligands act as positive reinforcers, whereas κ-agonists exert an opposing action and have strong dysphoric properties (Di Chiara and North, 1992). Repeated exposure to exogenous opiates results in profound adaptive changes (Nestler and Aghajanian, 1997) mainly mediated by μ-receptors, but there is also evidence for an involvement of κ- and δ-receptors (Cowan et al., 1988; Maldonado et al., 1992). Altogether the multiplicity of opioid receptors provides a basis for explaining the complex pharmacology of opioids.

Another level of complexity stems from the postulated existence of interactions between opioid receptors (Rothman et al., 1993; Traynor and Elliott, 1993). *In vivo*, the use of combinations of selective agonists or antagonists reveals responses that differ from those observed from a single compound. The cross-talk between μ- and δ-receptors is best documented, mainly from the observation that subeffective doses of δ-agonists modulate μ-mediated analgesia (Vaught et al., 1982). Computer analysis of binding data has suggested noncompetitive inhibition modes for μ- and δ-ligands at δ- and μ-receptor sites in brain, respectively, leading to the hypothesis of allosteric coupling between μ- and δ-receptors (Rothman et al., 1993). Recently, immunohistochemical studies have demonstrated colocalization of opioid receptors

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in some neurons (Ji et al., 1995), and biochemical cross-linking studies have suggested the possible existence of a receptor complex (Schoffelmeer et al., 1990; Cvejic and Devi, 1997), opening the possibility for physical interactions between the receptors. Therefore, there is indirect evidence that opioid receptors do not necessarily act independently from each other. The putative coordinated action of the receptors, which may have therapeutic implications, needs to be clarified.

We have recently disrupted the μ-opioid receptor (MOR) gene in mice by homologous recombination (Matthes et al., 1996). Although we cannot exclude the possibility that compensatory changes have occurred during knock-out mice development, we have shown that the expression levels and distribution of remaining components of the opioid system have not been markedly modified (Kitchen et al., 1997). Thus δ- and κ-receptor sites appear unchanged in mice lacking μ-receptors. Therefore these mutant mice provide a unique tool to determine whether opioid receptors interact functionally. If this is the case, it is expected that functional responses to δ- and κ-agonists will be altered in mice lacking μ-receptors. We have analyzed *in vitro* and *in vivo* functional properties of δ- and κ-receptors in mutant mice and here we show that functional activity of κ-receptors is maintained while some central δ-receptor-mediated responses are impaired in the absence of μ-receptors.

MATERIALS AND METHODS

Animal care. Animals were bred under standard animal housing conditions in a 12 hr dark-light cycle and had free access to food and water. All animals were 1:1 hybrids from 129/SV and C57Bl/6 mouse strains (Matthes et al., 1996) and were first or second generation descendants of heterozygous (MOR ±) founders. The animals were 8 to 16 weeks old and we matched groups of similar age. Also an equal number of males and females were used in each experimental group.

Chemicals and drugs. [^{35}S]GTPγS (46.1–51.5 TBq/mmol) was from New England Nuclear. GDP, [^{3}H]-D-Ala^2 MePhe^{4}Gly-ol^5 enkephalin (DAMGO), pCl-cyclic[D-penicillamine^2, D-penicillamine^5] enkephalin (pCl-DPDPE), and U-50,488H were from Sigma (St. Louis, MO); DPDPE, deltorphin II was from Sigma or Neosystems, Strasbourg, and [5A-(5a,7a,8b)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro [4.5]dec-8-yl] benzo [b] furan-4-acetamide (CI-977) was a gift from John Hughes (Parke-Davis Neuroscience Research Center, Cambridge, UK). [^{3}H] DAMGO and [^{3}H] CI-977 were purchased from Amersham, and [^{3}H] naltrindole was from Tocris. Morphine was from Francopia, France, and norbinaltorphimine (norBNI) was from Research Biochemical International, France.

Ligand binding. Membranes were prepared from wild-type 129/SV x C57Bl/6 hybrid mice, as described previously (Matthes et al., 1996). μ-, δ-, and κ-receptor sites were labeled using [^{3}H] DAMGO (2.5 nM), [^{3}H] naltrindole (0.08 nM), and [^{3}H] CI-977 (0.3 nM), respectively, and competition experiments were performed using 40–100 μg of membrane protein per assay in the presence of variable concentrations of opioid ligand (Table 1). K_i values were calculated using the EBDA/Ligand program (G. A. McPherson, Biosoft, UK), and results are shown as mean values ± SEM from at least two experiments performed in duplicate.

Agonist-stimulated [^{35}S]GTPγS binding autoradiography. Wild-type or MOR knock-out mice were decapitated. The brains and spinal cords were removed, and brains were immediately immersed in isopentane at −25°C. Spinal cords were immersed in molds containing OCT and frozen in dry ice powder. Coronal sections (20 μm) were cut on a cryostat at −20°C, thaw-mounted onto gelatin-coated slides, dried under vacuum, and stored desiccated at −80°C (Sim et al., 1995). Slides were then incubated in assay buffer (50 mM Tris-HCL, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.5) at 25°C for 10 min as described (Sim et al., 1995, 1996) with GDP (2 mM) in assay buffer for 15 min (25°C) and in the presence of GDP (2 mM) and [^{35}S]GTPγS and either DAMGO (μ, 3 μM), pCl-DPDPE (δ, 3 μM), DPDPE (δ, 3 μM), deltorphin II (δ, 3 μM), or CI-977 (κ, 1 μM) for 2 hr at 25°C. Sections were then rinsed twice in ice-cold 50 mM Tris-HCl pH 7.0 and rinsed briefly with ice-cold deionized water. Slides were dried in a desiccator for 15 min and exposed to a Kodak BioMax MR film overnight or for higher resolution to a DuPont Reflection film for 2–3 d. Each section was recorded using a dark-field microscope at 20× magnification. A Sun SPARC 10 work station and an Image technology digitizer were used to acquire and analyze the sections using a specifically designed imaging program (J.-L. Vonesch, unpublished observations). This system allows for the identification of 256 different intensity levels. All autoradiograms shown represent typical sections, which were performed at least three times.

Table 1. Affinity values of μ-, δ-, and κ-opioid agonists in 129/SV x C57B1/6 mice

	μ	δ	κ
DAMGO	1.34[a]	540	2180
Morphine	3.15	221	121
Deltorphin II	1488	0.36[b]	>100000
DPDPE	742	1.8	>100000
CI-977	165	972	0.21[a]
U-50,488H	837	14500	2.0

Opioid receptor sites were labeled using [^{3}H] DAMGO (μ), [^{3}H] naltrindole (δ), and [^{3}H] CI-977 (κ), and K_i values were determined by competition experiments. Affinity values (nM) are means from at least two separate experiments.

[a]From Matthes et al. (1996).

[b]Determined by Scatchard analysis of [^{3}H] deltorphin II binding.

Agonist-stimulated GTP[γ-^{35}S] binding in membranes. Wild-type or MOR knock-out mice were decapitated. The brains and spinal cords were removed and membranes were isolated as described (Sim et al., 1995). Protein levels were determined with a Bio-Rad assay system. Membranes (10 μg of protein) were incubated as described (Sim et al., 1995) for 60 min at 30°C in the presence of 10 μM GDP, 0.05 nM GTP[γ-^{35}S], DAMGO (0.3 μM), pCl-DPDPE (0.3 μM), DPDPE (0.3 μM), deltorphin II (0.3 μM), or CI-977 (0.1 μM) in assay buffer (see above). The incubation was terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters, followed by three washes with ice-cold 50 mM Tris-HCl, pH 7.4. Bound radiography was determined by liquid scintillation spectrophotometry after extraction overnight in Ultima Gold MV scintillation fluid (Pachard). Data are reported as mean ± SEM values of at least three experiments of each membrane preparation that were performed in triplicate. For brain and spinal cord membrane, results are from preparations of two series of three animals of each genotype. Specific binding is defined as the difference between total [^{35}S]GTPγS binding and [^{35}S]GTPγS binding in the presence of excess cold GTPγS (10 μM). Basal activity represents the extent of specific [^{35}S]GTPγS binding in the absence of agonist and is expressed as 100%. Two-tailed nonpaired Student's *t* test was used to compare agonist-induced activation levels between genotypes. To compare agonist-induced activation levels with basal levels, the two-tailed paired *t* test was used.

Adenylyl cyclase activity. Brains from MOR +/+ and MOR −/− mice were homogenized in 20 mM Tris-HCl, pH 7.4, 2 mM EGTA, 1 mM $MgCl_2$, and 250 mM sucrose. Amounts of 15–30 μg protein in 10 μl volume were added to assay tubes containing 80 mM Tris-HCl, pH 7.4, 10 mM theophyline, 1 mM $MgSO_4$, 0.8 mM EGTA, 30 mM NaCl, 0.25 mM ATP, and 0.01 mM GTP with either the drug being tested or water. Triplicate samples for each treatment were incubated at 30°C for 5 min. Adenylyl cyclase activity was terminated by placing the tubes into boiling water for 2 min. The amount of cAMP formed was determined by a [^{3}H] cAMP protein binding assay (Brown et al., 1971). Briefly, [^{3}H] cAMP (final concentration 4 nM) in citrate-phosphate buffer, pH 5.0, was incubated with cAMP binding protein prepared from bovine adrenal glands (90 min at 4°C). Charcoal was added, the mixture was centrifuged (1000 × *g* for 15 min at 4°C), and the amount of bound [^{3}H] cAMP found in the supernatant was determined by liquid scintillation. Radioactivity was converted to picomoles of cAMP by comparison with a standard curve, and basal adenylyl cyclase activity refers to picomoles of cAMP generated in 5 min. Results are expressed as percentage basal activity. Comparisons between dose–response curves of different genotypes were analyzed using the two-way ANOVA. If a significant effect was observed, a one-way ANOVA was used, followed by a Scheffé F test, to determine the significance of each concentration. The level of significance was set at $p < 0.05$.

Mouse vas deferens bioassay. Preparations from vas deferens of MOR +/+ and MOR −/− mice were performed as described previously (Hughes et al., 1975). Vasa were mounted in an organ bath of 10 ml

capacity, in Krebs' solution at 37°C gassed with 95% 0_2 and 5% CO_2. No protease inhibitor was added. Longitudinal contractions were recorded isometrically by a strain gauge transducer (DY 1, Basile, Milan, Italy) and displayed on a recording microdynamometer (Unirecord, Basile). Intramural nerves were stimulated with trains of rectilinear pulses, and stimulation trains were given at intervals of 20 sec and consisted of six stimuli of 1 msec duration with intervals of 10 msec (Melchiorri et al., 1991). Various concentrations (0.5–50 nM) of δ-agonist (DPDPE and deltorphin II) were added to inhibit electric-stimulated contractions. Results are expressed as IC_{50} values obtained from dose–response curves, and mean values ± SEM from 12 independent experiments are shown. Two-tailed nonpaired Student's *t* test was used to compare the IC_{50} values from both genotypes and for each compound.

Analgesia. The tail-immersion and hot-plate tests were used to evaluate antinociceptive responses in this study. Pharmacological tests were in accordance with standard ethical guidelines (National Institutes of Health, 1985) and approved by the local ethical committee. The number of animals in each group was between 6 and 12. Morphine was administered intraperitoneally 15 min before testing at the dose of 6 mg/kg. U-50,488H was administered subcutaneously at doses of 3 mg/kg, 10 mg/kg, and 30 mg/kg, 20 min before the test. Deltorphin II [3 μg (3.81 nmol), 10 μg (12.7 nmol), and 30 μg (38.1 nmol)] and DPDPE [5 μg (7.71 nmol), 15 μg (23.1 nmol), and 45 μg (69.4 nmol)] were administered intracerebroventricularly 10 min before testing. The opioid antagonists naltrindole (2.5 mg/kg) and norBNI (5 mg/kg) were administered subcutaneously 20 and 60 min, respectively, before testing. The volume of administration in the case of peripheral routes was 1 ml/100 gm of body weight. All the compounds were dissolved in saline (0.9%) for *in vivo* experiments. Vehicle and δ-agonists were injected slowly (15 sec) free-hand into the left lateral ventricle of each mouse using a modified Hamilton microliter syringe in a volume of 5 μl per animal, according to the method of Haley and McCormick (1957).

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. The maximum latency allowed was 10 sec.

Hot-plate test. A glass cylinder (16 cm high, 16 cm diameter) was used to maintain the mice on the heated surface of the plate, which was kept at a temperature of 50 ± 0.5°C using a thermoregulated water circulating pump. Two nociceptive thresholds were evaluated: licking of the paws and jumping. The cut-off was 30 and 240 sec, respectively, for licking and jumping responses. The endpoint for the licking response was the first paw lick, whether it was lick of the front or rear paw. The three nociceptive thresholds were evaluated in the same mouse, as reported previously (Matthes et al., 1996).

Respiration. Respiratory activity was measured using a barometric method (Bartlett and Tenney, 1970). The plethysmograph chamber (140 × 75 × 80 mm) equipped with a temperature sensor (Physitemp, Bat10) was connected to a reference chamber of the same volume. The pressure difference between the two chambers was measured with a differential pressure transducer (Validyne, DP-103-12) connected to a carrier demodulator (Validyne, CD15). The spirogram was stored on a PC computer (CED interface and ACQUIS1 software). Calibrations were made during each recording session by injecting 0.1 ml of air in the chamber. Each animal was placed in the chamber, which was kept hermetically closed and maintained at 26–27°C. Carbon dioxide concentration in the chamber was always <1% at the end of the session. The chamber was flushed between sessions with fresh humidified air. At the end of the exploring period (habituation time, 10–20 min) when the mouse presented periods of immobility, control data were collected during 3 min recording sessions and analyzed. Then saline or the opioid agonist was administered, and respiratory activity was measured again (12 min after morphine or deltorphin II; 15 min after U-50,488H). A computer-assisted method (Biological, ACQUIS1), was used to measure the duration of inspiration (Ti) and expiration (Te), from which respiratory frequency is derived, and the tidal volume from which minute volume is derived. Saline injection (subcutaneous) alone did not significantly affect respiratory parameters.

Statistical analysis. Data obtained from dose–response curves in wild-type mice were analyzed using a one-way ANOVA between subjects. *Post hoc* comparisons were made using Dunnett's test after significant effect of treatment by one-way ANOVA. Data from individual experiments realized in mutant and wild-type mice were analyzed using a two-way ANOVA between subjects (Tables 2, 3). The factors of variation were mutation and treatment. Individual treatment effects in each group (mutant and wild type) were analyzed using one-way ANOVA between subjects. *Post hoc* comparisons were made using Newman–Keuls or Dunnett's test after significant main effect of treatment by one-way ANOVA. The level of significance was set at $p < 0.05$ in all cases.

RESULTS

Functional signaling of δ- and κ-receptors in MOR-deficient mice

A first step in our evaluation of the functional properties of δ- and κ-receptors was to verify the selectivity of opioid agonists in hybrid 129/SV x C57Bl/6 mice, which are used throughout the study. Binding affinities of μ-compounds (morphine, DAMGO), δ-compounds (deltorphin II, DPDPE), and κ-compounds (CI-977, U-50,488H) were determined using brain membranes of wild-type animals (Table 1). K_i values are in good agreement with previous values (Corbett et al., 1993; Raynor et al., 1993) and confirm the weak μ-selectivity of morphine, compared with that of all other compounds, and the high receptor-subtype selectivity of DPDPE, deltorphin II, U-50,488H, and CI-977.

We have shown previously that δ- and κ-receptor sites are present and that their distribution and expression levels are not markedly altered in the brain of mice lacking μ-receptors (Kitchen et al., 1997). Here we have determined whether the absence of μ-receptors modifies coupling of δ- and κ-receptors to G-proteins. We have used a [^{35}S]GTPγS binding assay (Sim et al., 1995) to evaluate receptor-mediated activation of Gα subunits under agonist stimulation. Receptor–G-protein complexes remain functional in tissue sections, as well as in membrane preparations. We have therefore used [^{35}S]GTPγS autoradiography on brain sections to visualize receptor-activated G-proteins throughout the brain and have conducted [^{35}S]GTPγS binding on membrane preparations to further quantify the levels of functional opioid receptors in wild-type and mutant mice.

Previous studies have established that the μ-opioid-specific agonist DAMGO stimulates [^{35}S]GTPγS binding in brain sections of rats, with a distribution that parallels that of DAMGO binding (Sim et al., 1996). Our results show a similar pattern of G-protein activation in wild-type mice (Fig. 1*A*), with prominent labeling in striatal and thalamic areas, detectable labeling in the cortex and hypothalamus, and strong labeling of the superficial layers of the spinal cord (data not shown). In μ-receptor knock-out mice, [^{35}S]GTPγS labeling intensities were similar in the absence or presence of DAMGO (Fig. 1*A*), demonstrating the absence of any functional response to DAMGO, concordant with the lack of μ-receptor binding sites in those mice (Matthes et al., 1996; Kitchen et al., 1997).

Using the selective δ-agonists DPDPE (δ1) and deltorphin II (δ2) and the selective κ-agonist CI-977, we obtained specific [^{35}S]GTPγS binding on brain sections (Fig. 1*B*). δ-agonist-evoked labeling was intense in striatum and cortex, with a distribution similar to that described in rat (Sim et al., 1996). For CI-977, labeling was intense in the claustrum and endopiriform nucleus. The binding patterns correlate well with the distribution of binding sites that we have obtained previously using the same ligands under a radiolabeled form in an autoradiographic mapping study (Matthes et al., 1996; Kitchen et al., 1997). The anatomical distribution of activated G-proteins was similar in wild-type and mutant mice for the three agonists under study. This indicates that both δ- and κ-receptors are capable of activating G-proteins in brains of mice lacking the μ-receptor. Similar results were obtained from spinal cord sections (data not shown).

Table 2. Two-way ANOVA of analgesic responses

	Genotype		Treatment		Interaction	
	F	P	F	P	F	P
δ-Agonists						
Analgesic response						
Tail immersion	(1,2) = 3.00	N.S.	(2,44) = 8.46	<0.001	(2,44) = 0.38	N.S.
Hot plate						
Licking	(1,2) = 0.86	N.S.	(2,44) = 21.50	<0.0001	(2,44) = 0.12	N.S.
Jumping	(1,2) = 4.85	<0.05	(2,44) = 24.58	<0.0001	(2,44) = 1.33	N.S.
Naltrindole antagonism						
Tail immersion	(1,3) = 1.34	N.S.	(3,49) = 15.15	<0.0001	(3,49) = 3.03	<0.05
Hot plate						
Licking	(1,3) = 0.93	N.S.	(3,49) = 16.32	<0.0001	(3,49) = 0.93	N.S.
Jumping	(1,3) = 2.36	N.S.	(3,49) = 22.85	<0.0001	(3,49) = 2.49	N.S.
κ-Agonist						
Analgesic response						
Tail immersion	(1,1) = 3.74	N.S.	(1,28) = 37.53	<0.0001	(1,28) = 0.15	N.S.
Hot plate						
Licking	(1,1) = 0.35	N.S.	(1,28) = 111.02	<0.0001	(1,28) = 0.24	N.S.
Jumping	(1,1) = 0.13	N.S.	(1,28) = 42.65	<0.0001	(1,28) = 1.02	N.S.
Norbinaltorphimine antagonism						
Tail immersion	(1,3) = 0.48	N.S.	(3,63) = 27.65	<0.0001	(3,63) = 2.75	<0.05
Hot plate						
Licking	(1,3) = 0.81	N.S.	(3,63) = 84.21	<0.0001	(3,63) = 0.44	N.S.
Jumping	(1,3) = 1.87	N.S.	(3,63) = 29.38	<0.0001	(3,63) = 0.85	N.S.

The factors of variation (between subjects) were genotype and treatment. N.S., Not significant.

Table 3. Two-way ANOVA of respiratory responses

	Genotype		Treatment		Interaction	
	F	P	F	P	F	P
Morphine						
Respiratory frequency	(1,1) = 0.97	N.S.	(1,36) = 3.72	N.S.	(1,36) = 4.21	<0.05
Inspiratory time	(1,1) = 0.50	N.S.	(1,36) = 17.7	<0.005	(1,36) = 7.44	<0.01
Minute volume	(1,1) = 0.85	N.S.	(1,36) = 0.44	N.S.	(1,36) = 1.17	N.S.
δ-Agonist (Deltorphin II)						
Respiratory frequency	(1,1) = 4.35	<0.05	(1,44) = 0.12	N.S.	(1,44) = 7.35	<0.01
Inspiratory time	(1,1) = 0.66	N.S.	(1,44) = 0.01	N.S.	(1,44) = 3.54	N.S.
Minute volume	(1,1) = 0.12	N.S.	(1,44) = 2.70	N.S.	(1,44) = 4.68	<0.05
κ-Agonist						
Respiratory frequency	(1,1) = 0.12	N.S.	(1,38) = 3.21	N.S.	(1,38) = 0.10	N.S.
Inspiratory time	(1,1) = 1.79	N.S.	(1,38) = 64.7	<0.0001	(1,38) = 0.01	N.S.
Minute volume	(1,1) = 1.81	N.S.	(1,38) = 0.46	N.S.	(1,38) = 0.51	N.S.

The factors of variation (between factors) were genotype and treatment. N.S., Not significant.

We pooled brains from animals of each genotype and prepared membranes to quantify [^{35}S]GTPγS labeling. We used minimal agonist concentrations, that is, concentrations that produce significant stimulation with a minimal risk of nonselective activation across receptor subtypes. DAMGO and DPDPE concentrations were based on concentration-effect curves obtained by Sim et al. (1996), and concentrations of deltorphin II and CI-977 were adapted according to K_i values and selectivities obtained from binding experiments (Table 1). Thus, 0.3 μM for μ- and δ-ligands and 0.1 μM for CI-977 were used, and results are presented in Figure 2.

A first observation was that [^{35}S]GTPγS binding levels obtained in the absence of agonist were comparable in wild-type and mutant mice (data not shown), indicating that basal G-protein activity is unaltered in MOR-deficient mice. In the brain of wild-type mice, the stimulation induced by 0.3 μM DAMGO was 115% of basal activity, whereas it was undetectable in the knock-out mice. Because μ-sites are absent in MOR-deficient mice, these results confirm that DAMGO acts selectively at μ-receptors at a 0.3 μM concentration.

DPDPE, deltorphin II, and CI-977 induced a significant increase of [^{35}S]GTPγS binding in both mutant and wild-type mice.

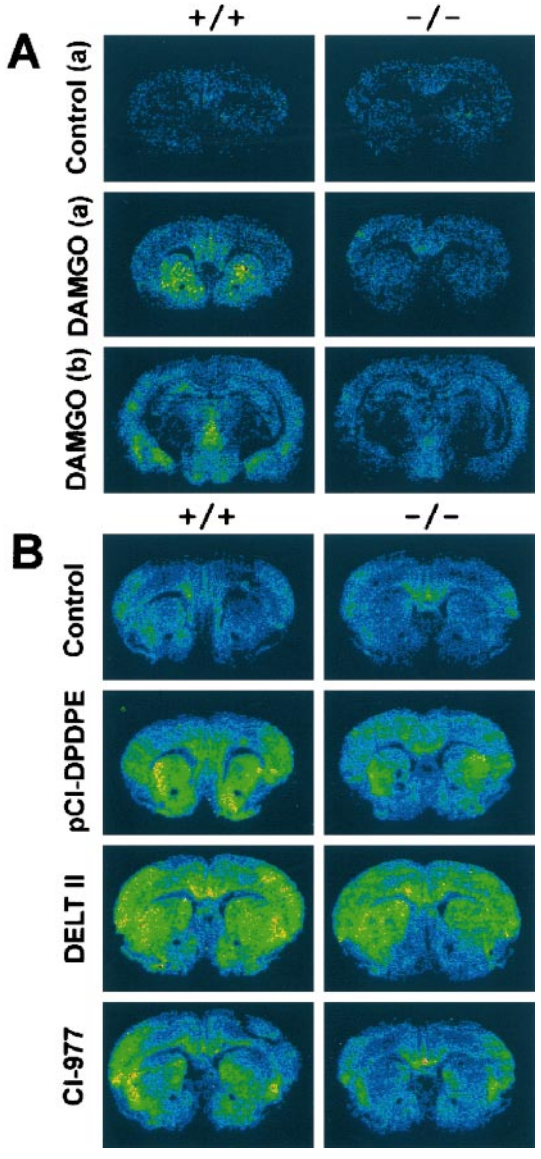

Figure 1. Autoradiographic study of opioid receptor coupling to G-proteins, by agonist-stimulated [^{35}S]GTPγS binding on brain sections. *A*, [^{35}S]GTPγS labeling induced by a μ-agonist. Coronal sections of brains from wild-type (+/+) and MOR-deficient (−/−) mice are shown. Slices were incubated in the absence (*Control*) or presence of 3 μM DAMGO and sections are shown at the level of caudate-putamen (*a*) or thalamus (*b*). *B*, [^{35}S]GTPγS labeling induced by δ- and κ-agonists. Coronal sections from brains are presented at the level of caudate-putamen of wild-type (+/+) and MOR-deficient (−/−) mice. Slices are incubated in the absence (*Control*) or presence of 3 μM pCl-DPDPE (δ1), 3 μM deltorphin II (δ2), or 1 μM CI-977 (κ). The signal increases as follows: *black* < *blue* < *green* < *yellow* < *red*.

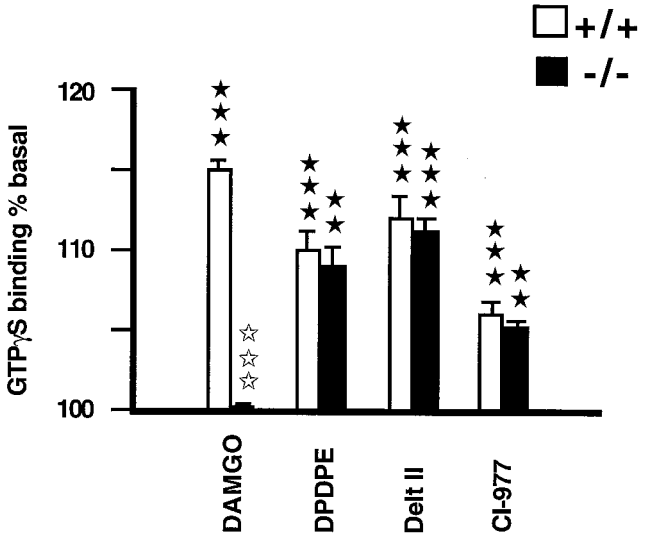

Figure 2. Agonist-induced [^{35}S]GTPγS binding on brain membrane preparations. Brain membranes were incubated in the absence or presence of the agonists DAMGO (0.3 μM, μ), DPDPE (0.3 μM, δ1), deltorphin II (0.3 μM, δ2), or CI-977 (0.1 μM, κ). Basal level (100%) represents the amount of specific [^{35}S]GTPγS binding (see Materials and Methods) in the absence of agonist, and values on the *y*-axis indicate the stimulation obtained using the various agonists. Results are expressed as mean ± SEM of at least three experiments performed in triplicate and conducted on at least two distinct membrane preparations. DPDPE, deltorphin II, and CI-977 significantly increase [^{35}S]GTPγS binding above basal levels in both wild-type and mutant mice (*black stars*, comparisons with basal levels for the same genotype; *three stars*, $p < 0.001$; *two stars*, $p < 0.01$). There is no significant difference between genotypes, except for DAMGO-induced [^{35}S]GTPγS binding (*white stars*, comparisons between wild-type and mutant groups receiving the same treatment; *three stars*, $p < 0.001$).

At the chosen concentrations (0.3 μM for DPDPE and deltorphin II and 0.1 μM for CI-977), there was no significant difference between labeling levels in MOR +/+ and MOR −/− preparations. Stimulation was 109–110% (DPDPE), 111–112% (deltorphin II), and 106% (CI-977) for both wild-type and mutant mice. This finding further confirms that both δ- and κ-receptors remain functionally coupled to G-proteins in the absence of μ-receptors. Altogether the data indicate that functional coupling of δ- and κ-receptors is not markedly altered in the absence of μ-receptors.

The maintenance of δ- and κ-receptor signaling properties in MOR-deficient mice was further confirmed by examining the ability of δ- and κ-agonists to inhibit adenylate cyclase, a well known downstream effector of the opioid receptor–G-protein complex. Adenylyl cyclase activity was measured on brain homogenates in the presence of increasing concentrations of DPDPE, deltorphin II, and U-50,488H, and results are shown in Figure 3. All three agonists were found to significantly inhibit enzyme activity in a dose-dependent manner, as described previously in rat (Noble and Cox, 1995), with maximal inhibition ranging from 29.3% (deltorphin II, MOR +/+) to 47.1% (DPDPE, MOR −/−) of basal activity. There was no significant difference between results obtained for wild-type and mutant mice.

δ and κ analgesia in MOR-deficient mice

We first investigated morphine selectivity *in vivo* by examining the ability of δ- and κ-antagonists to reverse morphine analgesia in wild-type mice, under conditions that produce no morphine analgesia in MOR −/− mice (6 mg/kg, i.p.) (Matthes et al., 1996). Results from both the tail-immersion and hot-plate tests indicate no reduction of morphine analgesia in the presence of norBNI (κ) or naltrindole (δ) administered at doses known to block κ-receptors (Ossipov et al., 1996) and δ-receptors (Kalso et al., 1992), respectively (data not shown). This indicates that antinociceptive responses produced by the administration of fairly low doses of morphine are attributable to the activation of μ-opioid receptors only and explains the complete absence of morphine analgesia in μ-receptor-deficient mice (Matthes et al., 1996).

We then investigated the antinociceptive responses induced by

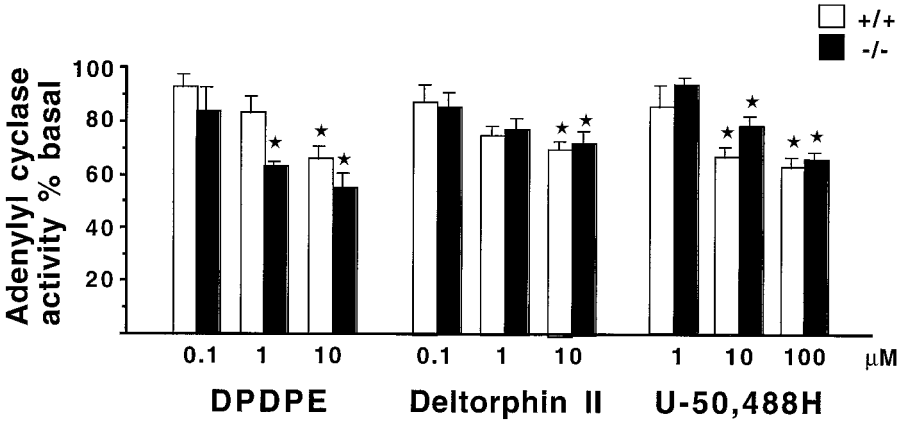

Figure 3. δ- and κ-induced inhibition of adenylyl cyclase activity in brain. Adenylyl cyclase activity was measured in brain homogenates (see Materials and Methods) in the absence or presence of increasing concentrations of selective δ-agonists (DPDPE and deltorphin II) and κ-agonists (U-50,488H). Basal activity (100%) refers to the amount of cAMP generated in the absence of opioid agonist. Data are mean ± SEM values from three to four independent experiments, each performed in triplicate. *Black stars*, comparisons with cyclase inhibition level in the absence of agonist for the same genotype; $p < 0.05$. There is a significant dose-dependent inhibition of adenylyl cyclase activity for all three agonists in both MOR +/+ and MOR −/− mice, with no significant difference between mouse genotypes.

the selective δ-opioid agonists deltorphin II and DPDPE and the selective κ-opioid agonist U-50,488H in wild-type and mutant mice. Two different antinociceptive models were used: the tail-immersion test and the hot-plate test (see Materials and Methods). In an attempt to determine the doses of each opioid compound to be administered in mutant mice, a preliminary dose–response experiment was performed in wild-type animals with genetic background similar to that of mutant animals (data not shown). Three different doses of each opioid agonist—DPDPE (5, 15, 45 μg, i.c.v.), deltorphin II (3, 10, 30 μg, i.c.v.), and U-50,488H (3, 10, 30 mg/kg, i.p.) were tested, and doses producing a submaximal antinociceptive effect were chosen as follows: 15 μg (i.c.v.) for DPDPE, 10 μg (i.c.v.) for deltorphin II, and 30 mg/kg (s.c.) for U-50,488H. Using those conditions we then compared the analgesic action of the δ- and κ-agonists in mutant and wild-type mice, and results are shown in Figures 4 (δ analgesia) and 5 (κ analgesia).

First, basal pain perception after thermal stimuli could be reevaluated (Matthes et al., 1996) by comparing responses of mice from both genotypes in the control experiments (saline injection) (Figs. 4*A,B*, 5*A,B*). We have previously reported the absence of modification in nociceptive thresholds in mutant mice. Here we have used lower temperatures in both hot-plate (50°C instead of 54°C) and tail-flick (50°C instead of 52°C) tests to reveal possible subtle alterations that may occur in basal nociception. In the tail-immersion test and the paw-lick latency evaluated in the hot-plate test, MOR −/− mice showed spontaneous nociceptive thresholds similar to those of their wild-type littermates, in agreement with our previous study. In contrast, the latency of the jumping response in the hot-plate test at 50°C was lower in mutant than in wild-type mice (Fig. 4*A*), although this difference in nociceptive threshold did not reach a significant level in all the experiments (Figs. 4*B*, 5*A,B*). These data indicate a slightly higher sensitivity of MOR-deficient mice to perceive thermal noxious stimuli, which was only revealed in the jumping response in the hot-plate test. One should note that mouse exposure to the hot plate may represent a stressful situation. Therefore, we cannot exclude that different responses of MOR +/+ and MOR −/− mice to the nociceptive stimulus are attributable to a difference in stress-induced analgesia. Using similar experimental settings, Sora et al. (1997a) also showed small but significant changes in spontaneous nociceptive thresholds (tail-withdrawal in the tail-flick test and paw-lick latencies in the hot-plate test) in another μ-receptor-deficient mutant mouse strain.

The selective δ-opioid agonist deltorphin II (10 μg, i.c.v.) produced a significant antinociceptive effect in both wild-type and mutant mice, considering tail withdrawal, paw-lick, and jump latencies (Fig. 4). The analgesic response of deltorphin II in the tail-immersion test tended to be higher in the wild-type group in one experiment (Fig. 4*A*), and this difference was significant in a second experiment (Fig. 4*B*). The selective δ-opioid agonist DPDPE (15 μg, i.c.v.) induced a significant antinociceptive response in the hot-plate test in the two groups of mice in both paw-lick and jump responses. The effect of this compound tended to be higher in the wild-type group on the jump response (Fig. 4*A*). In the tail-immersion test, DPDPE produced a significant antinociceptive effect in wild-type mice, but the response was not significant in mutant mice. Altogether the data indicate that some of the antinociceptive effects of the two δ-agonists are reduced in MOR −/− mice, mainly in the tail-withdrawal response.

To confirm that the antinociceptive responses induced by δ-agonists are indeed mediated by δ-opioid receptors, we evaluated the ability of the selective δ-antagonist naltrindole (Portoghese et al., 1988) to prevent the responses induced by deltorphin II. Deltorphin II, rather than DPDPE, was chosen in this experiment because it produced a significant and more reliable antinociceptive response in both nociceptive tests in mutant mice. We therefore repeated our experiment by pretreating (or not) the animals with naltrindole (2.5 mg/kg, i.p.) (Fig. 4*B*). The antagonist, administered alone, did not produce any intrinsic effect in the nociceptive thresholds evaluated in the tail-immersion and hot-plate tests. Pretreatment with naltrindole completely blocked the antinociceptive responses induced by deltorphin II for both genotypes in the tail-immersion test, as shown by the significant difference between groups treated with deltorphin II alone or associated with naltrindole. In the hot-plate test, naltrindole was able to decrease the analgesic effects of deltorphin II in both groups of mice. Although the dose of antagonist was higher than doses required in previous studies to block δ-mediated responses (from 0.1 to 1 mg/kg) (Gacel et al., 1990; Baamonde et al., 1992; Kalso et al., 1992), one should note that a residual response seems to remain in the case of the hot-plate test (jumping response). Furthermore, no significant difference between groups treated with deltorphin II alone or associated with naltrindole was observed for the licking response in MOR +/+ mice. Higher doses of naltrindole were not used here to avoid cross-reactivity with other receptors.

The selective κ-opioid agonist, U-50,488H (30 mg/kg, s.c.) produced a significant antinociceptive effect in the tail-immersion and hot-plate (paw-lick and jump responses) tests in mutant and wild-type mice (Fig. 5). In this experiment, there was no significant difference between these two groups of animals in any of the

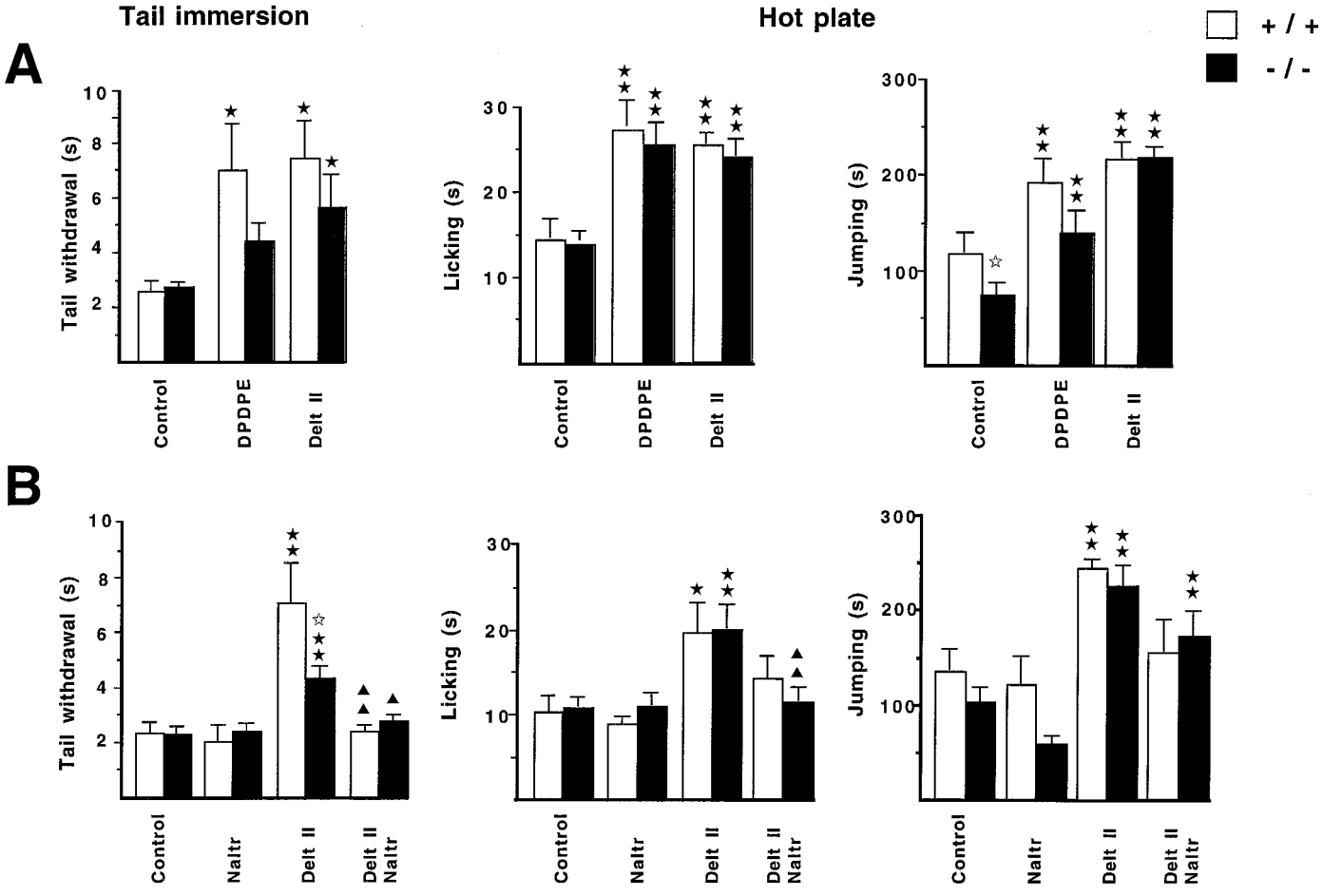

Figure 4. δ analgesia. *A*, Antinociceptive responses induced by the selective δ-opioid agonists deltorphin II (*Delt II*, 10 μg, i.c.v.) and DPDPE (15 μg, i.c.v.), in MOR-deficient (−/−) and wild-type (+/+) mice (8 animals per group). *B*, Reversal of deltorphin II (*Delt II*, 10 μg, i.c.v.) antinociception by the selective δ-opioid antagonist naltrindole (*Naltr*, 25 mg/kg, s.c.) in MOR-deficient (−/−) and wild-type (+/+) mice (6–8 animals per group). Tail-immersion (tail-withdrawal latency) and hot-plate (licking and jumping latencies) tests were used. Values on *y*-axis represent the latencies in seconds of different nociceptive thresholds expressed as mean ± SEM. *Black stars* indicate comparisons with saline-treated animals of the same genotype. *White stars* indicate comparisons between wild-type and mutant groups receiving the same treatment. *Black triangles* in *B* represent comparisons with deltorphin II-treated animals of the same genotype. *One symbol*, $p < 0.05$; *two symbols*, $p < 0.01$.

nociceptive thresholds evaluated, indicating that κ-analgesia is preserved in MOR-deficient mice.

As for δ-analgesia, we also verified that the effect of U-50,488H is mediated by the κ-receptor by using a selective κ-opioid antagonist, norBNI. We conducted the experiment in the absence or presence of norBNI (5 mg/kg, i.p.) in the treatment (Fig. 5*B*). The κ-opioid antagonist administered alone did not produce any intrinsic pharmacological response in mutant and wild-type mice in any of the tests. The effects induced by U-50,488H were antagonized by norBNI in both groups of animals in two of the antinociceptive tests, confirming that U-50,488H analgesia is essentially mediated by κ-receptors. Of note is the fact that latencies obtained in wild-type animals, but in not mutant animals, after co-administration of U-50488H and norBNI were always slightly higher than their respective saline controls. This difference could be attributable to a slight nonspecific antinociceptive response of U-50,488H mediated by μ-receptors in wild-type animals.

Opioid-induced respiratory depression in MOR-deficient mice

Respiratory depression is considered a major unwanted side-effect of opioid analgesics, and the implication of the opioid system in the modulation of respiratory function has been largely documented (Shook et al., 1990). The activation of μ-, δ-, and κ-opioid receptors has been reported to depress ventilation, and interactions between opioid receptors have been described on the respiratory function (Morin-Surun et al., 1984; Yeadon and Kitchen, 1989; France et al., 1994; Denavit-Saubié and Foutz, 1997). We have coupled the study of the analgesic action of deltorphin II and U50,488H with measurements of several respiratory parameters, including respiratory frequency, inspiration time, and minute volume. We have also examined the action of morphine in this experiment, and results are shown in Figure 6 and Table 3. Under basal conditions, mutant mice showed respiratory patterns similar to those of their wild-type littermates, and there was no significant difference for any of the three respiratory responses analyzed. The dose of morphine that produced potent analgesia in wild-type mice (6 mg/kg) also decreased respiratory frequency by increasing inspiratory time. This effect was not observed in MOR-deficient mice, demonstrating a main implication of μ-receptors in morphine respiratory depression. Deltorphin II also significantly altered respiration in wild-type mice, by decreasing breathing frequency and minute volume. Interestingly,

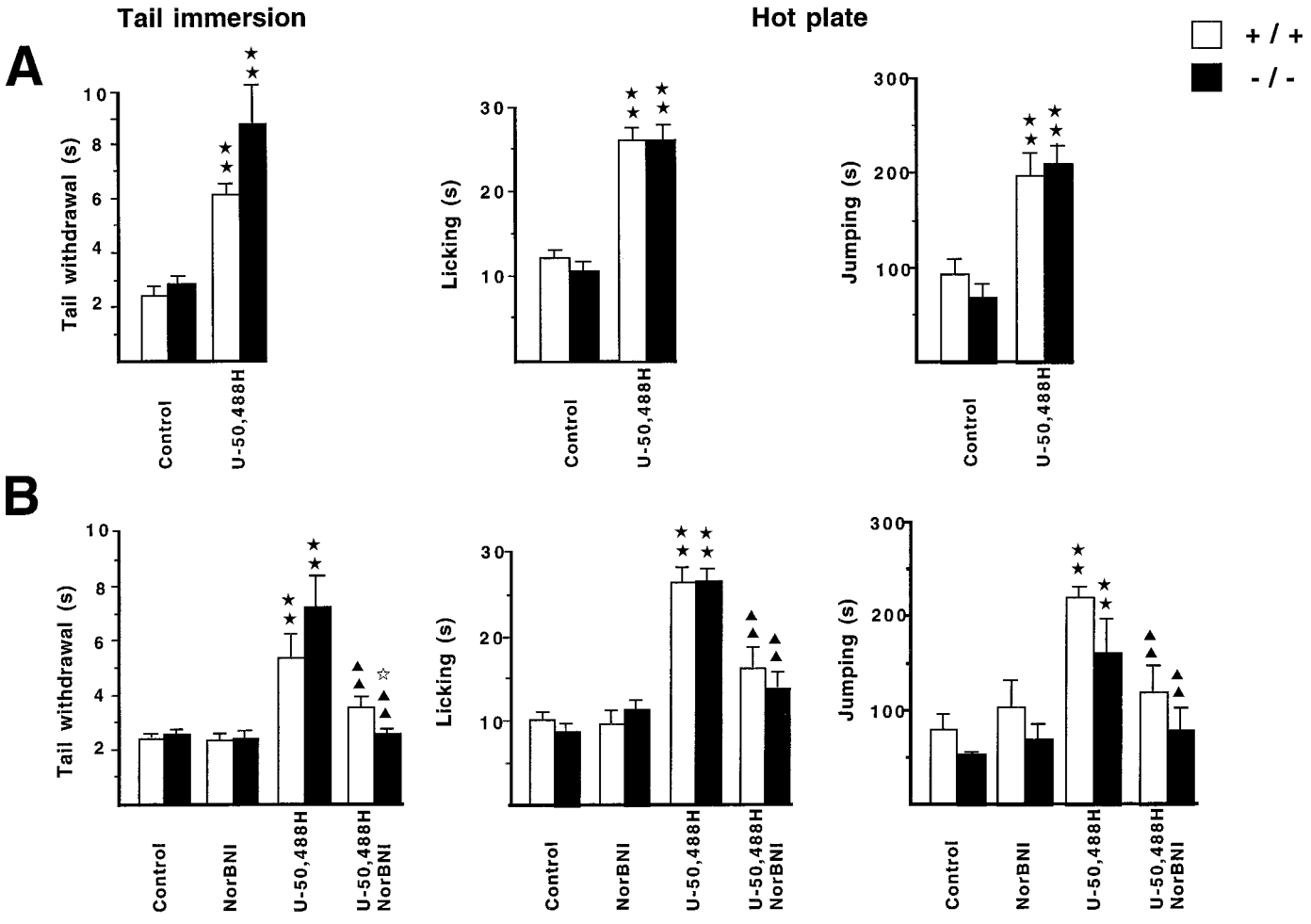

Figure 5. κ analgesia. *A*, Antinociceptive responses induced by the selective κ-opioid agonist U-50,488H (30 mg/kg, s.c.) in MOR-deficient (−/−) and wild-type (+/+) mice (8 animals per group). *B*, Reversal of U-50,488H (30 mg/kg, s.c.) antinociception by the selective κ-opioid antagonist norbinaltorphimine (*NorBNI*, 5 mg/kg, s.c.) in MOR-deficient (−/−) and wild-type (+/+) mice (10–12 animals per group, excepting groups receiving opioid antagonists alone, where the number of animals was 6). Tail-immersion (tail-withdrawal latency) and hot-plate (licking and jumping latencies) tests were used. Values on the *y*-axis represent the latencies in seconds of different nociceptive thresholds expressed as mean ± SEM. *Black stars* represent comparisons with saline-treated animals of the same genotype. *White stars* represent comparison between wild-type and mutant groups receiving the same treatment. *Black triangles* in *B* represent comparisons with U-50,488H-treated animals of the same genotype. *One symbol*, $p < 0.05$; *two symbols*, $p < 0.01$.

this effect was not observed in mutant mice, indicating that the δ-mediated modulation of respiration is absent in μ-deficient mice. Finally, U-50,488H did not change respiratory frequency and minute volume in either wild-type or mutant animals and induced a similar increase of the inspiratory time and decrease of the expiratory time in both groups of mice. Therefore, the absence of μ-receptor does not seem to modify the action of the κ-agonist on respiration. Taken together, these results indicate that κ-receptors, but not δ-receptors, modulate respiration independently from the μ-receptor.

Inhibition of the vas deferens twitch in MOR-deficient mice

A wide variety of peripheral tissues have been shown to be sensitive to the action of opioids. The predominant effect of opioid receptor activation in the periphery is to reduce smooth muscle contraction. This is commonly studied on isolated organ preparations, by measuring the inhibitory action of opioids on electrically stimulated contractions of the vas deferens. These excised organ preparations contain heterologous populations of opioid receptor subtypes that vary across species (for review, see Smith and Leslie, 1993), and δ-agonists have been shown most potent in the mouse vas deferens (Porrecca et al., 1990). We have therefore examined the biological activity of the two prototypic δ-agonists DPDPE and deltorphin II in vas deferens preparations of MOR +/+ and MOR −/− mice. The IC_{50} values obtained in preparations from wild-type mice were 13.89 ± 2.16 nM for DPDPE and 1.87 ± 0.30 nM for deltorphin II. A similar activity profile of the two agonists was reported previously in Albino Swiss mice (Melchiorri et al., 1991), although vas deferens preparations from this mouse strain were more responsive to δ-opioid agonists than those from hybrid 129/SV x C57Bl/6 mice used in the present study. In mutant mice, IC_{50} values were 22.44 ± 4.59 nM for DPDPE and 3.85 ± 0.90 nM for deltorphin II. Although IC_{50} values showed a tendency to be slightly higher in mice lacking μ-receptors, our statistical analysis indicated no significant difference between MOR +/+ and MOR −/− mice, neither for DPDPE ($t_{(1, 21)} = 1.728$, NS) nor for deltorphin II ($t_{(1, 20)} = 2.048$, NS). The regression analysis indicated that the slope of

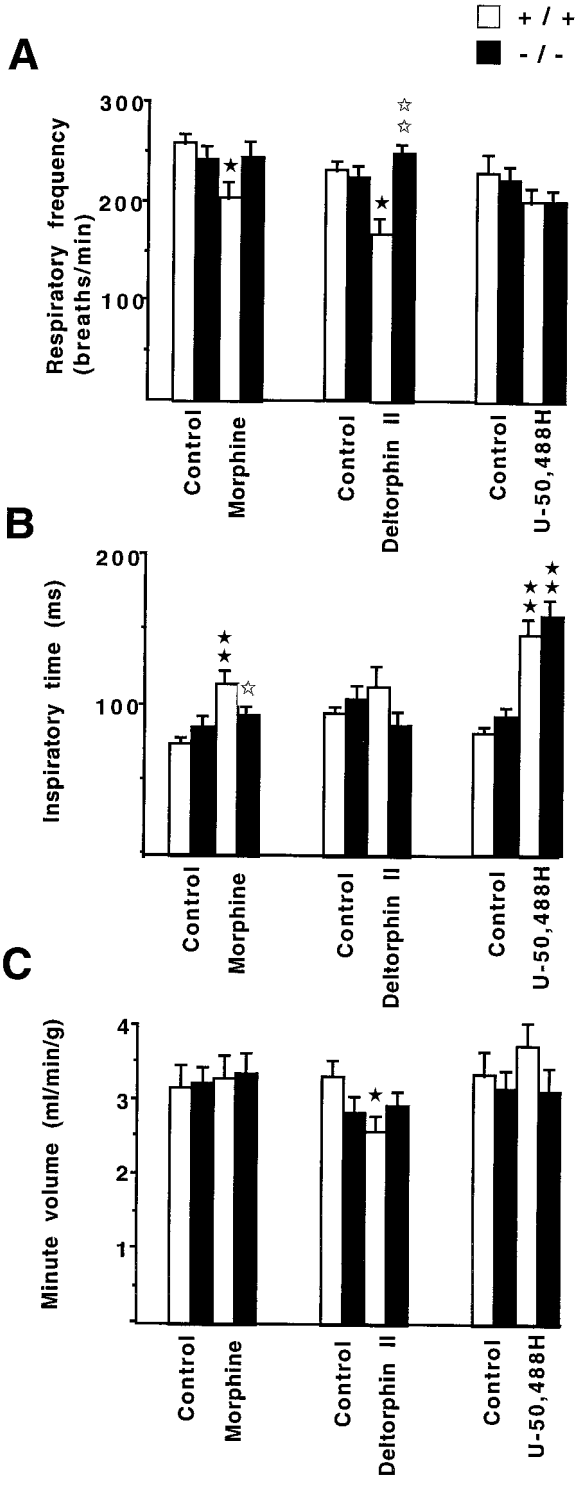

Figure 6. Respiratory depression. Respiratory responses induced by morphine (6 mg/kg, s.c.), the selective δ-opioid agonist deltorphin II (10 μg, i.c.v.), and the selective κ-opioid agonist U-50,488H (30 mg/kg, s.c.) in MOR-deficient (−/−) and wild-type (+/+) mice. Values on the *y*-axis represent respiratory frequency (*A*), inspiratory time (*B*), and minute volume (*C*) expressed as mean ± SEM. *Black stars* represent comparisons with the same animal before subcutaneous drug injection (controls for morphine and U-50,488H) or with saline-injected (intracerebroventricular) animals of the same genotype (controls for deltorphin II). *White stars* represent comparison between wild-type and mutant animals receiving the same treatment. *One symbol*, $p < 0.05$; *two symbols*, $p < 0.01$ (10–12 animals per group).

DPDPE was higher in MOR +/+ (1.853 ± 3.51; linear trend: $F = 16.83$, $p < 0.01$) than in MOR−/− mice (0.938 ± 1.94; linear trend: $F = 23.21$, $p < 0.001$). A similar result was obtained when the slope of deltorphin II was calculated for MOR +/+ (11.39 ± 2.47; linear trend: $F = 27.33$, $p < 0.001$) and MOR−/− mice (5.97 ± 1.43; linear trend: $F = 31.44$, $p < 0.001$). However, the efficacy of δ-opioid agonists was comparable in both genotypes, because the intercept was similar for DPDPE (MOR +/+ = 22.65; MOR−/− = 28.04) and deltorphin II (MOR +/+ = 28.21; MOR−/− = 27.58) for both groups of animals. Altogether our results suggest that the inhibition of smooth muscle contraction mediated by peripheral δ-receptors is preserved in MOR −/− mice.

DISCUSSION

The molecular mechanism of action of morphine

Our previous behavioral studies of MOR-deficient mice have focused on responses to the prototypic opiate morphine. We have shown that morphine analgesia, reward, and physical dependence are abolished in μ-deficient mice (Matthes et al., 1996). In another study, we have demonstrated that morphine immunosuppression is absent in mutant mice (Gavériaux-Ruff et al., 1998). Here we have investigated respiratory depression, another important action of morphine, which is generally considered one of the most adverse side-effects in the treatment of severe pain. Our results show that morphine does not affect respiratory function in mutant animals, providing the first genetic evidence for the essential involvement of the MOR gene product in mediating morphine respiratory effects. This result adds to the notion that both desired (analgesia) and adverse (addiction, immunosuppression, respiratory depression) actions of morphine are mediated by the same receptor protein, which clearly limits the usefulness of MOR as a therapeutic target.

Morphine is weakly μ-selective *in vitro* and may partially act via δ- and κ-receptors *in vivo*. Although the complete absence of morphine responses in mutant mice is most probably attributable to the absence of its main molecular target, we cannot exclude the possibility that it could also arise partly from an alteration of δ- and κ-receptor function. In addition, an impairment of δ- and κ-receptor activity in those mice would reflect the existence of functional interactions between opioid receptors, a hypothesis that was suggested previously but could not be demonstrated by molecular approaches. It is therefore of critical importance to examine the functional activity of δ- and κ-receptors in MOR-deficient mice.

κ-Receptor activity is preserved in mice lacking the μ-receptor

To investigate responses produced by the activation of κ-opioid receptors in mutant mice, we have used two structurally related arylacetamide compounds, CI-977 and U-50,488H, described as highly κ-selective agonists. CI-977 was chosen for *in vitro* studies because of its high affinity, selectivity (Table 1), and efficacy to induce [^{35}S]GTPγS labeling. U-50,488H was selected for analgesic and respiratory studies because pharmacological responses to this compound *in vivo* (Lahti et al., 1982) have been widely reported in multiple experimental models, and its action was shown to be mediated essentially by κ-receptor (Piercey et al., 1982; von Voigtlander et al., 1983). Accordingly, in our study, pretreatment with the κ-selective antagonist norBNI, at a dose that does not modify morphine-induced analgesia (data not shown), and was previously reported to block κ-mediated re-

sponses (Ossipov et al., 1996), completely antagonized the analgesic responses of U-50,488H in all the tests. It is therefore reasonable to assume that responses to κ-agonists evaluated in this study indeed reflect the functional activity of κ-receptors.

We have shown that κ-receptors functionally activate G-proteins and inhibit adenylate cyclase in the brain of MOR-deficient mice. Further we have observed a comparable analgesic action of U-50,488H in wild-type and mutant mice, which indicates that κ-receptor-mediated antinociception is not altered by the absence of μ-receptors, at least in response to an acute noxious thermal stimulus. Finally, the κ-agonist U-50,488H affected the timing of respiratory phases similarly in wild-type and mutant mice, suggesting that the modulation of respiratory function by the κ-receptor is not altered in mice lacking the μ-receptor. Altogether, these results strongly suggest that functional properties of the κ-receptor are maintained in mutant mice and that the κ-receptor mainly acts independently from the μ-receptor. From the literature indicating possible cooperativity between opioid receptors (Rothman et al., 1993), there are few indications for μ-/κ-receptor interactions compared with μ-/δ-receptor interactions. Our results demonstrate that μ-/κ-receptor interactions, if they exist, do not seem to have any influence on receptor coupling or the control of nociception and respiration *in vivo*.

δ-Receptor activity is slightly reduced in mice lacking the μ-receptor

The existence of two different subtypes of δ-opioid receptors has been proposed to explain the analgesic responses of δ-agonists. Although the existence of receptor subtypes that would be distinct molecular entities is controversial (Zaki et al., 1996), the existence of two functionally distinct receptor sites is well documented from *in vivo* pharmacological studies (Rothman et al., 1993; Traynor and Elliot, 1993). We have therefore used two δ-agonists proposed to activate each of the two receptor subtypes, DPDPE (δ1) and deltorphin II (δ2) (Mattia et al., 1991), to ensure the functional investigation of all δ-receptor subpopulations. Our binding data (Table 1) have confirmed the high δ-selectivity of the two compounds in the mouse strain under study. In our *in vivo* experiments, we have verified that deltorphin II analgesia is mediated by δ-opioid receptors by using the selective δ-antagonist naltrindole at a dose reported to be selective of δ-opioid receptors (Gacel et al., 1990; Kalso et al., 1992). Thus, as for κ-agonists, we have good indications that the δ-agonists act in a selective manner under our experimental conditions.

The analysis of agonist-stimulated [^{35}S]GTPγS binding and adenylyl cyclase inhibition shows that δ-receptors functionally activate intracellular effectors in the absence of μ-receptors. Another preserved δ-response is the inhibition of the vas deferens twitch in the isolated organ preparation, a bioassay that classically evaluates the functional activity of peripheral δ-opioid receptors (Smith and Leslie, 1993). Therefore these aspects of δ-receptor activity are independent from the μ-receptor.

The *in vivo* study shows that some of the antinociceptive actions of deltorphin II and DPDPE are less effective in mutant than in wild-type mice. Accordingly, a different response was observed in the tail-immersion test between both genotypes, suggesting that δ-mediated spinal analgesia is diminished in the absence of μ-receptors. Of note, however, is the fact that a large part of δ-analgesia is preserved in MOR-deficient mice, because no significant difference was observed in the analgesic action of deltorphin II and DPDPE when the hot-plate test was used. Therefore δ-receptor-mediated analgesia seems to be influenced by the existence of MOR-encoded receptors, essentially at the spinal level in the CNS. A reduction of DPDPE analgesia in both tail-flick and hot-plate tests was reported recently (Sora et al., 1997b). When comparing the two studies, it seems that the reduction of DPDPE analgesia is less pronounced in our study. However, results are not necessarily discordant, because data presentation is different in our study (latencies in seconds) than in the previous study (maximal possible analgesia). Moreover, the possibility of different baseline latencies between mutant mice, which are used to calculate the maximal possible analgesia, hampers the comparison of present data from both studies. Both findings, however, support the notion of a μ-/δ-receptor cooperativity in opioid analgesia, as suggested previously by pharmacological experiments (Jiang et al., 1990). In addition, our data indicate that μ-/δ-receptor interactions are not equally involved in response to different painful stimuli, possibly as a consequence of regionally selective mechanisms.

Our results further suggest that μ-/δ-receptor cooperativity occurs in other responses to opioids *in vivo*. Specifically, the action of deltorphin II on respiratory function is impaired in MOR-deficient mice, although δ sites are present in respiratory centers (Kitchen et al., 1997). This indicates that the presence of μ-receptors is necessary to allow δ-receptor-mediated modulation of respiration. Therefore, our data provide genetic evidence for synergistic interactions between μ- and δ-receptors at the level of respiratory pathways. μ-/δ-receptor interactions may not be limited to these aspects of opioid physiology, and other behavioral studies are currently under investigation in MOR-deficient mice.

The question of whether receptor interactions take place between distant receptors located on separate neurons, or arise from receptor cross-talk at the cellular level, is not clear yet. Binding data have provided indications for allosteric coupling between the receptors (Rothman et al., 1993), colocalization of opioid receptors in dorsal root ganglia has been suggested by immunohistochemistry (Ji et al., 1995), and both μ- and δ-receptors have been identified on bulbar respiratory neurons (Morin-Surun et al., 1984). If μ-/δ-receptor cross-talk occurs between populations of μ- and δ-receptors co-expressed in the same neurons, we may expect that δ-receptor transduction properties would be altered in mice lacking μ-receptors. However, our investigation of δ-receptor signaling in MOR-deficient mice did not reveal any marked alteration of agonist-induced G-protein activation or adenylate cyclase inhibition that would support this hypothesis. Therefore, we would rather suggest that the synergistic activity of μ- and δ-receptors, evidenced in this study, involves distinct cells that are functionally associated within the neuronal network. The precise mode of interaction between the two receptors should be clarified further, and the parallel study of mice with a genetic disruption of the δ-opioid receptor gene will allow us to definitely identify δ-receptors involved in μ-/δ-receptor cooperativity at the molecular level.

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