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Anesthetic Potency and Influence of Morphine and Sevoflurane on Respiration in μ-Opioid Receptor Knockout Mice

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Background: **The involvement of the μ-opioid receptor (μOR) system in the control of breathing, anesthetic potency, and morphine- and anesthesia-induced respiratory depression was investigated in mice lacking the μOR.**

Methods: **Experiments were performed in mice lacking exon 2 of the μOR gene ($\mu OR^{-/-}$) and their wild-type littermates ($\mu OR^{+/+}$). The influence of saline, morphine, naloxone, and sevoflurane on respiration was measured using a whole body plethysmographic method during air breathing and elevations in inspired carbon dioxide concentration. The influence of morphine and naloxone on anesthetic potency of sevoflurane was determined by tail clamp test.**

Results: **Relative to wild-type mice, μOR-deficient mice displayed approximately 15% higher resting breathing frequencies resulting in greater resting ventilation levels. The slope of the ventilation–carbon dioxide response did not differ between genotypes. In $\mu OR^{+/+}$ but not $\mu OR^{-/-}$ mice, a reduction in resting ventilation and slope, relative to placebo, was observed after 100 mg/kg morphine. Naloxone increased resting ventilation and slope in both genotypes. Sevoflurane at 1% inspired concentration induced similar reductions in resting ventilation and slope in the two genotypes. Anesthetic potency was 20% lower in mutant relevant to wild-type mice. Naloxone and morphine caused an increase and decrease, respectively, in anesthetic potency in $\mu OR^{+/+}$ mice only.**

Conclusions: **The data indicate the importance of the endogenous opioid system in the physiology of the control of breathing with only a minor role for the μOR. The μOR gene is the molecular site of action of the respiratory effects of morphine. Anesthetic potency is modulated by the endogenous μ-opioid system but not by the κ- and δ-opioid systems.**

OPIOID peptides are composed of a family of structurally related endogenous peptides that act at three receptor types known as μ-, κ-, and δ-opioid receptors (ORs),[1] which are involved in physiologic responses to pain, stress, and emotion and have a modulatory influence on various physiologic functions such as thermoregulation, nociception, and the immune response.[1] Endogenous opioid peptides and ORs are found in high concentrations in areas of the central and peripheral nervous system, which play a role in the control of breathing,[2–4] and exogenously administered opioids are potent depressants of respiration.[3–15] Despite a multitude of studies, the (relative) involvement of the different ORs in the physiology of the control of breathing and the mechanism(s) of respiratory depression from exogenous opioids remains unclear. With respect to the latter, previous animal studies considered both OR and non-OR mechanisms. For example, it is believed that morphine exerts its respiratory depressant effects through μ- and δ-receptors,[4,6] whereas respiratory depression from morphine is (partially) antagonized by physostigmine, a centrally acting anticholinesterase.[15] Furthermore, some researchers proposed that respiratory depression from μ-ligands is mediated by interaction with δ-receptors.[9,11] To complicate things further, some animal studies indicate that μ- and δ-opioid agonists have a stimulatory effect on respiration, especially at relatively low doses.[16,17]

Recently, mice lacking the various ORs have been generated by inactivation of the specific receptor genes by genetic engineering.[1,18–22] This enables the assessment of the necessity of the OR gene product, (*i.e.*, the OR) for mediating specific processes. In this study, we examined respiration in mice lacking exon 2 of the μOR gene ($\mu OR^{-/-}$)[18] and wild-type mice ($\mu OR^{+/+}$) of the same strain to determine the involvement and contribution of the ORs (*i.e.*, the μOR *vs.* the κ- and δ-ORs) in the physiology of the control of breathing.

Studies in μOR knockout mice have implicated the μOR gene product as molecular site of action of morphine analgesia.[18–21] Morphine remains a valuable analgesic in alleviating acute pain despite its many side effects, such as sedation, nausea, and respiratory depression. Respiratory depression from morphine can be life-threatening, especially when the balance between respiratory stimulation from chemoreflexes, arousal, and pain, and respiratory depression from morphine is shifted to the latter. We tested the hypothesis that the μOR is the primary target for both morphine-induced respiratory depression and morphine analgesia by examining morphine antinociception and the influence of systemic morphine on the control of breathing in the μOR-deficient mouse model.

In anesthetic practice, combining opioids and anesthetics has various advantages, one being the synergistic interaction on anesthetic potency (defined as the con-

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centration anesthetic causing suppression of somatic responses and movement in response to a noxious stimulus—*e.g.*, tail clamp—in 50% of subjects).[23,24] This suggests the modulation of neuronal networks involved in anesthesia-induced suppression of somatic responses by the μ-opioid system. Whether such an interaction exists between neuronal systems involved in anesthesia-induced depression of breathing and the μ-opioid system remains unclear. We therefore determined the anesthetic potency of sevoflurane (and the influence of morphine and naloxone on the potency of sevoflurane) and the influence of sevoflurane anesthesia on ventilatory control in mice lacking the μOR gene. Taking into account the data on suppression of somatic responses,[23,24] we expected a lower anesthetic potency and less anesthetic-induced depression of breathing in $\mu OR^{-/-}$ mice relative to their wild-type littermates (*i.e.,* mice with functional μORs).

Methods

Mice with a disruption of exon 2 of the μOR gene and wild-type animals of the same mouse strain have been reported previously.[18] All animals are 1:1 hybrids from 129/SV and C57BL/6 mouse strains, and hence both genotypes have an identical genetic background. The mice were genotyped using Southern blot analysis on mouse tail DNA using specific external probes to distinguish deletion of the μOR gene from the intact gene (see Matthes *et al.*[18] for the details and results of the genotyping). Experiments were performed in mice of either sex (weight, 21–41 g) while the animals were 2–6 months of age. All experiments were conducted after obtaining approval for the protocol from the local Animal Ethics Committee (Dier Ethische Commissie, Leiden University Medical Center). Animal care was conducted in accordance with institutional guidelines.

Measurement of Respiratory Variables

Respiratory activity was measured using whole body plethysmography, originally described by Drorbaugh and Fenn,[25] with an important adaptation allowing the continuous flow of dry gas through the measurement and referential chambers. A barometric measurement of ventilation is based on the principle that when an animal in a closed chamber inhales a tidal volume, this volume is warmed to body temperature and saturated with water vapor. On expiration, the gas is cooled down to ambient temperature and desaturated to the conditions in the chamber. The temperature and water vapor changes are the cause of small pressure changes in the chamber, which are measured with a pressure transducer. The pressure changes are proportional to the inspired and expired tidal volumes. By using a differential pressure measurement between the chamber that contains the animal (measurement chamber) and the reference chamber, pressure changes in the room where the experiment is taking place are canceled out. Both chambers are transparent and are approximately 600 ml each. The flow and composition of the gases was set by three mass flow controllers (Bronkhorst High Tec, Veenendaal, The Netherlands). The chambers were kept at room temperature (24–26°C). Data acquisition was started after an animal was placed in the chamber and had ample time for habituation.

Antinociception

Two nociceptive tests were performed: the tail-immersion test and the hot-plate test. In the tail-immersion test, the tails of the mice were immersed approximately 2 cm in water that was 54°C, and the latency time to a rapid tail flick was recorded. The cutoff time for this test was 15 s. In the hot-plate test, mice were placed on a plate heated to 52°C. The latency time to paw licking was determined, with a cutoff of 30 s.

Determination of Anesthetic Potency

Anesthetic potency was determined by assessing the minimum alveolar concentration (MAC) of sevoflurane according to the following method.[26,27] Mice were anesthetized in an acrylic cylinder. The temperature of the mice was monitored and kept between 36 and 38°C with a heating lamp. Initially, mice randomly received one of two sevoflurane concentrations (1.3 and 4.0% in oxygen) for 20 min. A tail clamp (alligator clip) was applied to the tail for 30 s, and the mice were observed for movement in response to the stimulation. In case of a positive–negative motor response, the anesthetic was increased–decreased in steps of 0.3% until the positive–negative response reversed. Consecutive tail clamps were applied proximal to the previous test site, and only the middle third part of the tail was used for MAC assessment. After reversal of response had occurred, the study ended. The MAC was defined as the concentration midway between the concentration that permitted movement and the concentration that prevented movement. Twenty-minute inhalation periods were allowed for equilibration after each change in anesthetic concentration.[28,29] Body temperature was measured *via* a probe inserted approximately 1 cm rectally.

Experimental Protocols

Assessment of Resting Ventilation and Control Responses to Carbon Dioxide. Breathing was measured while the animals breathed a gas mixture of 21% oxygen in nitrogen. Subsequently, to obtain a measure of the hypercapnic ventilatory response (HCVR), two to three elevations in inspired carbon dioxide were applied (inspired carbon dioxide concentrations: 3, 5, and 7%). When online analysis showed that a ventilatory steady state had not been reached, the duration of hypercapnia

Table 1. Protocols and Number of Animals Involved

	$\mu OR^{-/-}$	$\mu OR^{+/+}$
Respiratory studies at rest*	8	10
Morphine effect on respiration	8	10
Naloxone effect on respiration	6	6
Sevoflurane effect on respiration	8	8
Influence of morphine on anesthetic potency	8	8
Influence of naloxone on anesthetic potency	6	6
Morphine antinociception	8	10

* Without administration of any agent, data were collected on five occasions.
μOR = μ-opioid receptor.

was extended. Minute ventilation, tidal volume, and respiratory frequency were calculated per breath. These data were averaged over 50 breaths and stored for further analysis. The HCVR was determined by the slope of the relation between ventilation and inspired carbon dioxide using least-squares regression analysis. In each animal (table 1), measurements were performed on five separate occasions, with at least 3 weeks between measurements.

Influence of Morphine on Ventilation and Responses to Carbon Dioxide. Morphine (intraperitoneal administration of 6, 20, and 100 mg/kg; volume for each dose, 0.12 ml) and placebo (0.9% NaCl, 0.12 ml) were administered in random order to each animal (table 1), with at least 3 weeks between studies. Twenty minutes after the drug was administered, respiratory measurements as described above were performed.

Influence of Naloxone on Ventilation and Responses to Carbon Dioxide, and the Influence of Morphine in Naloxone Pretreated Animals. After naloxone (intraperitoneal administration of 100 μg/kg, 0.1 ml) respiratory measurements as described above were performed. After a resting period, morphine (intraperitoneal administration of 100 mg/kg, 0.1 ml) plus naloxone (intraperitoneal administration of 100 μg/kg, 0.1 ml) were given and respiratory measurements repeated.

Influence of Sevoflurane on Ventilation and Responses to Carbon Dioxide. After assessment of control respiratory measurements as described above, 1% sevoflurane was added to the gas mixture flowing through the chambers. After an equilibration period of at least 20 min,[28,29] and the observation that ventilation had reached a new steady state, another set of respiratory measurements was obtained.

Morphine Antinociception. After intraperitoneal administration of saline, 6, 20, or 100 mg/kg morphine (volume, 0.12 ml), and an equilibration period of 20 min, the nociceptive tests were performed by a researcher blinded to the doses, with at least 3 weeks between studies (table 1).

Anesthetic Potency. In one set of animals (table 1), baseline anesthetic potency was assessed, followed by MAC determination after the intraperitoneal administration of 20 mg/kg morphine. In another set of animals (table 1), baseline anesthetic potency was assessed, followed by MAC determination after intraperitoneal administration of 100 μg/kg naloxone.

Statistical Analysis

The control respiratory data obtained during air breathing and the slope of the HCVR were compared between genotypes using analysis of variance. Because morphine doses of 6 and 20 mg/kg had little effect on the slope of the hypercapnic ventilatory response curve and respiratory depression (in terms of a depression of the slope of ventilation *vs.* carbon dioxide) was observed only after a dose of 100 mg/kg (see Results), we performed a statistical analysis on the saline and 100-mg/kg morphine data using two-way analysis of variance (factors: genotype and treatment) and least-significant differences test.[30] Data from naloxone-treated mice were compared with data from placebo-treated mice of the same genotype using Student *t* tests. In wild-type animals, the significance of morphine-induced changes in naloxone pretreated mice were tested using paired *t* tests. Sevoflurane-induced changes in respiratory parameters were compared between genotypes with Student *t* tests. Statistical analysis of the analgesic and MAC data was conducted using two-way analysis of variance and least significant differences test. P values < 0.05 were considered significant. All values reported are mean $\pm$ SD.

Results

There were no obvious morphologic or behavioral abnormalities in the animals, apart from a tendency for exaggerated aggressive behavior in the male mutant mice. A total of 20 $\mu OR^{-/-}$ and 22 $\mu OR^{+/+}$ mice were used in the experiments. The two groups were matched with respect to weight and sex. During resting, morphine, and naloxone respiratory studies, the mice were relatively quiescent with their eyes open.

Assessment of Resting Ventilation and Control Responses to Carbon Dioxide

A total of 90 experiments were performed (50 and 40 in wild-type and mutant mice, respectively). Representative sample tracings of respiration during air breathing in $\mu OR^{+/+}$ and $\mu OR^{-/-}$ mice are shown in figure 1. There were subtle differences in breathing pattern between genotypes (values are the mean of animal means obtained at five sessions): tidal volume, 48 $\pm$ 4 μl in $\mu OR^{+/+}$ and 44 $\pm$ 3 μl in $\mu OR^{-/-}$ mice (nonsignificant [NS]); respiratory frequency, 188 $\pm$ 14 breaths/min in $\mu OR^{+/+}$ and 220 $\pm$ 16 breaths/min in $\mu OR^{-/-}$ mice ($P < 0.0001$); ventilation, 8.4 $\pm$ 0.9 ml/min in $\mu OR^{+/+}$ and 9.5 $\pm$ 0.9 ml/min in $\mu OR^{-/-}$ mice ($P = 0.03$). The slope of the ventilatory response to inspired carbon dioxide

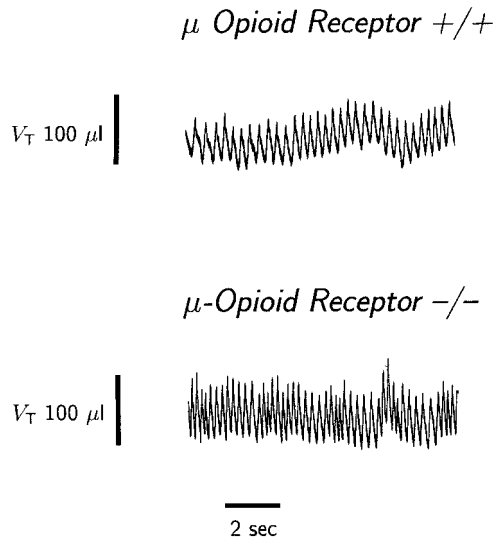

Fig. 1. Representative sample tracings of control (*i.e.*, no drugs administered) respiratory responses to air breathing in a μ-opioid receptor (OR)$^{+/+}$ mouse (*top*) and a μOR$^{-/-}$ mouse (*bottom*). The vertical bars represent a tidal volume of 100 μl. Note the higher breathing frequency in the μOR$^{-/-}$ mouse compared with its wild-type littermate. V_T = tidal volume.

did not differ between genotypes (μOR$^{+/+}$ 1.7 ± 0.4 ml · min^{-1} · %$^{-1}$ *vs.* μOR$^{-/-}$ 1.6 ± 0.8 ml · min^{-1} · %$^{-1}$; NS).

Influence of Morphine on Ventilation and Responses to Carbon Dioxide

Representative sample tracings of the influence of 100 mg/kg morphine on respiration at two levels of inspired carbon dioxide in a μOR$^{+/+}$ mouse and a μOR$^{-/-}$ mouse are given in figure 2. Note the reduced breathing frequency in the μOR$^{+/+}$ mouse relative to the μOR$^{-/-}$ mouse at both carbon dioxide concentrations. Examples of the influence of 100 mg/kg morphine on the HCVR in a mutant mouse and a wild-type mouse are shown in figure 3A. In the mutant mouse, the response to inspired carbon dioxide was not affected by morphine. In contrast, in the mouse with an intact μOR gene, morphine caused the decrease of resting ventilation and the slope of the HCVR. The influences of the three tested morphine doses (6, 20, and 100 mg/kg) and saline on the slope of ventilation *versus* carbon dioxide are shown in figure 4A. In μOR$^{-/-}$ mice, 6, 20, and 100 mg/kg morphine caused no change in slope of the carbon dioxide response curve relative to saline. In μOR$^{+/+}$ animals, morphine at 6 and 20 mg/kg morphine had no effect on the slope of the carbon dioxide response curve relative to saline, whereas an approximately 50% depression was observed at 100 mg/kg.

Table 2 and figure 4B give the averaged effects of 100 mg/kg morphine on respiration relative to saline. Morphine, at a dose at which respiratory depression was obvious in μOR$^{+/+}$ mice, had no effect on any of the measured variables in μOR$^{-/-}$ knockout mice. In wild-type animals, ventilatory depression (as observed by a decrease in ventilation during air breathing) was caused by a reduction of breathing frequency but not tidal volume. The reduction in slope of the HCVR was caused by reductions of the tidal volume and respiratory frequency responses to carbon dioxide.

Influence of Naloxone on Ventilation and Responses to Carbon Dioxide, and the Influence of Morphine in Naloxone Pretreated Animals

μ-Opioid Receptor$^{+/+}$ Mice. Ventilatory parameters after naloxone were as follows: ventilation, 11.6 ± 2.0 ml/min (P = 0.002 *vs.* saline-treated animals of the same genotype); respiratory frequency, 278 ± 40 breaths/min (P = 0.001); tidal volume, 43 ± 11 ml (NS); slope of the HCVR, 2.3 ± 0.5 ml/min per percent carbon dioxide (P = 0.02).

Morphine in naloxone pretreated animals did not affect any of the measured respiratory variables: ventilation, 11.2 ± 5.5 ml/min (NS *vs.* naloxone-treated animals); respiratory frequency, 256 ± 47 breaths/min (NS); tidal volume, 43 ± 15 ml (NS); slope of the HCVR, 2.1 ± 0.6 ml/min per percent carbon dioxide (NS) (figure 4B).

μ-Opioid Receptor$^{-/-}$ Mice. Ventilatory parameters after naloxone were as follows: ventilation, 15.1 ± 1.6 ml/min (P < 0.0001 *vs.* saline-treated animals of the same genotype); respiratory frequency, 294 ± 32 breaths/min (P = 0.002); tidal volume, 52 ± 6 ml (P = 0.01); slope of the HCVR, 2.1 ± 0.3 ml/min per percent carbon dioxide (P = 0.04) (figure 4B).

Influence of Sevoflurane on Ventilation and Responses to Carbon Dioxide

Examples of the influence of 1% sevoflurane on the HCVR in a mutant mouse and a wild-type mouse are shown in figure 3B. In both mice, sevoflurane caused a decrease in resting ventilation and slope of the HCVR.

The changes (Δ) in tidal volume, timing parameters, and ventilation caused by inhalation of 1% sevoflurane did not differ between genotypes: Δ tidal volume, −1 ± 3 and −6 ± 11 μl in μOR$^{+/+}$ and μOR$^{-/-}$ mice, respectively (NS); Δ respiratory frequency, −36 ± 25 and −26 ± 27 breaths/min in μOR$^{+/+}$ and μOR$^{-/-}$ mice, respectively (NS); Δ ventilation, −1.5 ± 1 and −1.3 ± 2 ml/min in μOR$^{+/+}$ and μOR$^{-/-}$ mice, respectively (NS). Relative to control, sevoflurane reduced the slope of the HCVR by approximately 40% in both genotypes (Δ slope = −1.1 ± 0.3 and −1.0 ± 0.3 ml · min^{-1} · %$^{-1}$ in μOR$^{+/+}$ and μOR$^{-/-}$ mice, respectively; NS).

Morphine Antinociception

In both nociceptive tests (hot plate and tail immersion), μOR$^{-/-}$ mice displayed the absence of morphine-induced analgesia. At 6, 20, and 100 mg/kg morphine, latencies for hind paw licking and tail withdrawal were not different from those after saline (fig. 5). In contrast,

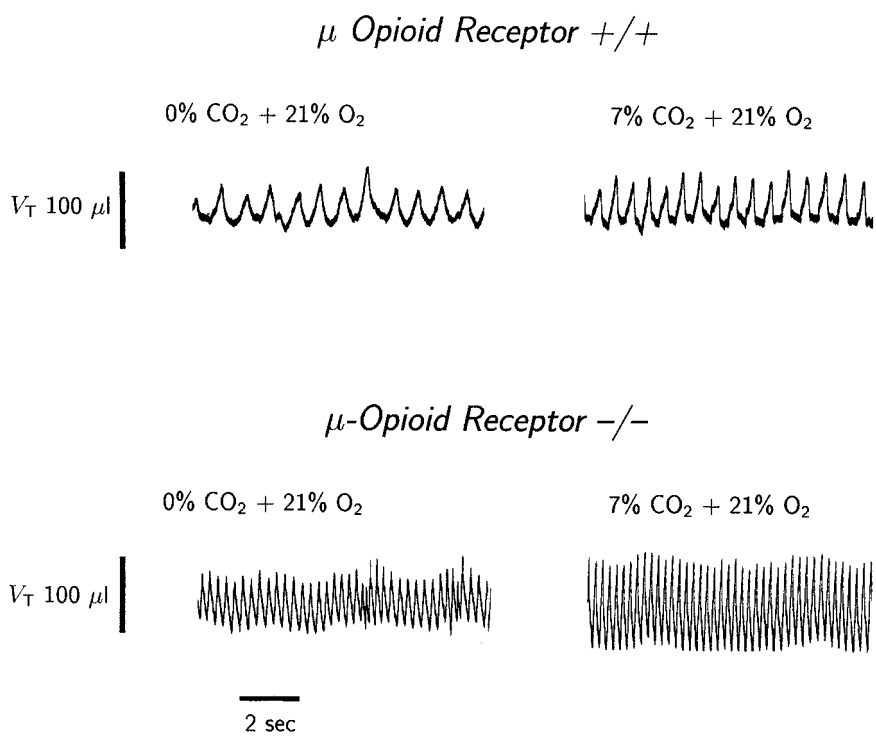

Fig. 2. Representative sample tracings of respiratory responses to two levels of inspired carbon dioxide in a wild-type mouse (*top*) and a μ-opioid receptor (OR) knockout mouse (*bottom*) after intraperitoneal administration of 100 mg/kg morphine. The vertical bars represent a tidal volume of 100 μl. Note the decrease in breathing frequency in the wild-type animal. Breathing frequency in the $\mu OR^{-/-}$ mouse was identical to control values. V_T = tidal volume.

in $\mu OR^{+/+}$ animals, morphine showed a dose-dependent increase in latencies, with cutoff values reached for both tests at 100 mg/kg morphine (fig. 5). At all morphine doses tested, latencies were greater in $\mu OR^{+/+}$ mice compared with $\mu OR^{-/-}$ mice, whereas saline latencies did not differ between genotypes.

Anesthetic Potency

In the first set of mice (table 1), baseline sevoflurane MAC values differed by approximately 20% between genotypes: MAC in $\mu OR^{+/+}$ mice = 2.7 ± 0.2 vol% *versus* $\mu OR^{-/-}$ mice = 3.3 ± 0.5 vol% (P = 0.02). Morphine reduced the sevoflurane MAC by approximately 50% in wild-type animals (MAC after morphine = 1.4 ± 0.4%; $P < 0.001$ *vs.* untreated animals of the same genotype) but did not affect the MAC of sevoflurane in mutant mice (MAC after morphine = 3.6 ± 0.6%; NS *vs.* untreated animals of the same genotype) (figure 6).

In the second set of mice, baseline sevoflurane MAC values differed by approximately 20% between genotypes: MAC in $\mu OR^{+/+}$ mice = 2.9 ± 0.2 vol% *versus* $\mu OR^{-/-}$ mice = 3.5 ± 0.2 vol% (P = 0.02). Naloxone increased the sevoflurane MAC by 18% in wild-type animals (MAC after naloxone = 3.4 ± 0.2%; $P < 0.01$ *vs.* untreated animals of the same genotype) but had no effect in mutant mice (MAC after naloxone = 3.6 ± 0.2%; NS *vs.* untreated animals of the same genotype; fig. 6).

Discussion

Using a knockout mouse model, we examined the influence of the μOR gene product on the control of breathing in the absence and presence of morphine, naloxone, and sevoflurane, and on the anesthetic potency of sevoflurane in the absence and presence of morphine and naloxone. Our main findings are as follows. (1) Relative to mice that do posses the μOR, mice that lack the μ-receptor displayed an approximately 10% greater level of ventilation (because of greater breathing frequencies). The slope of the ventilatory response to inspired carbon dioxide did not differ between genotypes. (2) Morphine, at a dose that caused analgesia and overt respiratory depression in wild-type animals, had no effect on ventilation and the ventilatory response to inspired carbon dioxide in mice with a deficient μOR. (3) Naloxone, at a dose that blocks μ-, κ- and δ-receptors,[31,32] caused an increase in ventilation (by 50–90%) and slope of the ventilatory response to inspired carbon dioxide (by 25–35%) in mice with and without intact μ-receptors. (4) Morphine had no respiratory effect in naloxone-pretreated wild-type mice. (5) The volatile anesthetic sevoflurane caused respiratory depression independent of the μOR. (6) Morphine analgesia was absent in μOR-deficient mice. (7) Anesthetic potency was reduced in $\mu OR^{-/-}$ mice relative to mice that do possess the μ-receptor. (8) While morphine increased and naloxone decreased anesthetic potency in wild-type mice, these agents had no effect in μ-receptor–deficient mice. Our observations indicate an important function for the endogenous opioid system in the physiology of the control of breathing with, however, only a modest role for the μOR gene product. Our findings implicate the μOR as sole mediator of the respiratory and analgesic actions of morphine. They further indicate the modulation of

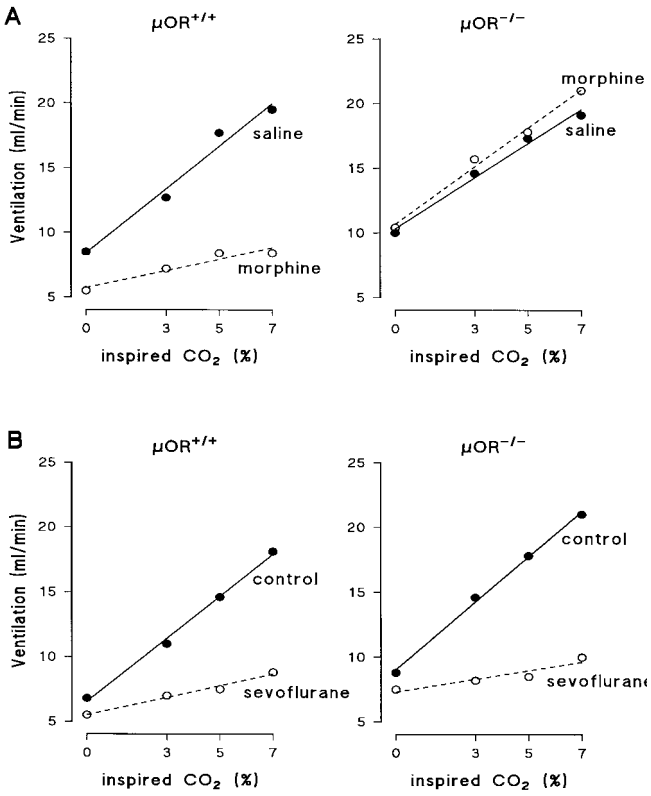

Fig. 3. (*A*) Examples of the influence of 100 mg/kg morphine on the hypercapnic ventilatory response in a mutant mouse (μ-opioid receptor [OR]$^{-/-}$) and wild-type mouse (μOR$^{+/+}$). In the mutant mouse, the response to inspired carbon dioxide was not affected by morphine. In contrast, in the mouse with an intact μOR gene, morphine caused the decrease of resting ventilation and the slope of the hypercapnic ventilatory response. (*B*) Examples of the influence of 1% sevoflurane on the hypercapnic ventilatory response in a mutant mouse (μOR$^{-/-}$) and wild-type mouse (μOR$^{+/+}$) are shown. In both mice, sevoflurane caused a decrease in resting ventilation and slope of the hypercapnic ventilatory response.

anesthetic potency but not anesthesia-induced respiratory depression by the endogenous μ-opioid system. Our studies confirm and extend our earlier studies in the same mouse strain, which showed the involvement of the μOR in morphine spinal and supraspinal analgesia, reward, physical dependence, respiratory depression, and immunosuppression.[18,33,34]

We remained uninformed on the arterial or end-tidal carbon dioxide and oxygen tensions in the animals. As a consequence, we calculated the slope of the HCVR from the inspired carbon dioxide tension. The major drawback of this approach is that a reduction of the slope of the relation between ventilation and inspired carbon dioxide (as was observed after 100 mg/kg morphine in wild-type animals only and during sevoflurane inhalation in both genotypes) may be related not only to opioid- and anesthetic-induced depression of respiratory neurons, but also to a reduction in metabolic rate. The differences in effect of morphine on slope between genotypes should therefore be viewed as the action of the μOR either to cause respiratory depression or to reduce metabolism or, most probably, to both. Note, however, that although at this stage we are unable to discriminate between these two mechanisms, there is ample evidence for a direct respiratory-depressant effect of opioids on brainstem neurons independent of metabolism.[5,8] Hence, it is not unreasonable to attribute the reduction of slope predominantly to an effect of morphine on respiratory neurons in the brainstem. The same reasoning applies to the influences of sevoflurane on slope, although non–μ-opioid mechanisms are responsible for the respiratory effects of sevoflurane. Furthermore, during sevoflurane inhalation, because of overt hypoventilation or atelectasis, the measured carbon dioxide drive may reflect the combination of a hypoxic plus hypercapnic drive. Because this may have occurred

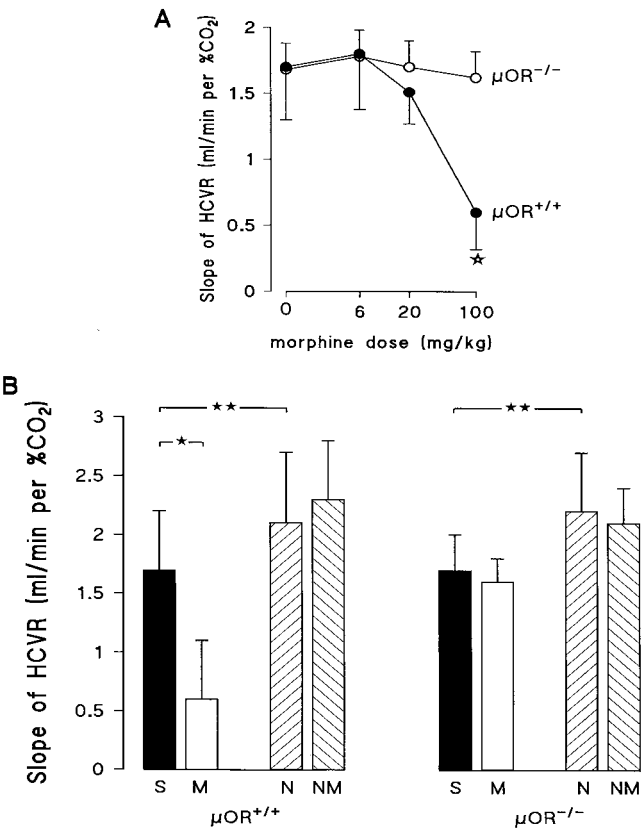

Fig. 4. (*A*) The influence of saline (0 mg/kg morphine) and three morphine doses on the slope of the hypercapnic ventilatory response. Values are mean ± SD. Analysis of variance: **P* < 0.05 *versus* all other treatment levels. (*B*) Mean values ± SD of the slope of ventilation *versus* carbon dioxide in μ-opioid receptor (OR)$^{-/-}$ and μOR$^{+/+}$ mice after various treatments. S = saline; M = 100 mg/kg morphine; N = 100 μg/kg naloxone; NM = combined administration of 100 mg/kg morphine and 100 μg/kg naloxone. Morphine reduced the slope of the hypercapnic ventilatory response (HCVR) in wild-type mice only, whereas naloxone increased the slope in mutant and wild-type animals. Note the absence of effect of morphine after naloxone pretreatment in wild-type animals (pharmacologic knockout). **P* < 0.0001 (paired comparison); ***P* = 0.02 (unpaired comparison).

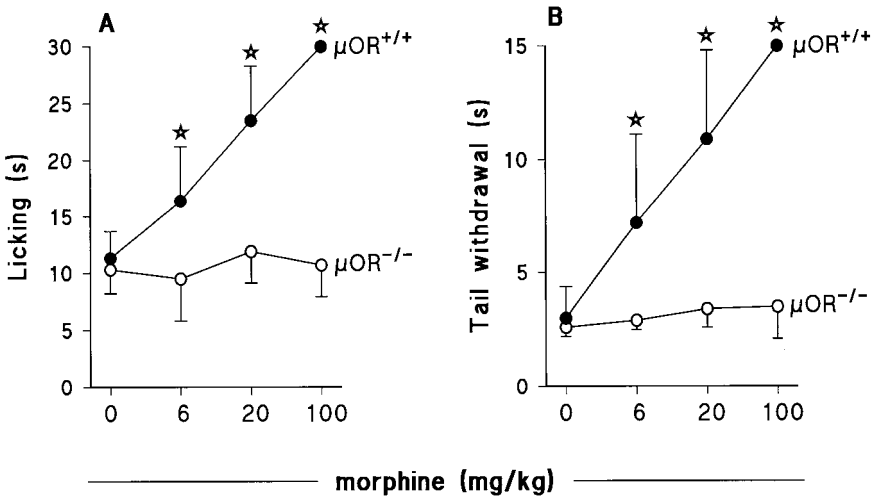

Fig. 5. Morphine antinociceptive responses in μ-opioid receptor (OR)$^{-/-}$ (open circles) and μOR$^{+/+}$ (closed circles) mice (0 mg/kg morphine = NaCl 0.9%). (*A*) Hot-plate test; (*B*) tail-immersion test. Values on the *y*-axis represent the latencies in seconds of the nociceptive thresholds. Cutoff latencies were 30 s for the hot-plate test and 15 s for the tail-immersion test. Values are mean ± SD. **P* < 0.05 *versus* saline and μOR knockout mice (two-way analysis of variance).

in both genotypes, we believe that this did not influence our conclusions.

Our findings of a difference in resting ventilation between the two tested genotypes (table 2) and increase in ventilation after naloxone in both genotypes (fig. 4B) suggest the involvement of the opioid system in the physiology of the control of breathing. The genotype differences and the naloxone effect were primarily in breathing frequency. This is not surprising considering that endogenous opioid peptides and ORs are found in areas of the central nervous system involved in respiratory rhythmogenesis and frequency control, such as the preBötzinger complex (see below).[5] Taking into account the finding that naloxone increased breathing frequency in mice with and without functional μ-receptors, and the small differences in resting values between μOR mutant mice (table 2), our data suggest only a minor role for the μ-opioid system. This may be related to the fact that the knockout mice in our study lacked the μOR gene throughout development. Functional compensation may have (partly) masked the phenotype (*i.e.*, the frequency generated by the respiratory rhythm generator) resulting from the long-term absence of the targeted gene. Furthermore, it may well be that differences in breathing frequencies between genotypes and naloxone effect are accentuated during stressful stimulation of the ventilatory control system, which may occur during exercise, chronic hypoxia, chronic elevations of arterial carbon dioxide concentration, pain, and suffering. For now, our *in vivo* data do indicate the existence of an inhibitory tonic drive on respiratory rhythmogenic neurons from opioid peptides acting predominantly through non-μORs. A plausible candidate receptor is the δOR. Further studies in δOR knockout mice and studies using specific δ-opioid antagonists are needed to elucidate this matter.

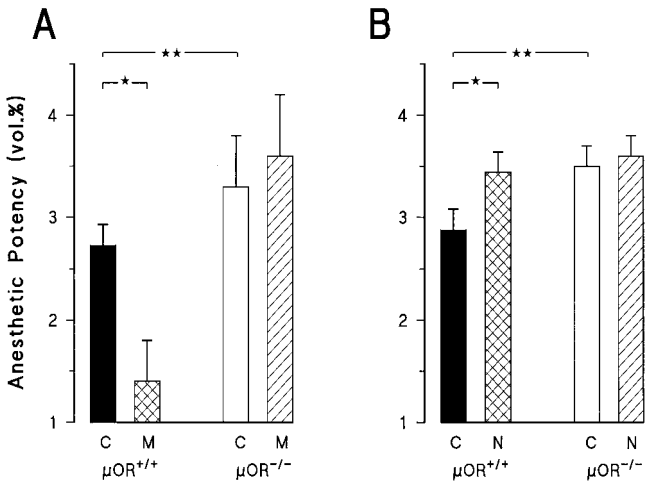

Fig. 6. Anesthetic potency (*i.e.*, sevoflurane minimum alveolar concentration [MAC]) in mutant (μ-opioid receptor [OR]$^{-/-}$) and wild-type animals (μOR$^{+/+}$). (*A*) The influence of morphine on MAC. C = control (no treatment); M = 20 mg/kg morphine. **P* < 0.001; ***P* = 0.02. (*B*) The influence of naloxone on sevoflurane anesthetic potency. C = control (no treatment); N = 100 μg/kg naloxone. **P* < 0.01; ***P* = 0.02. In both data sets, sevoflurane MAC was greater in mutant mice relative to the wild-type animals. In wild-type mice, morphine reduced and naloxone increased the MAC value. In contrast, neither morphine nor naloxone had any effect on MAC in mutant mice.

Morphine, even at 100 mg/kg, had no effect on ventilatory control in mice lacking the μOR. This stands in contrast with the data from μOR-intact mice, showing reduction in ventilation, breathing frequency, and slopes of the ventilation, tidal volume, and respiratory frequency responses to carbon dioxide at 100 mg/kg morphine (table 2 and figs. 2, 3A, and 4). These data show that the μ-opioid gene and receptor are essential in generating the respiratory effects of morphine. Finding no respiratory depression from 100 mg/kg morphine in μOR$^{-/-}$ mice suggests further the absence of involvement of δ- and κ-receptors in morphine respiratory depression or, in analogy to the finding that δ-analgesia is dependent on functional μ-receptors,[33] the need for functional μ-receptors in the production of respiratory depression from μ-receptors.

The site at which morphine induces bradypnea needs

Table 2. Influence of 100 mg/kg Morphine and Saline on Respiration in μ-Opioid Receptor Knockout ($\mu OR^{-/-}$) Mice and Their Wild-type Littermates ($\mu OR^{+/+}$)

	$\mu OR^{+/+}$ Mice		$\mu OR^{-/-}$ Mice		Two-way ANOVA*	
	0.9% NaCl	Morphine	0.9% NaCl	Morphine	Genotype	Genotype × Treatment
V_T (μl)	44.8 ± 7.6	46.4 ± 12.5	44.3 ± 7.2	43.6 ± 10.7	NS	NS
RR (min^{-1})	187 ± 41	124 ± 35†	192 ± 13	192 ± 38	0.03	< 0.01
$\dot{V}$ (ml/min)	8.3 ± 1.9	5.8 ± 2.2‡	8.8 ± 1.3	8.3 ± 3.1	0.05	< 0.01
S ($ml \cdot min^{-1} \cdot \%^{-1}$)	1.7 ± 0.5	0.6 ± 0.5§	1.7 ± 0.3	1.6 ± 0.2	< 0.001	< 0.001

Values are mean of the animal means ± SD.

* *P* values. Treatment effects (least significant differences test): † $P < 0.02$; ‡ $P < 0.01$; § $P < 0.001$.

ANOVA = analysis of variance; NS = not significant; V_T = tidal volume; RR = breathing frequency; $\dot{V}$ = minute ventilation; S = slope of the hypercapnic ventilatory response.

to contain neurons that express μORs and that are involved in the control of breathing frequency. We propose the preBötzinger complex as a possible site of the effect of morphine on breathing frequency. Recently, Gray *et al.*[5] studied the *in vitro* effects of injections of DAMGO, a μOR agonist, into the preBötzinger complex of rodents. DAMGO decreased endogenous respiratory-related rhythm. The preBötzinger complex is part of a narrow column of respiratory neurons in the ventromedial medulla extending from the facial nucleus to the spinal cord (the ventral respiratory group) and involved in respiratory rhythmogenesis and frequency control.[5,35] We are aware that this may be just one of many sites in the peripheral and central nervous system *via* which exogenous administered opioids may exert an effect on the control of breathing (other sites include the locus coeruleus and the tractus nucleus solitarius).[3,4] Note also that the carotid bodies contain μORs.[2] These small organs, part of the peripheral nervous system, hold the peripheral chemoreceptors and are located at the bifurcations of the carotid arteries and are responsible for 20–30% of ventilatory drive at rest and for more than 80% during hypoxia.[36] Further studies are needed to identify sites involved in exogenous opioid-induced reduction of breathing frequency and tidal volume.

Between genotypes, we observed no differences in sevoflurane-induced changes in resting ventilation and slope of the HCVR (fig. 3B). The respiratory depression caused by 1% sevoflurane is in agreement with observations in other species.[37] Anesthetics and opioids affect ventilatory control *via* distinct neuronal pathways. Recent studies indicate that inhalational anesthetics, such as sevoflurane, cause respiratory depression *via* reduction of glutamatergic excitation in the brainstem[38] and possibly also *via* stimulation of an oxygen-sensitive background K^+ channel in type-I cells of the carotid bodies.[39] Our data indicate the absence of functional interaction between the endogenous μ-opioid system and neuronal networks involved in anesthesia-induced respiratory depression. On the other hand, we did observe the modulation—*i.e.,* potentiation—of anesthetic potency (defined as the concentration sevoflurane necessary to suppress a motor response to noxious stimulation of the tail) by μ-opioid-receptors. Mice with active μ-receptors had a 20% lower MAC relative to the $\mu OR^{-/-}$ mice, and naloxone increased the MAC in wild-type animals exclusively (fig. 6). This further indicates the absence of involvement of δ- and κ-ORs in the modulation of anesthetic potency. Our data are in agreement with clinical observations of synergistic μ-opioid–anesthetic interaction on anesthetic potency.[23,24] We hypothesize that the differences in μ-receptor involvement in the respiratory system and in anesthetic potency are a result of different neuronal networks and possibly also different receptor systems involved in anesthesia-induced respiratory depression, and anesthesia-induced suppression of somatic responses (MAC is predominantly mediated by spinal pathways),[40] with absent and active interactions with the endogenous μ-opioid system, respectively.

In conclusion, our data indicate that the endogenous opioid system plays an important role in the control of breathing frequency. The finding that knockout mice, without μ-receptors, were unaffected by morphine, showing neither respiratory depression nor analgesia, confirms the concept that μORs are the essential targets of morphine on both respiratory control and pain response.

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