The pro-nociceptive effects of remifentanil or surgical injury in mice are associated with a decrease in delta-opioid receptor mRNA levels: Prevention of the nociceptive response by on-site delivery of enkephalins

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\textbf{Abstract}

The ultra-short-acting mu-opioid receptor (MOR) agonist remifentanil enhances postsurgical pain when used as main anesthetic in animal models and man. Although the mechanisms involved are poorly characterized, changes in opioid receptor expression could be a relevant feature. Using a mouse model of postoperative pain, we assessed the expression of MOR and delta opioid receptors (DORs) and the efficacy of Herpes Simplex vector-mediated proenkephalin release (SHPE) preventing postoperative nociceptive sensitization induced by remifentanil or surgical incision. We determined MOR and DOR expressions in the dorsal root ganglia and the spinal cord after remifentanil or surgery in CD1 mice, using real-time PCR and Western blotting. We also assessed the effect of SHPE on nociception induced by remifentanil, surgery, and their combination (2 and 7 days after manipulation), using thermal and mechanical tests. Both remifentanil and surgery decreased DOR mRNA levels (up to days 2 and 4, respectively) in the dorsal root ganglia, but not in the spinal cord. No changes were observed in MOR mRNA, or in receptor-protein levels (Western) of either receptor. Pre-treatment with SHPE 7 days before manipulation prevented remifentanil-induced thermal hyperalgesia and mechanical allodynia and the increase in incisional pain observed when surgery was performed under remifentanil anesthesia. SHPE also prevented surgically induced allodynia but not hyperalgesia, which was blocked by the additional administration of RB101, an enkephalinase inhibitor. The study suggests that down-regulation of DOR contributes to remifentanil and surgery-induced nociception, and that postoperative pain is completely reversed by increasing enkephalin levels in the spinal cord and the periphery.

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1. Introduction

Short-acting MOR-agonists are analgesic drugs commonly used as anesthetics; however these drugs also induce delayed pro-nociceptive effects, increasing pain intensity and analgesic requirements in the postoperative period in humans [1]. Inflammatory injury and MOR agonists induce similar neuroplastic changes [34], responsible for the increase in acute [22] and chronic [8] pain after injury. In a mouse model of postoperative pain, we have shown that post-incisional hyperalgesia and allodynia are enhanced when the ultra-short-acting opioid remifentanil is administered during anesthesia [6].

The activation of MOR and DOR by endogenous agonists has been implicated in the suppression of different pain-related behaviors [9,16]. Some authors propose structural and functional interactions between these receptors [13,36], and experiments performed in MOR knockout mice suggest that MORs are required for the analgesic effects of DOR agonists [32,40]. However, MOR activation is also essential for the manifestation of the pronociceptive effects of opioids [26]. Additionally, increased [23] or decreased [37] DOR levels have been reported in animal models of neuropathic and inflammatory pain. Since receptor expression, rather than ligand availability, seems to be responsible for the antinociceptive effects of endogenous opioids [5], we undertook the present study to clarify the role of MOR and DOR in nociceptive sensitization after surgical incision or remifentanil administration. Our results show that both remifentanil and incision significantly...
decrease DOR mRNA levels in the dorsal root ganglia, while MOR mRNA remains unaltered. Overstimulation of DOR by enkephalins seemed a logical way to compensate the reduction of DOR mRNA in our model.

The Herpes Simplex Virus-based vector expressing pro-enkephalin (SHPE) induces antinociception in animal models of inflammatory [15], neuropathic [17,25] and cancer pain [14]. The vector is released both peripherally [2] and in the spinal cord [28]. Enkephalins are endogenous opioid peptides with a higher affinity for DOR than MOR [19]. Increased levels of enkephalins can also be achieved by preventing their catabolism at the synapses [11]. Compound RB101 is a complete inhibitor of neutral endopeptidases and aminopeptidase-N that induces short-lived but efficient antinociception in different animal models, and enhances DOR-agonist analgesia [33]. Aiming at intensifying antinociception, we combined the SHPE vector and RB101. We hypothesized that increased levels of enkephalins could prevent the pronociceptive effects of surgery when performed under remifentanil anesthesia, in a mouse model of postoperative pain that mimics human clinical conditions.

2. Methods

2.1. Animals

CD1 male mice (25–30 g) obtained from Charles-River (CRIFFA, France) were used in all experiments. Animals were housed five per cage and maintained in a room with a 12 h light–dark cycle (light between 8 AM and 8 PM), at controlled temperature (21 ± 1 °C) and humidity (55 ± 10%). Food and water were available ad libitum except during behavioral evaluation. All procedures and animal handling met the guidelines of the International Association for the Study of Pain [41] and the European Communities directive 86/609/EEC regulating animal research. The protocol used in the study was endorsed by the Ethical Committee of the institution (CEEA-PRBB, Comité Ético de Experimentación Animal PRBB, Barcelona, Spain).

2.2. Surgery

We used the incisional postoperative pain model recently validated in our laboratory [6]. Animals were anesthetized with sevoflurane delivered for 30 min via a nose mask (induction, 3.5 %/v/v; surgery, 3 %/v/v) in a sterile operating room. A 0.7 cm longitudinal incision was made with a number 20 blade through the skin and fascia of the plantar surface of the right hind paw, starting 0.3 cm from the proximal edge of the heel extending toward the toes. The underlying plantaris muscle was exposed and incised longitudinally, keeping the muscle origin and insertion intact. After hemostasis with slight pressure, the skin was closed with two 6-0 silk sutures and the wound covered with povidone–iodine antiseptic ointment. After surgery, the animals were allowed to recover under a heat source in cages with sterile bedding. Control animals (non-operated mice), underwent a sham procedure that consisted of the administration of anesthesia (sevoflurane ± saline or remifentanil) for 30 min, without incision.

2.3. Drug administration

Remifentanil (Ultiva®; GlaxoSmithKline, Madrid, Spain) and sevoflurane (Sevorange®; Abbot Laboratories SA, Madrid, Spain) were supplied by the Department of Anesthesiology of the Hospital del Mar (Barcelona, Spain). Remifentanil (0.04 mg/kg) was dissolved in saline (NaCl 0.9%) and infused subcutaneously over a period of 30 min (rate 0.8 ml/h) using a Harvard Apparatus pump (Biosis S.L., Biologic Systems, Barcelona, Spain). Control animals received the same volume of saline in identical conditions. This dose of remifentanil has been shown by our group to induce delayed pronociceptive effects in the same strain of mice [6].

RB101 (N-[(R,S)-2-benzyl-3-(S)-(2-amino-4-methylthio)butyl-dithio]-1-oxopropyl]-l-phenylalanine benzyl ester) was provided by Prof. B.P. Roques. The drug was dissolved in 0.3 ml of vehicle containing ethanol (10%), Cremophor EL (10%) (Sigma Chemicals, Saint-Quentin-Fallavier, France), and distilled water (80%), and injected intravenously at a dose of 20 mg/kg, 5 min before nociceptive testing. Control animals received vehicle alone in identical conditions. The dose of RB101 was selected on the basis of previous studies in mice that show the antinociceptive effects of the drug in the presence of thermal stimulation [33].

2.4. Messenger RNA quantification by real-time PCR

Messenger RNA levels of MOR and DOR were determined in different experimental conditions (see below). Tissues from the lumbar spinal cord and dorsal root ganglia were removed after sacrifice, and frozen in liquid nitrogen. All tissues were homogenized in ice-cold buffer (homogenizer Ultra-Turf, T8; Ika Werke, Staufen, Germany) and the total RNA isolated with TRIzol (Invitrogen, Renfrewshire, England).

2.4.1. Reverse transcription

In all experiments, 2 µg of total RNA was transcribed into cDNA using SuperScript II RNAse H– reverse transcriptase (Invitrogen, Renfrewshire, England).

2.4.2. TaqMan probe real-time polymerase chain reaction (PCR)

The expression of the MOR and DOR was determined by relative real-time PCR using pre-made TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA) for these genes (Mm00440568_m1 (MOR), Mm01180757_m1 (DOR)). A probe against PGK-1 (Mm00435617_m1) was used as endogenous control. PCR reactions were set up in 384-well plates containing the corresponding cDNA, 2X universal master mix (Applied Biosystems, Foster City, CA), the forward and reverse primers and the TaqMan probe. The assays were conducted using the Applied Biosystems ABI PRISM 7900HT Sequence Detection System. Relative expression of the target genes was calculated by means of the comparative threshold cycle (CT) method [29].

2.5. Protein analysis by Western blot

After sacrifice, tissues from the lumbar spinal cord and dorsal root ganglia were removed and frozen in liquid nitrogen. Tissues were homogenized in a SDS sample buffer containing proteinase inhibitors. For Western analysis, protein samples were separated on a SDS–PAGE gel and transferred to PVDF filters. The filters were incubated with polyclonal anti-MOR and DOR antibodies (Chemicon Intl, Inc, Temecula, CA) followed by an HRP-conjugated secondary antibody (Amersham, Barcelona, Spain), developed in ECL solution (DuPont NEN, Boston, MA) and exposed onto hyperfilms (Amersham, Barcelona, Spain). To normalize the immunoreactive band density for minor variations in protein loading, all membranes were stripped and re-probed for β-actin (Sigma, Saint Louis, MO). Densitometry on scanned films was performed using QuantityOne software (BioRad, CA).

2.6. Tissue collection

The lumbar section of the spinal cord and L4–L6 dorsal root ganglia were removed after sacrifice, and frozen in liquid nitrogen.
In the experimental groups where animals had a surgical incision in the right hindpaw, the right hemi-cord and the right dorsal root ganglia were used for PCR and Western blotting, while the left hemi-cord and ganglia were used as control. In addition, sham-operated animals were also used for comparison.

2.7. Vector construction and inoculation

The vector SHPE expressing proenkephalin and control vector SHZ expressing lacZ were constructed as previously described [15]. Both the SHPE and SHZ were isolated and purified at $1.2 \times 10^4$ and $3.5 \times 10^5$ plaque forming units (p.f.u.)/ml, respectively. Under sevoflurane anesthesia, mice received a subplantar injection of 10 µl phosphate-buffered saline (PBS), containing $1 \times 10^3$ p.f.u./ml of either SHPE or SHZ. The vectors were injected in the subcutaneous tissue of the plantar aspect of the right hind paw, 7 days before surgery. The investigator performing the experiments was blinded to the injected vector (SHPE, or SHZ).

2.8. Nociceptive behavioral testing

Hyperalgesia to noxious heat stimulus and hyperalgesia to punctuated stimulus (which will be referred as mechanical allodynia throughout the text) served as measures of nociception (pain). We used the following nociceptive tests:

2.8.1. Heat hyperalgesia measurement

Heat hyperalgesia was evaluated as previously described [18]. Paw withdrawal latency in response to radiant heat was measured using the plantar test equipment (Ugo Basile, Varese, Italy). Briefly, mice were placed in Plexiglas® boxes (20 cm high, 9 cm diameter) positioned on a glass surface. Animals were habituated to the environment for 1 h before testing. The heat source was then positioned under the plantar surface of the hind paw and activated with a light beam intensity that in preliminary studies gave baseline latencies of 9–11 s in control mice. A cut-off time of 20 s was used to prevent tissue damage in the absence of a response. The mean paw withdrawal latencies for both hind paws were obtained from the average values of three separate trials, taken at 5–10 min intervals to prevent unspecific behavioral responses. When RB101 was administered, the plantar test was performed in hand-held mice, due to the pro-kinetic effects of the drug [10]. In this experiment the final value (average response) was obtained from the first two responses, due to the short-lasting effect of RB101; measurements of each hind paw were made alternately.

2.8.2. Mechanical allodynia measurement

Mechanical nociceptive thresholds were evaluated by measuring the hind paw withdrawal response to von Frey filaments stimulation [7]. Animals were placed in a Plexiglas® box (20 cm high, 9 cm diameter) with a wire grid bottom through which the von Frey filaments (bending force range from 0.008 to 2 g) (North Coast Medical, Inc., San Jose, CA) were applied. Animals were allowed to habituate for 1 h before testing in order to achieve immobility. The filament of 0.4 g was first used; then the strength of the next filament was increased or decreased according to the response. The upper limit value (2 g) was recorded even if there was no withdrawal response to this force. The threshold of response was calculated using the up-down method [7]. Clear paw withdrawal, shaking or licking were considered nociceptive-like responses. Both hind paws were alternately tested.

2.9. Experiments performed

All experimental groups received the same inhaled concentration of sevoflurane (3.5–3 %v/v) for 30 min. Special care was taken to reduce inter-individual variability and to use the smallest number of animals per group. In behavioral experiments, the animals were handled daily by the investigator and habituated to the experimental room environment (testing equipment without nociceptive stimulation) for a period of 1 week before beginning the actual testing.

We performed the following groups of experiments: Group 1: Determination of mRNA levels of MOR and DOR in the spinal cord and dorsal root ganglia, after the surgical incision (performed under sevoflurane anesthesia) or the infusion of remifentanil. After the procedure, animals were sacrificed at the following times: 4 h, and 2, 4 and 7 days. mRNA levels of both receptors were also determined 9 days after SHZ/SHPE inoculation. In order to assess mRNA levels of MOR and DOR in animals that had surgery under remifentanil anesthesia, we performed additional experiments in which animals were sacrificed on day 2 after manipulation. We used 4–5 animals per sample, and each experiment was repeated four times.

Group 2: Expression of MOR and DOR-protein in the spinal cord and dorsal root ganglia after the surgical incision or remifentanil infusion. The animals were sacrificed 2 and 5 days after the procedure and Western blot performed from tissue samples obtained from three animals. Experiments were repeated three times.

Group 3: Antinociceptive effects of the SHZ or SHPE vectors on thermal hyperalgesia and mechanical allodynia. Animals were tested 2 and 7 days after one of the following treatments, administered over a period of 30 min:

- sevoflurane + saline, a treatment that does not alter nociceptive thresholds [6]
- sevoflurane + infusion of remifentanil
- surgery performed under sevoflurane + saline anesthesia, and
- surgery performed under sevoflurane + remifentanil anesthesia.

After the habituation period, a baseline response was obtained in all experiments, as the average of the measures of three consecutive days in the following sequence: von Frey and plantar test. Afterwards, animals were injected either with the SHZ or with SHPE vectors into the right hind paw. Seven days later, a plantar incision (or sham operation) was performed in the same paw ($n = 5–8$ animals per group) under sevoflurane ± remifentanil anesthesia. Mice were tested using the same sequence as for the evaluation of baseline responses. The intra-operative analgesic effects of remifentanil were not assessed in the present investigation. The pro-nociceptive effects of the treatments were evaluated at time-points where previous experiments done in our laboratory have demonstrated the presence of hyperalgesia and allodynia [6].

Group 4: Effects of RB101 on incision-induced heat hyperalgesia in animals pretreated with SHZ or SHPE. Sham-operated animals served as control. The plantar incision was performed 7 days after vector inoculation, and the RB101 administered intravenously 2 days after surgery. Thermal hyperalgesia was evaluated 5 min after the injection of the drug ($n = 9–11$ animals per group).

2.10. Statistical analysis

In SHZ and SHPE-treated animals, a general linear-mixed model [35] was used to estimate the overall (days 2–7) pronociceptive effects induced by remifentanil, the surgical incision, and their combination (behavioral data). The overall magnitude of the treatment or manipulation was calculated by subtracting the area under the curve (AUC, values from days 2–7 after the procedure) obtained in the saline control group from the value obtained in each experimental condition. For the analysis of the data, the results are expressed as percentages (see Table 1). The statistical analysis was performed with S-PLUS functions using the nlme library [35].
model allowed multiple between-group comparisons to assess the effects of the treatments (remifentanil, surgery), the time, and the vectors (SHPE, SHZ) on the responses. The analysis also includes the evaluation of the interaction between the different factors and the animal hind paws (left vs. right), a factor particularly influenced by the incision. The time effect assesses differences at any day with respect to baseline values determined before vector inoculation, infusion and/or surgery. Estimation of coefficients, and their associated p values, was based on restricted maximum likelihood. A p value less than 0.05 was considered statistically significant.

The general linear-mixed model was also used to analyze the results of the real-time PCR experiments, in order to take into account the values of the triplicates performed with each sample. This allowed multiple comparisons between control and target samples, achieving greater statistical potency. The relative mRNA expression of the target genes was calculated using the comparative threshold cycle (CT) method, and the standard error of the fold change obtained using the Greenwood formula [4]. A p value less than 0.05 was considered statistically significant.

In Western blotting, the results are expressed as the mean of the intensity of the protein bands of three samples ±SEM, previously normalized to β-actin. For each membrane, statistical significances were assessed using the one-way analysis of variance (ANOVA). p values below 0.05 were considered significant.

### 3. Results

#### 3.1. Messenger RNA levels of MOR and DOR receptors in the spinal cord and dorsal root ganglia, in the different experimental conditions

These experiments were performed to assess whether the administration of remifentanil or the surgical incision would induce changes in the expression of DOR or MOR mRNA. In basal conditions, DOR mRNA levels in the dorsal root ganglia and spinal cord were 5.8 ± 0.93 (p < 0.001) and 11.78 ± 3.38 (p < 0.01) times higher than those of MOR (Fig. 1). The infusion of 0.04 mg/kg of remifentanil over a period of 30 min induced a significant decrease in DOR mRNA in the dorsal root ganglia at 4 h (36% decrease, p < 0.01 when compared to baseline) and 2 days (47% decrease, p < 0.001) after administration (Fig. 2); the values returned to baseline on day 4. No significant changes in MOR mRNA levels were observed.

In the spinal cord, DOR or MOR mRNA expression was unaltered after remifentanil infusion (data not shown).

After surgical incision, a significant decrease in DOR mRNA expression was observed in the ipsilateral dorsal root ganglia on days 2 (56% decrease, p < 0.001) and 4 (73% decrease, p < 0.001) after surgery (Fig. 2); the reduction observed 4 h after surgery (40% decrease) did not reach statistical significance (p = 0.05062), and DOR mRNA levels returned to baseline values at day 7. No changes were observed in the contralateral dorsal root ganglia. The surgical incision did not induce any changes in MOR mRNA levels in the dorsal root ganglia. In the spinal cord, the levels of mRNA of DOR and MOR were not affected by the surgery (data not shown).

When surgery was performed under remifentanil anesthesia, DOR and MOR mRNA levels were similar to those observed in ani-
mals receiving a single treatment (remifentanil or surgery). In these experimental conditions, we observed a 56% decrease in DOR mRNA expression in the ipsilateral dorsal root ganglia, 2 days after surgery (p < 0.001 compared to baseline).

In order to rule out that the SHPE/SHZ vectors could have an effect on opioid receptor expression, we determined DOR and MOR mRNA in the dorsal root ganglia and spinal cord of control animals, 2 days after sham operation (9 days after inoculation of the vectors, see Section 2). No changes in mRNA levels of MOR and DOR were seen in vector-injected animals when compared with the non-injected controls.

3.2. Effects of surgical incision and remifentanil infusion on the total DOR and MOR protein expression in the spinal cord and dorsal root ganglia

A complementary Western blotting protein analysis was performed to investigate whether the changes in the mRNA levels after remifentanil or incision would alter the total protein expression of MOR and DOR. In contrast to mRNA levels, no significant changes were observed in the dorsal root ganglia (Fig. 3) or the spinal cord, in the intensity of the protein bands (int. × mm) detected for DOR (approximately 84 and 41 KDa), 2 or 5 days after opioid administration or surgical incision. Likewise, MOR protein (54 KDa) did not change in any of the samples tested (data not shown).

3.3. Effects of the administration of SHZ and SHPE vectors on thermal hyperalgesia and mechanical allodynia

In these experiments, animals received an intra-plantar injection of SHZ or SHPE vector seven days before remifentanil and/or surgical incision; hyperalgesia and allodynia were tested 2 and 7 days later (Fig. 4 and Table 1). In sham-operated animals pre-treated with SHZ (Fig. 4A: Sham + Saline), sevoflurane did not induce significant changes in nociceptive thresholds in the plantar (mean values = 10.8 ± 0.52 s) and von Frey (mean values = 1.34 ± 0.07 g) tests. The administration of remifentanil induced a sustained and generalized mechanical allodynia in both paws (von Frey, p < 0.05) lasting over 7 days (Fig. 4A: Sham + Remifentanil); thus, 46% of the mechanical allodynia observed on day 2 was still present 7 days after remifentanil administration (p < 0.05; Fig. 4A: Sham + Remifentanil). A generalized thermal hyperalgesia was also observed after remifentanil (plantar test, p < 0.05). The decrease lasted over 1 week, since 52% of the thermal hyperalgesia present on day 2 was still present 7 days after opioid administration (p < 0.05). Remifentanil-induced pronociceptive effects were observed as early as 4 h after infusion with reductions of 42% and 55% in thermal and mechanical thresholds, respectively (p < 0.01). Animals were also hyperalgesic 4 days post-infusion as reported in a previous study by our group [8] with reductions of 27% and 45% for thermal hyperalgesia and mechanical allodynia [8]. No differences between the right and left paws were observed in any of the non-operated groups.

In animals pre-treated with SHZ, the surgical incision performed under sevoflurane anesthesia induced thermal hyperalgesia and mechanical allodynia in the operated paw (p < 0.05) that was completely recovered 7 days after surgery (Fig. 4A: Incision + Saline). The incision-induced pronociceptive effects were also observed as early as 4 h after surgery (with reductions of 75% and 89% in thermal and mechanical thresholds, respectively, p < 0.01). When surgery was performed under remifentanil anesthesia (Fig. 4A: Remifentanil + Incision), a significant increase in the magnitude and duration of postoperative hyperalgesia (49% overall reduction of nociceptive threshold) and allodynia (43% reduction) was observed when compared to the group that had surgery under sevoflurane anesthesia (p < 0.05). In these animals, 72% of the thermal hyperalgesia and 85% of the mechanical allodynia present in day 2 continued to be present 7 days after surgery (p < 0.05 in both tests). Significant mechanical allodynia lasting over 7 days (74%, p < 0.05) was also observed in the contralateral paw; no significant interaction between remifentanil and incision was present in the plantar or von Frey tests.

In animals pre-treated with the SHPE vector, a slight increase (not statistically significant) in nociceptive thresholds was observed in sham-operated mice (Table 1 and Fig. 4B: Sham + Saline) when compared to animals pre-treated with the SHZ control vector. Therefore, in control conditions, the SHPE vector did not induce relevant changes in nociceptive thresholds.

In sham-operated animals, pretreatment with the SHPE vector completely prevented remifentanil-induced thermal hyperalgesia and mechanical allodynia (Fig. 4B: Sham + Remifentanil). The SHPE vector also prevented mechanical allodynia induced by the surgical incision performed either under sevoflurane (Fig. 4 Incision + Saline) or remifentanil anesthesia (Fig. 4 Remifentanil +...
Incision). However, the SHPE vector did not prevent incision-induced thermal hyperalgesia, which was unaltered regardless of the type of anesthesia. Thus, in SHPE-treated animals, the incision-induced thermal hyperalgesia was not increased by remifentanil anesthesia (Fig. 4B: Remifentanil + Incision).

3.4. Effect of RB101 on the incision-induced thermal hyperalgesia, in animals pretreated with SHZ or SHPE

Since at the doses tested the SHPE vector did not prevent incision-induced thermal hyperalgesia, we assessed if the acute

Fig. 3. Effects of remifentanil and incision on total DOR-protein expression in the dorsal root ganglia. The total DOR-protein was determined by Western blotting in basal conditions and 2 or 5 days after opioid administration or surgical incision. Remifentanil (0.04 mg/kg) or saline were infused subcutaneously during a period of 30 min, and incision was performed on the right hind paw. Every lane corresponds to different samples, each one from a pool of three animals. Intensity of the protein bands is expressed as mean relative values with respect to the control samples (which values are set to 1). Immunoreactive band densities were standardized to β-actin. No significant changes were observed in the intensity (int. × mm.) of the 84 and 41 KDa protein bands of DOR.

Fig. 4. Effects of remifentanil or surgical incision on nociceptive thresholds in mice pre-treated with the inactive-SHZ (A) or active-SHPE (B) viral vectors. Nociceptive thresholds in both hind paws were evaluated by the plantar and Von Frey tests, on days 2 and 7 after opioid administration and/or incision. Surgery was performed in the right hindpaw (filled dots), while the left (unfilled dots) was used as control. Results are expressed as mean values obtained from 5 to 8 mice, and the vertical bars indicate the SEM. Remifentanil (0.04 mg/kg) or saline was infused subcutaneously over a period of 30 min. Sham-operated animals had general anesthesia (sevoflurane ± remifentanil) without surgery. The SHZ or SHPE (1 × 10⁶ p.f.u.) vectors were inoculated in the plantar aspect of the right hindpaw, 7 days before the experiments. The shadowed area indicates a significant pronociceptive effect.
administration of the enkephalinase inhibitor RB101 would enhance the anti-hyperalgesic effects of the active vector in the plantar test (Fig. 5). Experiments were performed on day 2 because the greatest pronociceptive effects of remifentanil and surgical incision are observed at this time point. Due to the pro-kinetic effects of RB101 [10], thermal hyperalgesia was assessed in hand-held animals (i.e. not restrained in polyethylene frames). In these experimental conditions, the SHPE vector did not change nociceptive thresholds in the plantar test as compared to the SHZ control group, supporting the results obtained in freely moving animals. The administration of RB101 to animals pre-treated with SHPE induced a slight but significant antinociceptive effect in the right (26.2% decrease, \( p < 0.05 \)) or SHPE (22.0% decrease, \( p < 0.05 \)) hindpaw 7 days before incision (same paw). RB101 (20 mg/kg) or vehicle was administered intravenously 5 min before testing. The letter a designates a \( p < 0.05 \) when compared to the control group (SHZ + Vehicle + Sham), and b indicates a \( p < 0.05 \), when comparing the left and right paws in the same group.

**Fig. 5.** Effects of the combined administration of SHPE and RB101 on thermal hyperalgesia induced by surgical incision. Nociceptive thresholds (plantar test) in both hind paws were measured on day 2 after incision in hand-held animals. Results are expressed as mean values of 7–9 animals per group, and the vertical bars indicate the SEM. The SHZ or SHPE vectors (1 x 10^6 p.f.u.) were inoculated in the right hindpaw 7 days before incision (same paw). RB101 (20 mg/kg) or vehicle was administered intravenously 5 min before testing. The letter a designates a \( p < 0.05 \) when compared to the control group (SHZ + Vehicle + Sham), and b indicates a \( p < 0.05 \), when comparing the left and right paws in the same group.

4. Discussion

In a mouse model of postoperative pain, we have recently shown that surgical injury (plantar incision) or remifentanil administration (0.04 mg/kg infused over 30 min) induced significant postoperative hyperalgesia and mechanical allodynia that lasted up to 7 days after manipulation [6]. Using the same model, the present investigation shows that similar treatments (incision and/or remifentanil) induce a significant decrease in DOR mRNA in the dorsal root ganglia that coincides with the early stages of nociceptive sensitization observed in the behavioral studies. The decrease in DOR mRNA levels did not correspond with changes in total DOR-protein expression in the dorsal root ganglia or in the spinal cord; however, changes in protein levels at the injured site cannot be excluded from the present data. Nociceptive sensitization induced by remifentanil, surgical incision, or their combination was completely prevented by increasing enkephalin levels through the administration of the SHPE vector (expressing the pro-enkephalin gene) plus RB101, a drug that blocks enkephalin inactivation. The results suggest that enkephalergic pathways play an important role in postoperative pain sensitization, suggesting novel therapeutic alternatives to improve postoperative analgesia.

Our aim was to assess if the same manipulations that induced nociceptive sensitization in the behavioral experiments (surgical incision and/or remifentanil) would alter MOR and DOR expressions in the peripheral (dorsal root ganglia) and central (spinal cord) nervous system. We observed that in untreated mice, the levels of DOR mRNA were significantly higher than those of MOR mRNA, both in the dorsal root ganglia and in the spinal cord. These findings are supported by in situ hybridization experiments in rat lumbar dorsal root ganglia that showed a higher expression of DOR in small and large neurons [39].

The decrease in DOR mRNA levels had an earlier onset (4 h) and a shorter duration (2 days) after remifentanil administration than after the incision (Fig. 2), and returned to baseline levels at 4 and 7 days, respectively. This decrease shows a positive correlation with the behavioral changes (hyperalgesia/allodynia) observed after similar treatments (Fig. 4A). The evaluation of the correlation between behavioral (Fig. 4) and DOR mRNA (Fig. 2) data was calculated using the 4 h, 2 and 7 days time-points. After incision, the coefficient correlation values (\( R^2 \)) were 0.92 and 0.68 for thermal hyperalgesia and mechanical allodynia, while after remifentanil infusion they were 0.52 and 0.98, respectively. The present results suggest that the suppression of DOR may contribute to opioid- and injury-induced pain sensitizations. On the behavioral experiments (Fig. 4, panel A), we observed a summation of effects in the remifentanil + incision group. However, the decrease in DOR mRNA levels observed in this group was of a similar magnitude to that when remifentanil and incision were assessed individually (Fig. 2). Thus, at this time point, reductions in DOR mRNA induced by each component are not additive, probably reflecting the limit or greatest extent of this alleged pronociceptive mechanism (in Fig. 2, reductions in DOR mRNA levels after incision in days 2 and 4 are not significantly different from each other). Our results are supported by recent studies performed in knockout mice for the DOR gene, reporting enhanced thermal hyperalgesia and mechanical allodynia in models of neuropathic [31] and inflammatory [12] pain. Moreover, a down-regulation of DOR protein in the dorsal root ganglia and the spinal cord has been described in models of inflammatory and neuropathic pain in rats [21,37]. Thus, while inflammatory or nerve injury has been reported to decrease DOR expression, our studies show for the first time that such an effect is observed after the administration of an opioid. However, when DOR protein was measured at 2 and 5 days after treatment in the dorsal root ganglia or the spinal cord, no changes were observed. This finding may have different possible explanations including an insufficient sensitivity of the Western blot technique to detect small decreases of DOR protein, a preferential axonal transport to the periphery, and the fact that DOR-protein levels may be maintained at constant levels due to the translation of DOR mRNA transcripts to compensate DOR-protein degradation. However, from the present experiments, a decrease in DOR-protein expression induced by surgery and/or remifentanil administration cannot be excluded.

Since DOR mRNA expression is reduced at approximately the same time than the manifestation of the pronociceptive effects of remifentanil or incision, we assumed that DOR de novo syntheses was also decreased (even if our Western blot results were negative). We attempted to compensate the DOR deficit by increasing the levels of enkephalins injecting a herpes simplex-derived vector (SHPE) that over-expresses enkephalins in the sensory neurons of the dorsal root ganglia, and induces nalozone-reversible antinociception in different animal models [14,15,17]. Furthermore, microdialysis experiments demonstrate that the SHPE vector induces the release of enkephalins in the spinal cord and the periphery [2]. Thus, in our experimental conditions, both spinal and peripheral
enkephalin release are likely to be present. However, since the reversal of the pronociceptive effect of systemic remifentanil after the injection of the SHPE vector was observed in both hindpaws (bilaterally), the results suggest a predominant release of enkephalins in the spinal cord. Enkephalins derived from the SHPE vector bind to spinal DOR and MOR, and could prevent changes in the central processing of sensory information involved in pain sensitization. It could be hypothesized that the increased enkephalin levels in the spinal cord would partially prevent the ascending stimulation, disrupting the spinal-bulbospinal loop involved in nociceptive sensitization [38]. The hypothesis is supported by the finding that spinal administration of DOR and MOR-agonists prevents substance P release in primary afferents [24], or could even displace substance P from NK-1 receptors [20].

SHPE also prevented surgically induced mechanical allodynia but not thermal hyperalgesia, which was completely reversed after co-administration of the enkephalainhase vector RB101 [11]. It is interesting to note that while the SHPE vector has mainly anti-hyperalgesic/anti-allodynic properties, RB101 can induce antinoiceptiception. It is has been postulated that after inoculation of the viral vector, enkephalin outflow to peripheral or spinal cord nerve terminals is conditioned by a prior stimulation of the nerve fibers [2]; in contrast, RB101 enhances the effect of extracellular enkephalins [27] without depending on a prior stimulus, inducing spinal and supraspinal analgesia.

The lower efficacy of enkephalins preventing surgical thermal hyperalgesia could be explained by the diversity of mechanisms and pathways involved in postoperative pain. However, down-regulation of DOR mRNA seems to be a relevant factor, since pretreatment with small interfering RNA to DOR blocks the anti-hyperalgesic effects of delta agonists in a dose-dependent manner [30]. It is likely that the inoculation of a higher quantity of vector p.f.u. would have completely reversed postoperative pain induced by surgery, since the vector-mediated effect is dose-dependent (unpublished observations). The complete reversal observed after the combined administration of SHPE and RB101 further supports and corroborates the hypothesis that increasing the concentration of enkephalin at the receptor sites can reverse the pronociceptive effects of remifentanil or surgery.

In summary, the present investigation suggests that the decrease in DOR mRNA in the dorsal root ganglia is a common mechanism involved in the development of remifentanil and surgical induced hyperalgesia and allodynia. DOR seem to play an important role modulating nociceptive sensitization, and its activation by endogenous agonists could be an efficient method to prevent or reverse postoperative pain sensitization. The results obtained with the proenkephalin encoding vector show that an increase in enkephalin levels could provide effective analgesia in the postoperative period, and supports the future application of gene therapy in the management of postoperative pain in humans.

Acknowledgements

The authors sincerely thank Ms. Carolina Zamora for her excellent technical assistance. We are grateful to Marta Pulido, MD, for editing the manuscript and for editorial assistance. This work was supported by grants from Fondo de Investigaciones Sanitarias, Instituto de Salud Carlos III, Madrid, Spain (PI030245 and PI060069), Marato de TV3 071110 and the Endowed Chair in Pain Management UAB-IMAS-MENARINI (MMP). The authors declare that they have no conflicts of interest in relation to the information presented here.

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