Anesthesiology 2009; 111:1334–45

Pronociceptive Effects of Remifentanil in a Mouse Model of Postsurgical Pain

Effect of a Second Surgery

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Background: **Remifentanil anesthesia enhances postoperative pain in animals and humans. The authors evaluated the impact of the dose ($\mu g \cdot kg^{-1} \cdot min^{-1}$) and duration of remifentanil infusion, and the effects of a second surgery on postoperative pain sensitization.**

Methods: **Mice received different doses of remifentanil over 30 or 60 min. The authors assessed thermal (Hargreaves) and mechanical hyperalgesia (von Frey) at 2, 4, 7, and 10 days. In other experiments, mice had a plantar incision during sevoflurane with or without remifentanil anesthesia that was repeated 27 days later, when nociceptive thresholds returned to baseline. Linear mixed models were used for statistical analysis.**

Results: **Remifentanil induced dose-dependent pronociceptive effects with calculated ED_{50}s of 1.7 (95% confidence interval, 1.3–2.1) and 1.26 (1.0–1.6) $\mu g \cdot kg^{-1} \cdot min^{-1}$ for thermal and mechanical hyperalgesia, respectively, which lasted longer with higher doses ($P < 0.001$). The duration of infusion did not alter the pronociceptive effects of remifentanil when administered at a constant dose of infusion. When given during surgery, high (2.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$) or low (0.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$) remifentanil increased the extent ($P < 0.05$) and duration ($P < 0.01$) of thermal and mechanical hyperalgesia. The latter was further enhanced after a second surgery performed in the same experimental conditions ($P < 0.05$). Surgery or remifentanil infusion, each one individually, induced significant mechanical hyperalgesia, which was greater when repeated ($P < 0.05$).**

Conclusions: **In this model of incisional pain, remifentanil induces pronociceptive effects, which are dose dependent but unaltered by the duration of administration. A second surgery performed on the same site and experimental conditions induces greater postoperative hyperalgesia that is enhanced when remifentanil is used as an anesthetic.**

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Received from the Anesthesiology Research Unit, Institut Municipal de Investigacions Mèdiques, Department of Anesthesiology, Hospital del Mar, Universitat Autònoma de Barcelona, Barcelona, Spain. Submitted for publication November 3, 2008. Accepted for publication July 30, 2009. Supported by grants from Fondo de Investigaciones Sanitarias, Instituto de Salud Carlos III, Madrid, Spain (PI060669); Marato de TV3, Barcelona, Spain (071110); and the Endowed Chair in Pain Management UAB-IMAS-MENARINI (Dr. Puig), Barcelona, Spain. Ms. Campillo holds a predoctoral fellowship (AP2006-4718) from the Spanish Ministry of Education, Madrid, Spain. Presented at the European Association of Veterinary Anesthesia Meeting, Barcelona, Spain, October 14–16, 2008.

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REMIFENTANIL is a potent short-acting μ-opioid receptor agonist widely used as anesthetic in humans. Its main advantage over other 4-anilidopiperidine derivatives (fentanyl, alfentanil, sufentanil) relates to its rapid inactivation by plasma and tissue esterases. When used as anesthetic, remifentanil has a fast and predictable onset and offset that is independent of the duration of infusion, and its metabolism is not affected by organ failure.[1] Many reports show that intraoperative remifentanil administration paradoxically enhances pain sensitization and increases analgesic requirements in the postoperative period.[2–5] Such opioid-induced hyperalgesia has been described in animal models and humans after several μ-opioid receptor agonists administered by different routes.[3,6] Animal studies also show that the magnitude of the pronociceptive effects of morphine, heroin, and methadone (among others) is influenced by the administration schedule.[7–9] In humans, it has been suggested that remifentanil-induced pain sensitization is greater with higher doses.[10–13] However, the design of such studies does not allow establishing whether the pronociceptive effects of remifentanil are related to the dose of infusion, its duration, or the total dose administered over time. This information could be useful when attempting to prevent or reduce the pronociceptive effects of remifentanil when used as the main anesthetic in humans.

Another relevant aspect of the use of remifentanil during surgery is its possible contribution to the development of long-term changes in pain sensitivity, leading to chronic postsurgical pain.[14] In a previous study using the same mouse model of incisional pain, we demonstrated an increase in postoperative pain in animals receiving intraoperative remifentanil.[4] However, the effect of a second surgery performed with or without remifentanil anesthesia was not evaluated. Therefore, the current experimental study was designed to assess the impact of the dose and duration of remifentanil infusion on nociceptive thresholds and to determine whether the intraoperative use of remifentanil may affect the magnitude of the postoperative pain after a second surgery (performed after full recovery from the first one).

Therefore, the current investigation has two distinct but related objectives: First, we aimed to establish whether the infusion dose ($\mu g \cdot kg^{-1} \cdot min^{-1}$), the duration of infusion (time), or the total dose adminis-

tered over time would alter the extent and duration of postoperative hyperalgesia. Second, after the hyperalgesic doses of remifentanil were established, we investigated the pronociceptive effects of remifentanil after two consecutive treatments to determine whether repeated surgery (performed 27 days after the first one) during remifentanil anesthesia would enhance postoperative hyperalgesia. These effects were compared with those obtained after a repeated incision or a repeated remifentanil infusion (each separated by 27 days). We also evaluated whether either a previous incision or an infusion of remifentanil would modify the hyperalgesia induced by surgery with or without remifentanil anesthesia performed 27 days later.

Materials and Methods

Animals

Male Swiss CD1 mice weighing 25–28 g at the beginning of the experiments were used. Animals were housed five per cage and maintained in a room with a 12-h light–dark cycle (light between 8:00 AM and 8:00 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 and the European Communities directive 86/609/EEC regulating animal research. The protocol used in the study was endorsed by the ethics committee of our institution (Comitè Ètic d'Experimentació del Parc de Recerca Biomèdica de Barcelona, Barcelona, Spain).

Drugs

Remifentanil (Ultiva®; GlaxoSmithKline, Madrid, Spain) and sevoflurane (Sevorane®; Abbott Laboratories SA, Madrid, Spain) were supplied by the Department of Anesthesiology at the Hospital del Mar (Barcelona, Spain). Remifentanil was dissolved in saline (0.9% NaCl) and infused subcutaneously at the nape of the neck over a period of 30 or 60 min using a KD Scientific pump (KD Scientific Inc., Holliston, MA). All infusions were made during sevoflurane anesthesia (3.0–3.5% vol/vol), an inhalational anesthetic drug that we have previously shown has no effect on nociceptive thresholds.[4] An intravenous catheter (22 gauge) was inserted in the posterior aspect of the neck and carefully pushed forward subcutaneously approximately 1 cm. After removal of the needle, the catheter was loosely fixed around the neck of the mice with adhesive tape. Because mice were immobile during sevoflurane anesthesia, the catheter remained in place during the procedure.

In all instances and regardless of the remifentanil dose, the infusion rate was kept constant at 0.8 ml/h.

Plantar Surgery

We used a mouse model of postoperative pain previously described in our laboratory.[4] In a sterile operating room, mice were anesthetized with sevoflurane (3.0–3.5% vol/vol) plus a constant infusion of remifentanil or saline, administered during a period of 30 min. 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 and extending toward the toes. The underlying plantaris muscle was then exposed and incised longitudinally, keeping the muscle insertions 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, animals were allowed to recover in cages with sterile bedding. Control animals underwent a sham procedure (sham incision) that consisted of the administration of sevoflurane plus saline for 30 min, without remifentanil or incision. When a second surgery was performed 27 days later, the same experimental protocol described above was used.

Nociceptive Behavioral Testing

Hyperalgesia to noxious heat stimulation and to mechanical punctuate stimulation were determined in each experimental condition. Before the experiments, animals were habituated to the environment (testing equipment without nociceptive stimulation) for 3 days. We used the following nociceptive tests.

Heat Hyperalgesia. Heat hyperalgesia was evaluated as previously described.[15] Paw withdrawal latency in response to radiant heat was measured using the Hargreaves test equipment (Ugo Basile, Varese, Italy). Briefly, mice were placed in methacrylate cylinders (30 cm high, 9 cm in diameter; Servei Estació, Barcelona, Spain) positioned over a glass surface. Animals were habituated to the environment for 2 h before testing. The heat source was then positioned under the plantar surface of the hind paw and activated with a light beam intensity set to elicit baseline latencies of 9–11 s in control mice. A cutoff 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- to 10-min intervals, to reduce the possible influence of thermal sensitization on the response.

Mechanical Hyperalgesia. Mechanical nociceptive thresholds were evaluated measuring the hind paw withdrawal response to von Frey filament stimulation.[16] Animals were placed in methacrylate cylinders (30 cm high, 9 cm in diameter) with a wire grid bottom, through which the von Frey filaments were applied (bending force range from 0.008 to 2 g; North Coast Medical, Inc., San Jose, CA). To minimize stress during the experimental procedure, animals were allowed to habituate for 2 h before testing. The filament of 0.4 g was first used; then

the strength of the next filament was increased or decreased according to the response (up–down method[16]). The results of the evaluation were obtained in grams, which is a continuous variable that can be analyzed with parametric methods. The upper limit value (2 g) was recorded even if there was no withdrawal response to this force. Clear paw withdrawal, shaking, or licking were considered nociceptive-like responses. Both hind paws were alternatively tested.

Groups of Experiments

Special care was taken to reduce interindividual variability while using the smallest number of animals per group. Before the study, the animals were habituated by the same investigator for 3 days, and mechanical and thermal thresholds were determined daily during 3 additional days to obtain baseline values. All experimental groups received the same inhaled concentration of sevoflurane (3.0–3.5% vol/vol) during a remifentanil or saline infusion (rate of 0.8 ml/h).

In all experiments, the investigator recording the data was blinded to the treatment and the doses of remifentanil administered.

To establish whether the infusion dose ($\mu g \cdot kg^{-1} \cdot min^{-1}$), the duration of infusion (time), or the total dose administered over time would alter the extent and duration of postoperative hyperalgesia, we performed the following experiments.

Dose–Response Curves of Remifentanil Administered over a Fixed Period of Time. Remifentanil was administered to different groups of mice (8–10 animals/group) at total doses of 20, 40, 80, or 100 μg/kg infused over a period of 30 min (corresponding to infusion doses of 0.66, 1.33, 2.66, or 3.33 $\mu g \cdot kg^{-1} \cdot min^{-1}$). Higher doses of remifentanil could not be used because of motor impairment and/or respiratory depression. Control mice received saline. Nociceptive thresholds (thermal and mechanical hyperalgesia) were determined 2, 4, 7, and 10 days after the procedure.

Effect of Dose and Duration of Infusion. To establish the effect of the dose and time of infusion on the pronociceptive effects of remifentanil, we performed two sets of experiments: First, we determined whether a nonhyperalgesic dose of remifentanil, infused over an extended period of time (60 min), would induce significant hyperalgesia. We infused 0.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$ remifentanil for 60 min (total dose 40 μg/kg) and compared the effects with those observed after the administration of the same total dose (40 μg/kg) infused over a 30-min period (infusion dose of 1.33 $\mu g \cdot kg^{-1} \cdot min^{-1}$, positive control group). In both groups, the volume of infusion was kept constant at 0.8 ml/h. The dose of 40 μg/kg was selected because induced a distinct pronociceptive effect lasting several days when performing the dose–response curves. To ensure uniformity of the volume infused, the positive control group received a 30-min infusion of remifentanil (1.33 $\mu g \cdot kg^{-1} \cdot min^{-1}$ in 0.4 ml), followed by a 30-min infusion of 0.4 ml saline. Nociceptive thresholds were determined 2, 4, 7, and 10 days after remifentanil.

A second set of experiments was performed to establish whether increasing the infusion time would enhance the magnitude and duration of a pronociceptive dose of remifentanil. We compared the effects of remifentanil at the same infusion dose (1.33 $\mu g \cdot kg^{-1} \cdot min^{-1}$) but administered during 60 min (total dose 80 μg/kg) or 30 min (total dose 40 μg/kg). The volume of infusion was kept at 0.8 ml/h. Nociceptive thresholds were determined 2, 4, 7, and 10 days after the opioid.

Pronociceptive Effects of Remifentanil after Two Consecutive Treatments. Experiments evaluating the effects of a single treatment (incision, remifentanil administration, or their combination), have been previously reported by our group, using the same strain of mice and experimental protocol.[4] In the current experiments, all animals received two treatments (first and second), separated by a period of 27 days, a time when nociceptive thresholds were completely recovered. The following treatments were used:

- Sham incision (without surgery) and saline infusion (control group, sham incision + saline)
- Incision and saline infusion performed during sevoflurane anesthesia (incision + saline)
- Remifentanil infusion and sham incision (sham incision + remifentanil)
- Incision performed during remifentanil anesthesia (incision + remifentanil)

After each treatment (first and second), nociceptive thresholds were measured at baseline and 1, 7, 10, 14, 18, 21, and 25 days later. When a second incision was performed, it was always on the same surgical site as the first one. The dose of remifentanil used was 2.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$ infused over a period of 30 min, except when testing a nonhyperalgesic dose of remifentanil (0.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$) on repeated surgery (table 1).

Under these experimental conditions, we assessed whether repeated surgery during remifentanil anesthesia would enhance postoperative hyperalgesia. The observed effects were compared with those obtained after a repeated incision or a repeated remifentanil infusion (each separated by 27 days). (table 1). The following groups of experiments were performed:

- Control group, where thermal and mechanical hyperalgesia were measured in nontreated animals; served as reference for the other groups (sham incision + saline)
- Repeated incision (incision + saline)
- Two surgeries performed each during 2.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$ remifentanil (incision + remifentanil)
- Two surgeries performed each during 0.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$ remifentanil (incision + remifentanil)

Table 1. Mechanical Hyperalgesia after Two Successive Treatments

Group	First Treatment		Second Treatment		*P* Value (First *vs.* Second)
1	Sham incision + saline	0.84 (−2.4 to 3.2)	Sham incision + saline	0.04 (−3.4 to 2.4)	0.49
2	Incision + saline	1.29 (−0.3 to 5.7)	Incision + saline	3.07 (2.6 to 5.3)	0.03
3	Incision + remifentanil	9.02 (7.8 to 10.1)	Incision + remifentanil	13.17 (10.3 to 16.7)	0.01
4	Incision + remifentanil*	6.47 (3.1 to 9.6)	Incision + remifentanil*	8.63 (5.6 to 10.6)	0.05
5	Sham incision + remifentanil	4.52 (2.8 to 5.1)	Sham incision + remifentanil	8.25 (3.9 to 11)	0.02
6	Sham incision + remifentanil	5.64 (5.3 to 6.4)	Incision + saline	4.27 (1 to 9.4)	0.53
7	Sham incision + remifentanil	5.62 (5.2 to 9.1)	Incision + remifentanil	9.13 (7.3 to 11.7)	0.01
8	Incision + saline	2.23 (1.7 to 6.7)	Incision + remifentanil	9.39 (7.6 to 13.1)	0.02

Results are expressed as median value of the area above the time–effect curves and interquartile range (lower quartile to upper quartile) from days 0 to 25 after each treatment. Values represent the overall variation of nociceptive thresholds. Each group of mice received two consecutive treatments (first and second), separated by a period of 27 days. All groups treated with remifentanil received a dose of 2.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (total dose 80 $\mu g/kg$), except in *, where the dose of remifentanil was 0.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (total dose 20 $\mu g/kg$). *P* values comparing first and second treatments were analyzed using the Wilcoxon Mann–Whitney test for repeated measures.

- Repeated remifentanil infusion at 2.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (sham incision + remifentanil)

We also evaluated whether either a previous incision or an infusion of remifentanil would modify the hyperalgesia induced by surgery with or without remifentanil anesthesia performed 27 days later (table 1), with the subsequent groups of experiments:

- Sham incision + remifentanil infusion (as first treatment), followed by incision + saline infusion (second treatment)
- Sham incision + remifentanil, or incision + saline (as first treatment), followed by incision + remifentanil (second treatment)

Statistical Analysis

The mean area above the time- effect curves (AACs; 0–10 days) obtained with the different doses of remifentanil were plotted in figure 1 to show the correlation between the infused doses and their overall effects. In this figure, each graph includes the equation corresponding to the represented linear regression, where y is the overall effect and x is the infused dose. Pearson coefficients, R^2, are included as a measure of the relation between both variables. The ED_{50} values of remifentanil for thermal and mechanical hyperalgesia were calculated by nonlinear regression analysis with a sigmoidal dose–response equation (variable slope) using GraphPad Prism 4 (GraphPad Software Inc, San Diego, CA).

All data presented in the time-course graphs (figs. 2–5) are expressed as mean values ± SD of 5–10 mice. For each mouse and time point, the responses in seconds (Hargreaves test) or grams (von Frey) are expressed as the changes with respect to the baseline values, normalized (subtracted) to the mean value of the corresponding control group (represented in the figures by a broken line). This calculation facilitates the graphic representation and interpretation of the data, where negative values indicate net pronociceptive effects and positive values indicate antinociception.

The time course of the effects of the infusion dose, the duration of infusion, and the total dose were analyzed using a linear mixed model with two factors, the experimental condition (infusion dose, infusion duration, or total dose) and the time of evaluation (day), as well as their interaction. A random intercept was considered, but random effects were not included. For the covariance structure of the repeated measures, a diagonal matrix was chosen. If the interaction between experimental condition and time was statistically significant,

Table 2. Decrease in Thermal Thresholds and Duration of Pain Sensitization after Increasing Doses of Remifentanil

Dose, $\mu g \cdot kg^{-1} \cdot min^{-1}$	% Decrease in Thermal Latency	*P* Value Compared with Saline	Duration of Effect, Days
0.66	—	0.999	—
1.33	24.5	0.075	4–7
2.66	34.7	0.005	> 10
3.33	34.1	0.003	> 10

Remifentanil was administered at infusion doses of 0.66, 1.33, 2.66, and 3.33 $\mu g \cdot kg^{-1} \cdot min^{-1}$ in a constant volume of 0.4 ml (total doses administered were 20, 40, 80, and 100 $\mu g/kg$, respectively). Thermal hyperalgesia was assessed 2 days after remifentanil. Values are expressed as percent decrease when compared with basal values, and normalized to the control group (see Materials and Methods). Data were analyzed using linear mixed models.

Table 3. Decrease in Mechanical Thresholds and Duration of Pain Sensitization after Increasing Doses of Remifentanil

Dose, $\mu g \cdot kg^{-1} \cdot min^{-1}$	% Decrease in Mechanical Threshold	*P* Value Compared with Saline	Duration of Effect, days
0.66	—	0.999	—
1.33	21.7	0.003	7–10
2.66	39.7	< 0.001	> 10
3.33	41.8	< 0.001	> 10

Remifentanil was administered at infusion doses of 0.66, 1.33, 2.66, and 3.33 $\mu g \cdot kg^{-1} \cdot min^{-1}$ in a constant volume of 0.4 ml (total doses administered were 20, 40, 80, and 100 $\mu g/kg$, respectively). Mechanical hyperalgesia was assessed 2 days after remifentanil. Values are expressed as percent decrease when compared with basal values, and normalized to the control group (see Materials and Methods). Data were analyzed using linear mixed models.

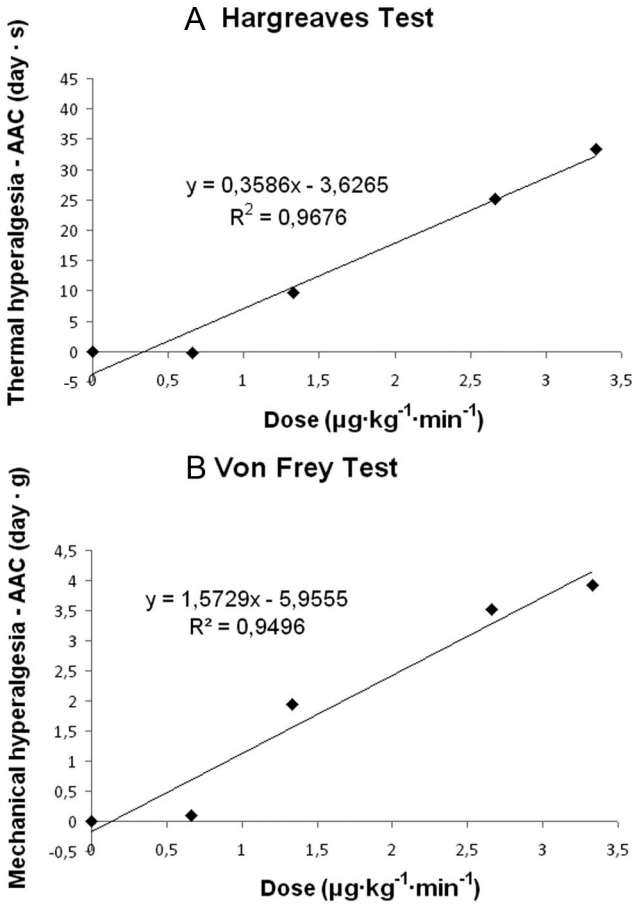

Fig. 1. Pronociceptive effects of remifentanil administered at different infusion doses. For the Hargreaves (*A*) and von Frey (*B*) tests, results are expressed as mean value of the area above the time–effect curves (AACs) of nociceptive thresholds over time (2, 4, 7, and 10 days) after remifentanil infusion. The number of animals in the saline group was n = 7, and those in the remifentanil groups were as follows: dose of 0.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (n = 8), 1.33 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (n = 8), 2.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (n = 7), and 3.33 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (n = 9). Values were normalized to the control group (saline). In this figure, each graph includes the equation corresponding to the represented linear regression, where y is the overall effect and x is the infused dose. The Pearson coefficient, R^2, is included as a measure of the relation between both variables.

multiple Tukey *post hoc* comparisons for repeated measures were performed at each time point. Analyses were performed with R (The R Foundation for Statistical Computing, Vienna, Austria), using its libraries nlme[17] and multcomp.[18] In figure 2, mean values ± SDs for the AACs are represented as histograms to illustrate the overall nociceptive sensitization corresponding to each experimental condition. Because the AAC data are reduced to a single value per mouse, the comparison of the AACs was performed with the nonparametric Wilcoxon Mann–Whitney test for independent samples, using SPSS version 13.0 (SPSS Inc., Chicago, IL).

To analyze the effects of the first and second treatment at each time point, nonparametric multiple test procedures were applied[19] using the R npmc library.[20] AACs and interquartile ranges (table 1) were calculated to illustrate the overall nociceptive sensitization corresponding to the first and second treatments. The comparison of the AACs was performed with the nonparametric Wilcoxon Mann–Whitney test for repeated measures, using SPSS version 13.0. Because of the duration of the pronociceptive effects observed after surgery, the AACs of the time effect were calculated for a period of 0–25 days (figs. 3–5).

Results

Dose–Response Curves of Remifentanil Administered over a Fixed Period of Time

Baseline thresholds to thermal and mechanical stimuli obtained before remifentanil administration were similar in all groups of study, with mean values of 11.28 ± 1.05 s and 1.21 ± 0.14 g, respectively. Saline administration (control group) did not induce significant changes in nociceptive thresholds over the 10-day period of evaluation. In contrast, increasing the dose of the remifentanil infusion induced dose-dependent pronociceptive effects in both the Hargreaves and von Frey tests. Maximal pronociceptive effects were observed on day 2, and these results are shown in tables 2 and 3.

In the Hargreaves test, the magnitude and duration of remifentanil-induced thermal hyperalgesia increased in a dose-dependent manner ($P < 0.001$, for the dose and time of evaluation; table 2). At this time point, the highest infusion doses (2.66 and 3.33 $\mu g \cdot kg^{-1} \cdot min^{-1}$) induced 34 ± 21% and 34 ± 9% decreases in thermal latency ($P < 0.01$ *vs.* saline) that remained statistically significant up to 10 days after remifentanil infusion (26 ± 21% to 33 ± 14% decrease; $P < 0.05$). At the same time point (day 2), the lower doses of remifentanil did not induce significant pronociceptive effects when compared with saline.

Comparison of the overall effects of remifentanil induced by the different doses was achieved using the AACs of the time–effect curves over the 10-day period of evaluation. Figure 1A shows a linear positive correlation between the different doses of remifentanil and the magnitude of thermal hyperalgesia, with a coefficient $R^2 = 0.9676$; the calculated ED_{50} of remifentanil was 1.7 (95% confidence interval, 1.3–2.1) $\mu g \cdot kg^{-1} \cdot min^{-1}$. These values could be underestimated because of the impossibility to test higher doses of remifentanil.

In the von Frey test, remifentanil also induced long-lasting mechanical hyperalgesia in a dose-dependent manner ($P < 0.001$). On day 2 after administration, the highest doses (2.66 and 3.33 $\mu g \cdot kg^{-1} \cdot min^{-1}$) induced 40 ± 15% and 42 ± 19% decreases in mechanical thresholds, respectively ($P < 0.01$ *vs.* saline; table 3). Mechanical hyperalgesia remained statistically significant 10 days after treatment ($P < 0.05$ compared with the saline group), with residual mean threshold reductions of 24 ± 18% and 27 ± 9%. At day 2, the dose of 0.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$ did not induce significant hypersensitivity to me-

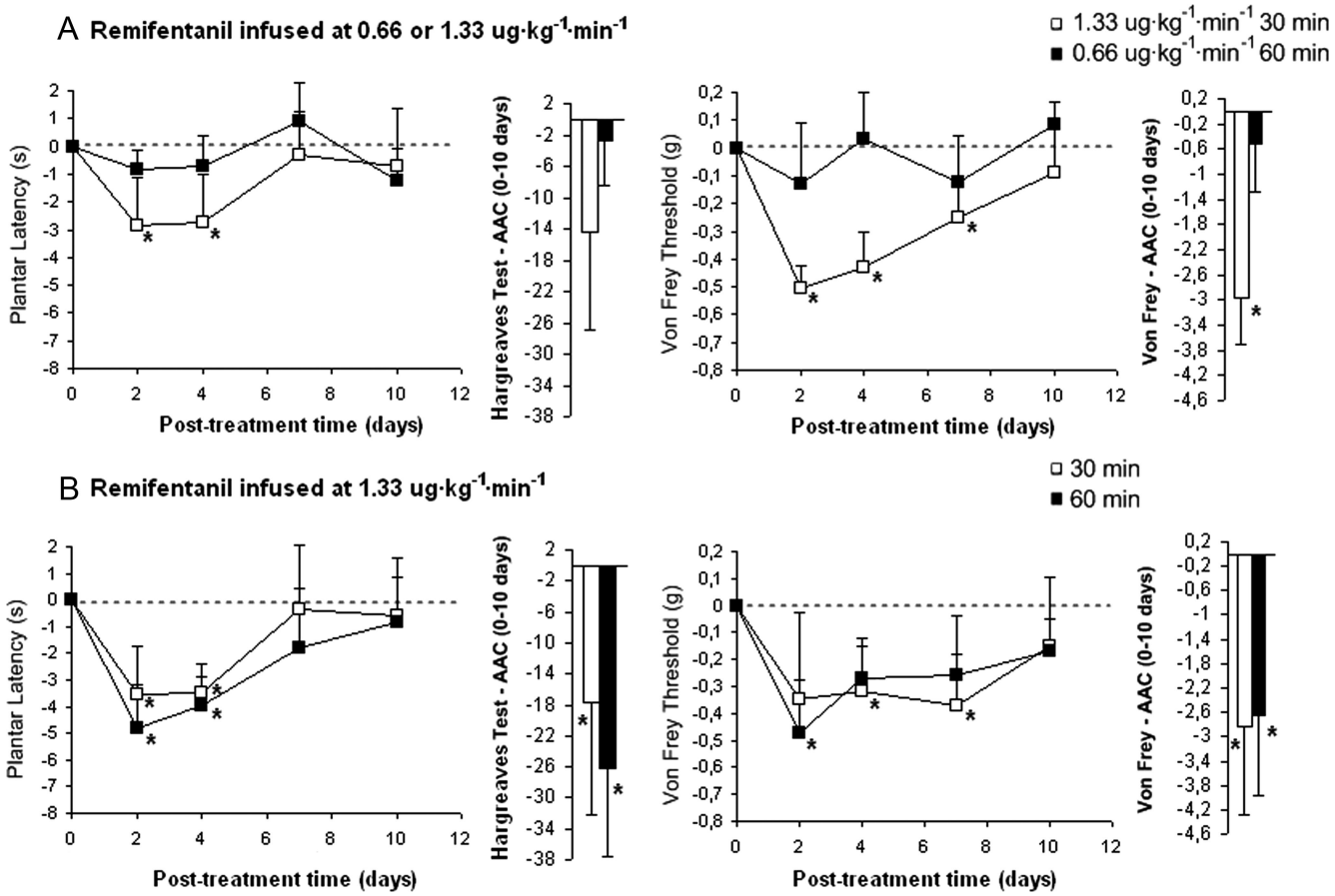

Fig. 2. Pronociceptive effects of remifentanil when infused over a 30- or 60-min period in the Hargreaves and von Frey tests. Results are expressed as mean value; *vertical bars* indicate SD. Each *point* indicates the variation of the response respect to the baseline, normalized to the control group (n = 6), represented by the *horizontal broken line*. In the graphs, *negative values* indicate pronociceptive effects. The histograms show the mean overall effects of the treatments obtained from the area above the time–effect curves (AACs). * $P < 0.05$ compared with control group. (*A*) Pronociceptive effects of 0.66 or 1.33 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (40 μg/kg) remifentanil, administered over 30 min (*empty squares*, n = 8) or 60 min (*filled squares*, n = 6). (*B*) Pronociceptive effects of remifentanil administered at 1.33 $\mu g \cdot kg^{-1} \cdot min^{-1}$ during 30 min (total dose 40 μg/kg, *empty squares*, n = 6) or 60 min (total dose 80 μg/kg, *filled squares*, n = 6).

chanical stimuli. A linear correlation between the dose and the extent of mechanical hyperalgesia (AAC) was also obtained, with a correlation coefficient $R^2 = 0.9496$ (fig. 1B) and a calculated ED_{50} of 1.26 (95% confidence interval, 1.0–1.6) $\mu g \cdot kg^{-1} \cdot min^{-1}$.

Effect of Dose and Duration of Infusion

In these experiments, we first assessed whether remifentanil at nonhyperalgesic doses could induce hyperalgesia when infused over an extended period of time. We infused the same total dose of 40 μg/kg remifentanil over a 60-min (0.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$) or 30-min period (1.33 $\mu g \cdot kg^{-1} \cdot min^{-1}$, positive control group). The results in the Hargreaves test show that the infusion of 40 μg/kg remifentanil in 30 min induced significant thermal hyperalgesia at days 2 and 4 ($P < 0.05$ compared with saline; fig. 2A, left). On the contrary, when the same total dose was infused over a period of 60 min (0.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$), no pronociceptive effects were observed. The AACs of the time–effect curves were 2.7 ± 6 and 14.5 ± 12 for the 0.66- and 1.33-$\mu g \cdot kg^{-1} \cdot min^{-1}$ doses ($P = 0.089$). These results are represented as histograms on the right side of the plantar latency graph shown in figure 2A.

Similar results were obtained in the von Frey test. The pronociceptive effects of 40 μg/kg remifentanil infused over 30 min (1.33 $\mu g \cdot kg^{-1} \cdot min^{-1}$) were maximal on day 2 and lasted approximately 7 days (fig. 2A, right). When the same total dose was administered over 60 min (0.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$), no significant pronociceptive effects were observed. The AACs were 0.51 ± 0.8 and 2.97 ± 0.7 for the 0.66- and 1.33-$\mu g \cdot kg^{-1} \cdot min^{-1}$ infusion doses ($P < 0.05$). This demonstrates a significant overall pronociceptive effect of the 1.33-$\mu g \cdot kg^{-1} \cdot min^{-1}$ dose ($P < 0.001$ compared with saline) but no significant effect of the 0.66-$\mu g \cdot kg^{-1} \cdot min^{-1}$ infusion dose (fig. 2A, histogram). The results show that administration of remifentanil at a low infusion dose over an extended period of time does not induce significant pronociceptive effects.

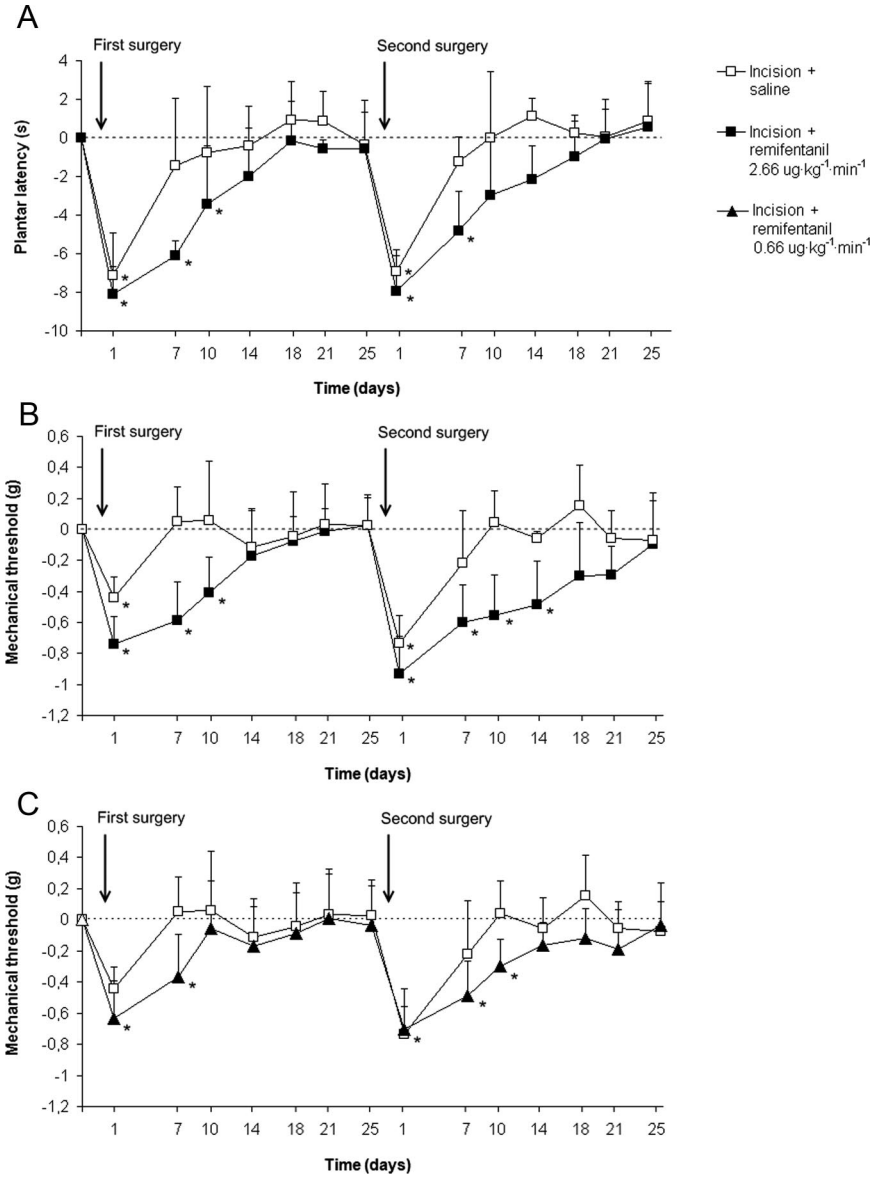

Fig. 3. Pain sensitization after two consecutive surgeries performed during sevoflurane (incision group, *empty squares*, n = 5) or remifentanil anesthesia at 2.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (*filled squares*, n = 9) or 0.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (*filled triangles*, n = 7). Each *point* represents the mean change in nociceptive thresholds compared with baseline, standardized to the control group (represented by the *broken line*, n = 9). *Vertical bars* show SD. *Negative values* indicate pronociceptive effects. Remifentanil was administered at a dose of 2.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (80 μg/kg; *A* and *B*) or 0.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (20 μg/kg; *C*) over 30 min. *A*, plantar latency (seconds); *B* and *C*, mechanical thresholds (grams). * $P < 0.05$ compared with control group.

We also assessed whether a dose of remifentanil that induced pronociceptive effects would increase hyperalgesia when infused over a prolonged period of time. We compared the effects of the same dose of remifentanil (1.33 $\mu g \cdot kg^{-1} \cdot min^{-1}$) infused over a period of 60 min (total dose 80 μg/kg) or 30 min (total dose 40 μg/kg). Both groups displayed significant thermal and mechanical hyperalgesia when compared with the saline group (fig. 2B; $P < 0.05$ for each test). In both groups, thermal hyperalgesia was statistically significant on days 2 and 4 when compared with saline ($P < 0.01$), lasting approximately 4 days. Mechanical hyperalgesia was observed on days 2, 4, and 7. For the 60-min group, $P < 0.05$ was obtained at day 2, when compared with saline, whereas for the 30-min group, differences were statistically significant from saline on days 4 and 7 ($P < 0.05$) and lasted approximately 7 days. In these experiments (fig. 2B, graphs), no significant differences between groups receiving remifentanil could be established at any time point or when comparing the AACs (fig. 2B, histograms). The results show that the increase in the infusion time did not alter the magnitude and duration of the pronociceptive effects of remifentanil.

Pronociceptive Effects of Remifentanil after Two Consecutive Treatments

In all of the groups, baseline nociceptive thresholds were similar, with mean values of 11.82 ± 0.8 s in the Hargreaves test and 1.16 ± 0.2 g in the von Frey test.

After a first surgery, significant thermal hyperalgesia was observed in the operated paw in both the incision + saline group and the incision + remifentanil group. Hyperalgesia was of similar magnitude (on day 1) but longer lasting in the latter (surgery + remifentanil [2.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$]; fig. 3A). On the first day after surgery, thermal nociceptive thresholds decreased 62 ± 19% and

Table 4. Thermal Hyperalgesia in Mice Receiving Two Successive Identical Treatments

Repeated Treatment	First Treatment	Second Treatment	*P* Value (First *vs.* Second)
Sham incision + saline	0.75 (−6.2 to 12.7)	9.17 (−10.8 to 9.2)	0.49
Incision + saline	24.5 (8.2 to 73.8)	30.7 (22 to 43.6)	0.42
Incision + remifentanil	83.4 (80.3 to 98.1)	83 (58.4 to 105.4)	0.42
P value (incision + saline *vs.* incision + remifentanil)	0.03	0.01	

Results are expressed as median value of the area above the time–effect curves and the interquartile range (lower quartile to upper quartile) from days 0 to 25 after each treatment. Values represent the overall variation of nociceptive thresholds. Remifentanil was administered at a dose of 2.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$.

67 ± 15% in the saline and remifentanil groups, respectively ($P < 0.01$ for each compared with untreated mice), with no significant differences between groups at this time point. In the incision + saline group, thermal hyperalgesia disappeared 7 days after surgery, but lasted up to 10 days in mice receiving incision + remifentanil anesthesia (28 ± 25% decrease at day 10; $P < 0.05$). Moreover, the comparison of the AACs showed significant differences between the incision + saline and incision + remifentanil groups after the first surgery ($P = 0.031$; table 4).

A second surgery performed 27 days later also induced sustained thermal hyperalgesia in the incision + saline and incision + remifentanil groups, with no statistical differences between groups on the first day after surgery (fig. 3A). The AAC showing the overall thermal hyperalgesia was significantly greater in the incision + remifentanil group than in the incision + saline group ($P = 0.01$). For each group, no significant differences in the magnitude and duration of thermal hyperalgesia were observed when comparing the first and second surgeries (fig. 3A and table 4).

In the von Frey test (fig. 3B and table 1), significant mechanical hyperalgesia was observed after the first surgery in the incision + saline and the incision + remifentanil [2.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$] groups ($P < 0.01$ *vs.* untreated mice), and was more severe and long lasting in the incision + remifentanil group. On the first day after surgery, mechanical thresholds decreased 43 ± 7% and 64 ± 11% in the incision + saline and incision + remifentanil groups, respectively ($P < 0.05$). Thresholds returned to baseline 7 days after surgery in the incision + saline group, but persisted up to 10 days in the incision + remifentanil group (35 ± 20% decrease on day 10; $P < 0.05$ *vs.* control). The comparison of the AACs showed significant differences between the incision + saline and incision + remifentanil groups ($P < 0.01$).

The second surgery also induced pronounced mechanical hyperalgesia in both groups. On day 1 after the second surgery, the decrease in mechanical thresholds was enhanced (72 ± 16% and 81 ± 13% for each group). The effects lasted approximately 7 days in the incision + saline group but were prolonged up to 14 days in the incision + remifentanil group (42 ± 19% decrease at day 14; $P < 0.01$ *vs.* control). Comparison of the AACs also demonstrated significant differences between the incision + saline and incision + remifentanil groups ($P < 0.01$). Therefore, in our experimental mouse model, the administration of a pronociceptive dose of remifentanil during two consecutive surgeries significantly increased the magnitude of postoperative mechanical (but not

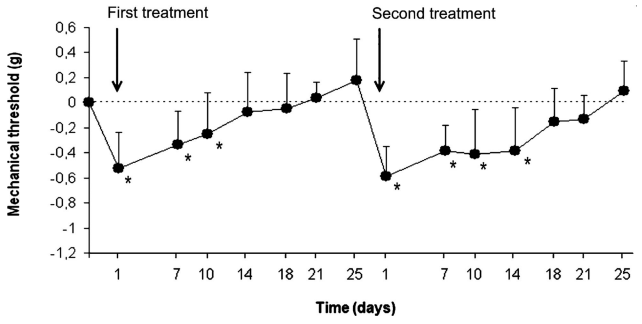

Fig. 4. Mechanical hyperalgesia in mice after two consecutive remifentanil infusions. Each *point* represents the mean change ± SD in nociceptive thresholds compared with baseline, standardized to the control group (represented by the *broken line*). Nine mice were used in control group, and seven were used in the remifentanil group. *Negative values* indicate net pronociceptive effects. Both treatments consisted of sham incision + remifentanil infusion at a dose of 2.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$ administered during a period of 30 min. * $P < 0.05$ compared with the sham incision + saline group.

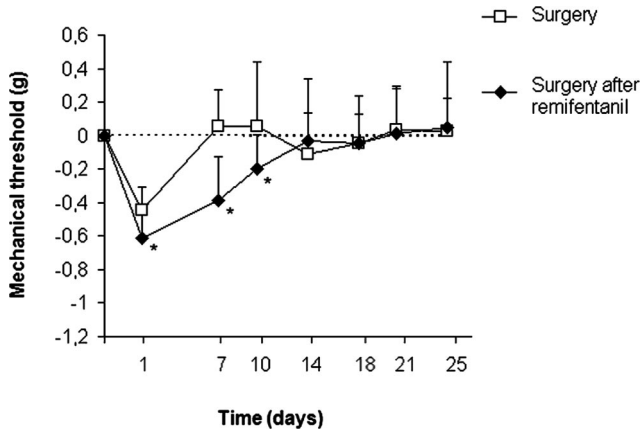

Fig. 5. Mechanical hyperalgesia in mice after a first incision preceded (or not) by a remifentanil infusion. Each *point* represents the mean changes in nociceptive thresholds compared with baseline, standardized to the control group (represented by the *broken line*, n = 5). *Vertical bars* show the SD. *Negative values* indicate pronociceptive effects. *Empty squares* represent results obtained after a first incision + saline without previous treatment (n = 5), whereas *filled rhombs* show the effects of a first incision + saline performed in mice previously exposed to 2.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$ remifentanil (n = 7). Remifentanil was administered 27 days before surgery (see Materials and Methods). * $P < 0.05$ compared with the control group.

thermal) hyperalgesia after the second surgery (tables 1 and 4).

The intraoperative administration of a nonhyperalgesic dose of remifentanil (0.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$) during two consecutive surgeries (fig. 3C and table 1) induced a significant mechanical hyperalgesia after each surgery when compared with untreated mice (sham incision + saline), and the second surgery produced a higher degree of hyperalgesia (table 1). The results also show that on day 1 after surgery, mechanical hyperalgesia thresholds decreased 48 ± 16% and 55 ± 18% after the first and second surgeries, respectively, lasting 7 and 10 days ($P < 0.05$ compared with the untreated group; fig. 3C). Table 1 shows the values of the AACs in the following groups: incision + saline, incision + remifentanil (2.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$), and incision + remifentanil (0.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$). A one-way analysis of variance followed by the Tukey test revealed a significant effect of the dose of remifentanil for both the first and the second surgeries ($P < 0.05$). Moreover, incision + nonhyperalgesic doses of remifentanil induced a longer-lasting hyperalgesia than incision + saline after the first and second surgeries (fig. 3C). Therefore, surgery performed during low doses of remifentanil still enhances postoperative hyperalgesia when compared with surgery + saline performed during sevoflurane anesthesia, supporting the results showing a dose-related hyperalgesic effect of remifentanil (fig. 1).

We also evaluated the effects of the administration of two consecutive doses of remifentanil (2.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$, 30 min) separated by a period of 27 days (table 1 and fig. 4, repeated sham incision + remifentanil). Mechanical hyperalgesia was similar on day 1 after each treatment (44 ± 20% and 50 ± 14% decreases for the first and second administrations). However, sensitization lasted 10 days after the first exposure (21 ± 28% decrease on day 10; $P < 0.05$ compared with controls) and 14 days after the second (32 ± 27% decrease; $P < 0.05$). The comparison of the AACs shows that overall hyperalgesia was greater after the second exposure (table 1).

In another group of experiments (table 1), we assessed whether a previous remifentanil exposure could enhance incision-induced hyperalgesia. In figure 5, we have plotted the effects of a first incision + saline treatment with and without a previous remifentanil infusion. The figure shows that surgery after remifentanil exposure induces a 51 ± 19% decrease in mechanical thresholds on day 1 that lasted up to 10 days (18 ± 25% decrease; $P < 0.05$ compared with control). On the contrary, hyperalgesia disappeared on day 7 when mice were not previously exposed to remifentanil. These results indicate that a previous exposure to remifentanil increases incisional pain. It is interesting to note that in table 1, when the overall pronociceptive effects of sham incision + remifentanil (as first treatment) are compared with those of the incision + saline (as a second treatment), they induce similar pronociceptive effects.

Finally, we assessed whether a previous surgery (incision + saline) or a remifentanil infusion (sham incision + remifentanil) would distinctly change postoperative hyperalgesia induced by a subsequent surgery performed during remifentanil anesthesia (table 1). In both groups, the second treatment induced greater hyperalgesia than the first one, but the extent of mechanical hyperalgesia after the second procedure was similar regardless of whether animals received an infusion of remifentanil or a surgical incision.

Discussion

The current study shows that the pronociceptive effects of remifentanil are determined by the dose rather than by the duration of infusion. Therefore, regardless of the time of exposure, drug concentration at the μ-opioid receptor effector sites seems to be the critical factor for the development of remifentanil-induced nociceptive sensitization. The study also shows, for the first time, that when a second surgery is performed after nociceptive thresholds are restored, a significant increase in postoperative mechanical (but not thermal) hyperalgesia is observed, regardless of the type of anesthesia. In all instances (first and second surgeries), the extent and duration of postoperative pain sensitization is significantly greater when surgery is performed during remifentanil anesthesia. Moreover, a previous exposure to remifentanil enhances the duration of incision- and remifentanil-induced hyperalgesia.

In the same mouse model of postoperative pain, we have previously reported that remifentanil induces delayed hyperalgesia and enhances postincisional pain when infused during surgery.[4,21] In the current investigation, we tried to assess whether the mode of remifentanil administration (infusion dose, time, total dose) could be a determinant of its pronociceptive effects. The objective was to provide answers to unsolved clinical questions that may help to reduce postoperative pain in patients undergoing surgery during remifentanil anesthesia. Remifentanil has been reported to induce dose-dependent pain sensitization in several experimental and clinical studies in humans.[6–9] However, these studies assessed simultaneously the effect of a given infusion dose of remifentanil ($\mu g \cdot kg^{-1} \cdot min^{-1}$) and the effect of the total dose of drug administered, which is proportional to the time of infusion when the infusion dose is kept constant. Consequently, the likely pronociceptive effects associated with each factor independently could not be established. In the current study, we were able to show that remifentanil-induced pronociceptive effects correlate positively with the infusion dose used, whereas the time of administration or the total dose has no major

effects. However, because remifentanil plasma levels after subcutaneous administration were not determined in our study, a different relation among dose, concentration, and time after intravenous administration cannot be excluded.

The acute administration of phenanthrene derivatives (morphine, methadone) by different routes induces delayed nociceptive sensitization lasting up to 2 days,[11–13] whereas hyperalgesia induced by piperidine derivatives such as fentanyl has been reported to persist up to 5 days.[22] In our experimental conditions, remifentanil-induced sensitization persisted for approximately 10 days, which is the longest period of time reported after a short exposure (30 min) to an opioid. However, because no direct comparison between the effects of matched doses of the different opioids was attempted in our study, no definite conclusions regarding the possible longer duration of the pronociceptive effects of remifentanil can be derived. The comparison of the postoperative pain sensitization induced by opioids following different schedules of administration has not been fully investigated, even though it could be a relevant factor in the development of chronic postsurgical pain. Although conclusions from animal studies cannot be precisely applied to humans, our results strongly suggest that the administration of low infusion doses of remifentanil in clinical practice would reduce the hyperalgesic effects regardless of the duration of infusion. To support this assumption, a recent study reported greater postoperative pain 1 month after breast surgery in patients who received high doses of opioids in the postanesthesia care unit.[23]

In our study, remifentanil had more effect decreasing mechanical than thermal thresholds, and lower doses were needed to induce mechanical (ED_{50} 1.26 $\mu g \cdot kg^{-1} \cdot min^{-1}$) than thermal hyperalgesia (ED_{50} 1.7 $\mu g \cdot kg^{-1} \cdot min^{-1}$). This is consistent with the greater sensitivity to opioids of pathways activated by mechanical rather than by thermal stimuli, as suggested in several studies.[3,4,12,24,25] In our model, the administration of 40 μg/kg remifentanil (1.33 $\mu g \cdot kg^{-1} \cdot min^{-1}$) did not induce cold allodynia in the acetone drop test[26] (data not shown), a result supported by clinical studies evaluating cold sensitivity after remifentanil anesthesia.[13]

The results demonstrate that a given remifentanil infusion dose induces the same degree of hyperalgesia regardless of the duration of infusion. However, prolonged infusion times may induce intraoperative acute antinociceptive tolerance to remifentanil,[27–29] an aspect that was not evaluated in the current investigation. It is likely that increasing remifentanil infusion doses to compensate the decrease in efficacy (acute tolerance) would enhance postoperative hyperalgesia.

The absence of cumulative effects of remifentanil when infused over extended periods of time is probably related to the rapid degradation of the opioid by plasma and tissue esterases[1]; this property would favor steady state levels of μ-opioid receptor occupancy during the infusion, partially explaining that the duration of anesthesia does not alter delayed remifentanil hyperalgesia. Time of infusion might be a more relevant factor when assessing the pronociceptive effects of opioids with slower metabolism/longer action.[30–32] This would imply that low doses of remifentanil could be infused for prolonged periods of time without inducing postoperative pain sensitization, and also that a single bolus of a high dose of remifentanil (*i.e.*, during the induction of anesthesia) could cause significant and long-lasting hyperalgesia in the postoperative period. Currently, we are testing these assumptions in our mouse model.

Establishing the effective doses of remifentanil that induce pronociceptive effects in our model was essential to select the optimal doses to study its effects after repeated surgery. Based on the current results, we were able to select the hyperalgesic (2.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$) and nonhyperalgesic doses (0.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$) used in the second part of the study.

The extent and duration of postoperative pain sensitization after a plantar incision were significantly increased in mice anesthetized with remifentanil at hyperalgesic doses, corroborating previous findings from our laboratory.[4,21] After complete recovery, a second surgery performed at the same site and in analogous experimental conditions induced more prominent and persistent changes in mechanical thresholds in remifentanil-treated mice, regardless of the remifentanil dose (table 1). Interestingly, repeated surgery performed during a low nonhyperalgesic dose of remifentanil increased the duration of postoperative hyperalgesia when compared with incision alone (fig. 3C and table 1). From these experiments, we could conclude that the magnitude and duration of the pronociceptive effects of remifentanil, when used during surgery in mice, are dose dependent, supporting the results of the first part of the study.

The repeated administration of two identical doses of remifentanil (2.66 $\mu g \cdot kg^{-1} \cdot min^{-1}$) separated by a 27-day interval increased hyperalgesia after the second dose, corroborating the results obtained after repeated administration of other opioids (heroin,[33] morphine,[34] or fentanyl[22]). Moreover, either an incision or an exposure to remifentanil increased the duration of hyperalgesia after a subsequent incision (figs. 3 and 5), regardless of surgery being performed during sevoflurane or remifentanil anesthesia. These results support previous studies performed in rats showing that chronic morphine administration increases incision-induced hyperalgesia and local cytokine release.[35,36] However, other studies show that acute administration of morphine reduces peri-incisional cytokine expression and neutrophil infiltration.[37] The reduction of opioid-containing leukocytes around the wound could increase incision-induced hyperalgesia.[38] In addition, the reported effects of opi-

oids delaying wound healing[39–41] could also favor the perpetuation of postoperative pain.

Taken together, our results suggest that, after nociceptive thresholds return to baseline values, a previous surgery and/or remifentanil infusion induces long-lasting persistent neuroplastic changes in nociceptive pathways that facilitate mechanical pain sensitization in future situations (pain memory[42]) A long-lasting imprint induced by acute nociceptive stimuli in the nervous system has been reported in models of inflammatory pain induced by carrageenan,[42–45] although substantial differences between postincisional and other pain models are likely to be present.[46–48]

Multiple central and peripheral mechanisms have been implicated in pain sensitization after tissue injury including C-fiber sensitization,[42] protein kinase C,[49] *N*-methyl-D-aspartate receptors,[43] nitric oxide,[4] and spinal dynorphin,[28,50] among others. Surprisingly, all of these mechanisms have also been implicated in opioid-induced hyperalgesia.[3] Our results in a postoperative pain model in mice put forward the clinical need to take preventive measures during surgical anesthesia, to avoid latent sensitization in future surgeries.

In conclusion, the current study illustrates that the infusion dose, but not the duration of infusion or the total dose administered, determines the pronociceptive effects of remifentanil. The study also shows for the first time that a second incision performed at the same surgical site during high- or low-dose remifentanil anesthesia increases postoperative mechanical hyperalgesia in mice. Moreover, either a previous exposure to remifentanil alone or a first surgery (performed with or without remifentanil) significantly enhances postoperative mechanical hyperalgesia after a second surgery. Although preclinical studies sometimes do not translate to the clinical bedside, the current results may be useful to design clinical studies testing the effects of remifentanil after repeated surgery in humans.

The authors thank Klaus Langohr, Ph.D. (Statistician, Grup de Recerca Clínica en Farmacologia Humana i Neurociències, Programa de Recerca en Neuropsicofarmacologia, Institut Municipal de Investigacions Mèdiques–Hospital del Mar, Barcelona, Spain), for his excellent assistance in the statistical analysis; Marta Pulido, M.D. (Servei d'Assessorament en Edició I Publicació Biomèdica, Institut Municipal de Investigacions Mèdiques–Hospital del Mar, Barcelona, Spain), for editorial assistance; and José Cerezo (Freelance Graphic Designer, Carcaixent, Valencia, Spain) for his help with the figure design.

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