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(-)-Pentazocine induces visceral chemical antinociception, but not thermal, mechanical, or somatic chemical antinociception, in μ-opioid receptor knockout mice

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Abstract

Background: (-)-Pentazocine has been hypothesized to induce analgesia via the κ-opioid (KOP) receptor, although the involvement of other opioid receptor subtypes in the effects of pentazocine remains unknown. In this study, we investigated the role of the μ-opioid (MOP) receptor in thermal, mechanical, and chemical antinociception induced by (-)-pentazocine using MOP receptor knockout (MOP-KO) mice.

Results: (-)-Pentazocine-induced thermal antinociception, assessed by the hot-plate and tail-flick tests, was significantly reduced in heterozygous and abolished in homozygous MOP-KO mice compared with wildtype mice. The results obtained from the (-)-pentazocine-induced mechanical and somatic chemical antinociception experiments, which used the hind-paw pressure and formalin tests, were similar to the results obtained from the thermal antinociception experiments in these mice. However, (-)-pentazocine retained its ability to induce significant visceral chemical antinociception, assessed by the writhing test, in homozygous MOP-KO mice, an effect that was completely blocked by pretreatment with nor-binaltorphimine, a KOP receptor antagonist. *In vitro* binding and cyclic adenosine monophosphate assays showed that (-)-pentazocine possessed higher affinity for KOP and MOP receptors than for δ-opioid receptors.

Conclusions: The present study demonstrated the abolition of the thermal, mechanical, and somatic chemical antinociceptive effects of (-)-pentazocine and retention of the visceral chemical antinociceptive effects of (-)-pentazocine in MOP-KO mice. These results suggest that the MOP receptor plays a pivotal role in thermal, mechanical, and somatic chemical antinociception induced by (-)-pentazocine, whereas the KOP receptor is involved in visceral chemical antinociception induced by (-)-pentazocine.

Keywords: Opioid receptor Knockout mice, Pentazocine, Antinociception

Background

The racemic compound (±)-pentazocine is used for the management of mild to moderate pain in humans. (-)-Pentazocine is known to act as an opioid analgesic, and (+)-pentazocine is a σ receptor agonist without analgesic effects. The antinociceptive effects of (-)-pentazocine are reportedly mediated by its agonist action at the κ-opioid (KOP) receptor [1]. A previous report showed that the antinociceptive effects of (-)-pentazocine were antagonized by nor-binaltorphimine (nor-BNI, a selective KOP receptor antagonist) but not by β-funaltrexamine (a selective μ-opioid [MOP] receptor antagonist) in the mouse tail-flick test [2]. However, (-)-pentazocine reportedly binds not only KOP receptors but also MOP receptors with high affinity [2] and acts as a MOP receptor partial agonist. Furthermore, the antinociceptive effects of (-)-pentazocine were antagonized by β-funaltrexamine in the mouse hot-plate test [3] and writhing test [4]. Thus, the role of the MOP receptor in the antinociceptive effects of

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(-)-pentazocine remains unclear. Moreover, the most selective ligands for specific opioid receptor subtypes (e.g., β-funaltrexamine for the MOP receptor, naltrindole for the δ-opioid [DOP] receptor, and nor-BNI for the KOP receptor) possess certain affinities for other opioid receptor subtypes [5]. Thus, the precise molecular mechanisms that underlie the antinociceptive effects of (-)-pentazocine have not been clearly delineated by traditional pharmacological studies that use only selective ligands.

Developing mice that lack the MOP receptor gene has made possible the discovery of the molecular mechanisms that underlie the effects of opioids [6-9]. Both the analgesic and rewarding effects of morphine are abolished in MOP receptor knockout (MOP-KO) mice [7-9]. Buprenorphine, a nonselective opioid receptor partial agonist, exerts no analgesic effects in the tail-flick and hot-plate tests but a significant rewarding effect in the conditioned place preference paradigm in homozygous MOP-KO mice [10]. These observations are especially interesting because the distributions of DOP and KOP receptors are not apparently altered in MOP-KO mice [6,7,9]. Furthermore, butorphanol, a nonselective opioid receptor partial agonist, exerts no thermal or mechanical antinociceptive effects but exerts visceral chemical antinociceptive effects that are sensitive to nor-BNI in MOP-KO mice [11]. Although several compensatory changes might occur in KO animals, these animal models have potential utility in the investigation of the *in vivo* roles of specific proteins. Thus, the use of MOP-KO mice has provided novel theories on the molecular mechanisms that underlie the effects of opioid ligands. The present study investigated the molecular mechanisms that underlie the antinociceptive effects of (-)-pentazocine using various types of nociceptive stimuli in MOP-KO mice.

Methods

Animals

The present study used wildtype, heterozygous, and homozygous MOP-KO mouse littermates from heterozygous/heterozygous MOP-KO crosses on a C57BL/6J genetic background (backcrossed at least 10 generations) as previously described [8]. The experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committee, and all animal care and treatment were in accordance with our institutional animal experimentation guidelines. Naive adult (>10 weeks old) male and female mice were group housed in an animal facility maintained at 22 ± 2°C and 55 ± 5% relative humidity under a 12 h/12 h light/dark cycle with lights on at 8:00 AM and off at 8:00 PM. Food and water were available *ad libitum*.

Drugs

(-)-Pentazocine and nor-BNI dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). For the *in vitro* assays, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin (DAMGO), a MOP-selective agonist, and [D-Pen^2,D-Pen^5]enkephalin (DPDPE), a DOP agonist, were purchased from Peninsula Laboratories Ltd. (Merseyside, UK). (+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspirol[4,5]dec-8-yl]benzeneacetamide (U69593), a KOP-selective agonist, was a gift from Upjohn (Kalamazoo, MI). [tyrosyl-3,5-^{3}H(N)]DAMGO (50.5 Ci/mmol), [phenyl-3,4-^{3}H]U69593 (47.5 Ci/mmol), and [tyrosyl-2,6-^{3}H(N)]DPDPE (33.0 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, MA).

Antinociceptive tests

Thermal antinociception was evaluated using the hot-plate and tail-flick tests. Hot-plate testing was performed according to the method of Woolfe and MacDonald (1944) [12] with slight modifications. A commercially available apparatus that consisted of an acrylic resin cage (20 × 25 × 25 cm, width × length × height) and a temperature-controlled aluminum plate (Model MK-350A, Muromachi Kikai Co., Tokyo, Japan) was used for this test. Mice were placed on a 52 ± 0.2°C hot-plate, and the latencies to lick the hind-paw and jump were recorded. We selected a relatively low temperature (52°C) to examine the mild thermal antinociceptive effects of opioid partial agonists [10]. The cut-off time was 60 s. Tail-flick testing was performed according to the method of D'Amour and Smith (1941) [13] with slight modifications using a commercially available apparatus that consisted of an irradiator for heat stimulation and a photosensor for the detection of tail-flick behavior (Model MK-330A, Muromachi Kikai Co., Tokyo, Japan). The mice were loosely wrapped in a felt towel. Their tails were heated, and tail-flick latencies were automatically recorded. The cut-off time was 15 s. The tail-flick test was followed by the hot-plate test, and both tests were conducted in the same mice. Mechanical antinociception was evaluated using the hind-paw pressure test according to the method of Randall and Selitto (1957) [14] with slight modifications using a commercially available apparatus (Pressure Analgesy-Meter, Model MK-201D, Muromachi Kikai Co., Tokyo, Japan). The mice were loosely wrapped in a felt towel. Their hind-paws were gradually pressed, and hind-paw withdrawal and struggle latencies were automatically recorded. The cut-off pressure was 250 mmHg. The drug injection volume was 10 ml/kg. (-)-Pentazocine was administered at doses of 3, 7, 20, and 26 mg/kg (s.c.), for cumulative doses of 3, 10, 30, and 56 mg/kg, respectively. Tail-flick, hot-plate, and hind-paw pressure tests were conducted

20 min after each drug injection, and then the next dose of drug was injected immediately after these tests.

The hot-plate, tail-flick, and hind-paw pressure responses of each mouse in the drug-induced antinociception tests were converted to the percentage of maximal possible effect (%MPE) according to the following formula:

$$\%MPE = (postdrug\ latency - predrug\ latency) / (cut - off\ time\ or\ pressure) - predrug\ latency) \times 100\%$$

Visceral chemical antinociception was evaluated using the writhing test (Collier et al., 1968) [15]. Acetic acid (0.6% v/v, 10 ml/kg) was injected intraperitoneally (i.p.), and the mouse was placed in a large plastic cage. The intensity of nociceptive behavior was quantified by counting the total number of writhes that occurred between 0 and 15 min after the acetic acid injection. The writhing response consists of contraction of the abdominal muscles. Nociception is expressed as a writhing score during the 15 min period. (-)-Pentazocine (10 mg/kg, s.c.) or saline was administered 10 min before the acetic acid injection in a blind manner. nor-BNI (10 and 20 mg/kg, s.c.) was administered 24 h before the (-)-pentazocine injection.

Somatic chemical antinociception was evaluated using the formalin test in a blind manner as previously described [16]. Formalin (5% v/v, 20 μl) was injected into the right hind-paw (intraplantar), and the mouse was placed in a large plastic cage. The amount of time the mouse spent elevating, licking, shaking, or biting the injected paw was measured for each 5 min period during a 60 min session. Nociception was quantified using a rating scale by assigning weights to the following categories of nociceptive behavior: category 0 (weight is evenly distributed among all paws), category 1 (injected paw is lifted), category 2 (injected paw is licked, shaken, or bitten). The nociceptive score was calculated for each 5 min (300 s) period using the following formula:

$$Nociceptive\ score = \{(time\ [s]\ spent\ lifting\ the\ injected\ paw) \times 1 + (time\ [s]\ spent\ licking,\ shaking,\ or\ biting\ the\ injected\ paw) \times 2\}/300\,s.$$

(-)-Pentazocine (10 mg/kg, s.c.) or saline was administered 10 min before formalin injection (intraplantar) in a blind manner.

Stable expression of human opioid receptors in Chinese hamster ovary cells

Chinese hamster ovary (CHO) cell lines that stably express human MOP, DOP, and KOP (MOP/CHO, DOP/CHO, and KOP/CHO, respectively) were established as previously described [10]. The K_d values of [^{3}H]DAMGO binding to MOP, [^{3}H]DPDPE binding to DOP, and [^{3}H]U69593 binding to KOP were 1.7 ± 0.3 nM (n = 4), 2.2 ± 0.2 nM (n = 4), and 2.5 ± 0.2 nM (n = 3), respectively. The B_{max} estimates of receptor densities in these cell lines were 2300 ± 160, 3000 ± 270, and 5000 ± 450 fmol/mg protein, respectively.

Radioligand binding assay

Binding assays were performed as previously described [17] with slight modifications. Expressing cells were harvested after 65 h in culture, homogenized in 50 mM Tris buffer (pH 7.4) that contained 10 mM $MgCl_2$ and 1 mM EDTA, pelleted by centrifugation for 20 min at 30000 × g, and resuspended in the same buffer. For the saturation binding assays, cell membrane suspensions were incubated for 60 min at 25°C with various concentrations of [^{3}H]DAMGO for the human MOP receptor, [^{3}H]DPDPE for the human DOP receptor, or [^{3}H]U69593 for the human KOP receptor. Nonspecific binding was determined in the presence of 10 mM unlabeled ligands. For the competitive binding assays, the cell membrane suspensions were incubated for 60 min at 25°C with 2 nM [^{3}H]DAMGO for the human MOP receptor, 2 nM [^{3}H]DPDPE for the human DOP receptor, or 3 nM [^{3}H]U69593 for the human KOP receptor in the presence of various concentrations of ligands. After incubation for 60 min, the membrane suspensions were rapidly filtrated, and the radioactivity of each filter was then measured by liquid scintillation counting. The K_d values of the radiolabeled ligands were obtained by Scatchard analysis of the data from the saturation binding assay. For the competitive binding assay, non-linear regression analysis using a one-competition model (GraphPad Prism, GraphPad, San Diego, CA) was conducted to estimate the inhibitory concentration at 50% (IC_{50}). K_i values were calculated from the IC_{50} values obtained from the competitive binding assay using the equation $K_i = IC_{50} / (1 + [radiolabeled\ ligand]/K_d)$, where IC_{50} is the concentration of unlabeled ligand that produces 50% inhibition of the specific binding of radiolabeled ligand. The binding assay results are expressed as the mean ± SEM of four independent experiments, each performed in duplicate.

cAMP assay

3',5'-Cyclic adenosine monophosphate (cAMP) assays were performed as previously described [17] with slight modifications. Briefly, 10^5 cells were placed into each well of a 24-well plate, grown for 24 h, washed, and incubated with 0.45 ml HEPES-buffered saline that contained 1 mM 3-isobutyl-1-methylxanthine for 10 min at 37°C. The cells were then stimulated for 10 min by the addition of 50 ml HEPES-buffered saline that contained 100 mM forskolin and 1 mM 3-isobutyl-1-methylxanthine in the presence or absence of various concentrations of opioid ligands and then disrupted by adding

0.5 ml ice-cold 10% trichloroacetic acid to each well. The concentrations of cAMP were measured by radioimmunoassay (Amersham, Buckinghamshire, UK). cAMP accumulation is expressed as a fraction of the control value obtained without the addition of opioids. Inhibition curves were generated using non-linear least-squares fit using GraphPad Prism (GraphPad, San Diego, CA). IC_{50} values were calculated as the concentration of ligand that produces 50% of maximal inhibition of cAMP accumulation. The IC_{50} values and maximal inhibitory effects (I_{max}) in the cAMP assays are expressed as mean ± SEM of four independent experiments, each performed in triplicate.

Statistical analyses

The dose-response functions of the thermal and mechanical antinociceptive effects of (-)-pentazocine were statistically evaluated by three-way, mixed-design analysis of variance (ANOVA) with two between-subjects factors (sex and genotype) and one within-subjects factor (drug dose). Differences among genotypes were statistically evaluated by two-way, mixed-design ANOVA followed by the Bonferroni *post hoc* test. The visceral chemical antinociceptive effects of (-)-pentazocine were analyzed by one-way and two-way factorial ANOVA followed by the Bonferroni *post hoc* test. The somatic chemical antinociceptive effects of (-)-pentazocine were statistically evaluated by four-way, mixed-design ANOVA with three between-subjects factors (drug treatment, sex, and genotype) and one within-subjects factor (time). The sum of the nociceptive scores during the 1st (0-15 min) and 2nd (15-60 min) phases were statistically evaluated by one-way factorial ANOVA followed by the Bonferroni *post hoc* test. The sum of the nociceptive scores of the (-)-pentazocine-treated groups were also analyzed by two-way factorial ANOVA with two between-subjects factors (genotype and sex). Values of $p < 0.05$ were considered statistically significant.

Results

Thermal antinociceptive effects

The thermal antinociceptive dose-response relationships of (-)-pentazocine were analyzed in wildtype, heterozygous, and homozygous MOP-KO mice (Figure 1). (-)-Pentazocine dose-dependently induced thermal antinociceptive effects in both wildtype and heterozygous MOP-KO mice but not in homozygous MOP-KO mice. Three-way, mixed-design ANOVA revealed that the thermal antinociceptive effects of (-)-pentazocine (% MPE) were significantly different among these genotypes in both the hot-plate test (significant difference between genotypes, $F_{2,31} = 34.39$, $p < 0.001$; significant genotype × dose interaction, $F_{8,124} = 13.53$, $p < 0.001$; Figure 1A, B) and tail-flick test (significant difference between genotypes, $F_{2,31} = 76.84$, $p < 0.001$; significant genotype × dose interaction, $F_{8,124} = 18.34$, $p < 0.001$; Figure 1C, D). The thermal antinociceptive effects of (-)-pentazocine were significantly different between male and female mice in the hot-plate test (significant difference between sexes, $F_{1,31} = 8.82$, $p < 0.01$; significant sex × dose interaction, $F_{4,124} = 4.16$, $p < 0.01$; Figure 1B) but not in the tail-flick test (no significant difference between sexes, $F_{1,31} = 2.30$, $p = 0.14$; no sex × dose interaction, $F_{4,124} = 0.78$, $p = 0.54$; Figure 1D). Although the thermal antinociceptive effects of (-)-pentazocine in the tail-flick test tended to be more pronounced in male mice than in female mice, these differences were significant only in the hot-plate test.

In the hot-plate test, two-way, mixed-design ANOVA revealed that the thermal antinociceptive effects of (-)-pentazocine were significantly different among genotypes in both males ($F_{2,18} = 18.23$, $p < 0.001$) and females ($F_{2,13} = 27.05$, $p < 0.001$; Figure 1B). The thermal antinociceptive effects of (-)-pentazocine in heterozygous and homozygous MOP-KO female mice were significantly lower than in wildtype female mice ($p < 0.05$, Bonferroni *post hoc* test). By contrast, these effects only in homozygous MOP-KO male mice were significantly lower than in wildtype male mice ($p < 0.05$, Bonferroni *post hoc* test). Furthermore, two-way, mixed-design ANOVA also revealed that the thermal antinociceptive effects of (-)-pentazocine were significantly different between male and female heterozygous MOP-KO mice ($F_{1,10} = 8.31$, $p < 0.05$) but not in wildtype and homozygous MOP-KO mice.

In the tail-flick test, two-way, mixed-design ANOVA revealed that the thermal antinociceptive effects of (-)-pentazocine were significantly different among genotypes ($F_{2,34} = 78.85$, $p < 0.001$; Figure 1C, D). The thermal antinociceptive effects of (-)-pentazocine in heterozygous and homozygous MOP-KO mice were significantly lower than in wildtype mice ($p < 0.05$, Bonferroni *post hoc* test).

Mechanical antinociceptive effects

The mechanical antinociceptive effects of (-)-pentazocine were then analyzed in wildtype, heterozygous, and homozygous MOP-KO mice (Figure 2). (-)-Pentazocine showed dose-dependent mechanical antinociceptive effects in both wildtype and heterozygous MOP-KO mice but not in homozygous MOP-KO mice. Three-way, mixed-design ANOVA revealed that the mechanical antinociceptive effects of (-)-pentazocine were significantly different among these genotypes in the hind-paw pressure test (significant difference between genotypes, $F_{2,19} = 233.2$, $p < 0.001$; significant genotype × dose

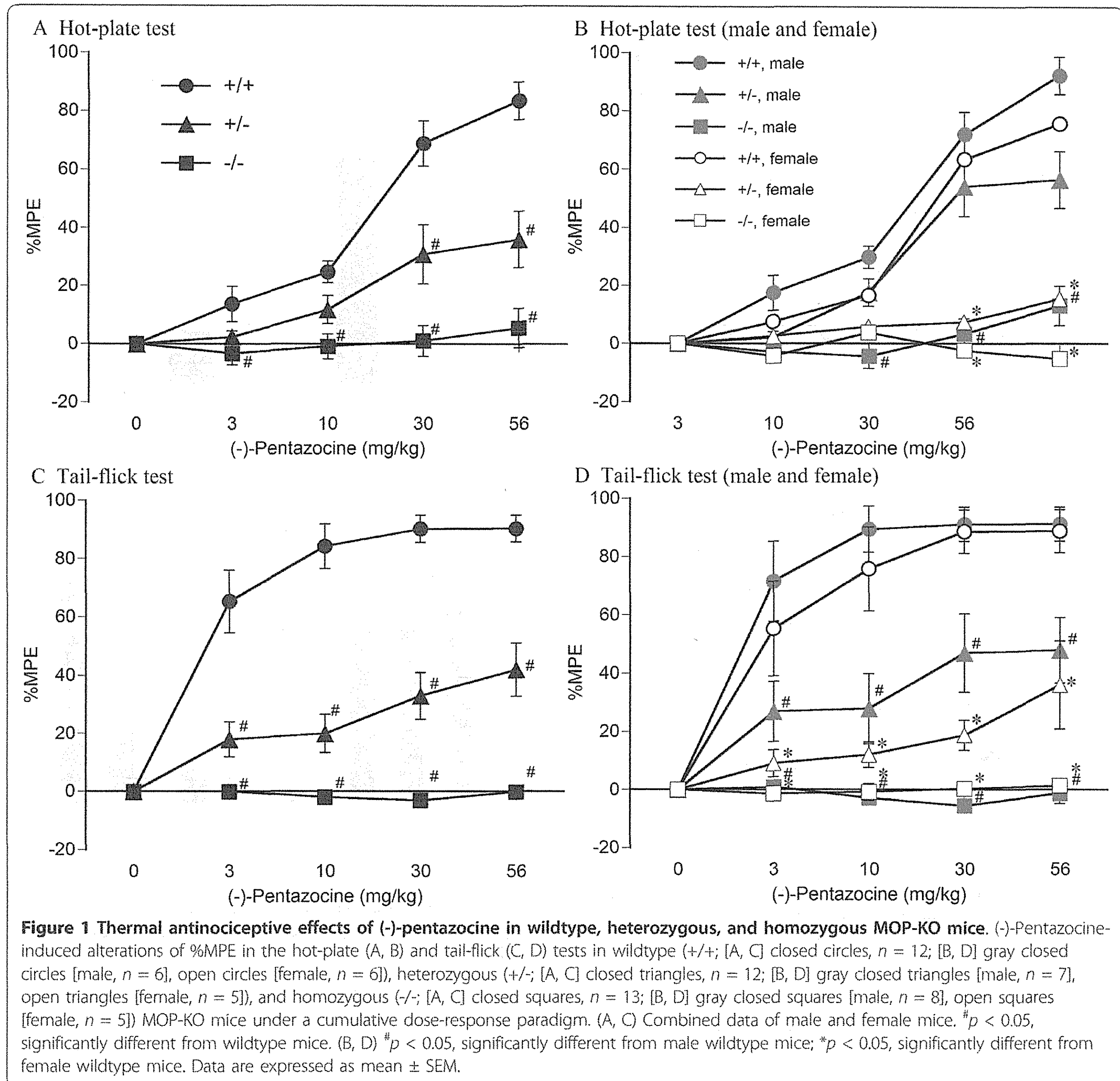

Figure 1 Thermal antinociceptive effects of (-)-pentazocine in wildtype, heterozygous, and homozygous MOP-KO mice. (-)-Pentazocine-induced alterations of %MPE in the hot-plate (A, B) and tail-flick (C, D) tests in wildtype (+/+; [A, C] closed circles, n = 12; [B, D] gray closed circles [male, n = 6], open circles [female, n = 6]), heterozygous (+/-; [A, C] closed triangles, n = 12; [B, D] gray closed triangles [male, n = 7], open triangles [female, n = 5]), and homozygous (-/-; [A, C] closed squares, n = 13; [B, D] gray closed squares [male, n = 8], open squares [female, n = 5]) MOP-KO mice under a cumulative dose-response paradigm. (A, C) Combined data of male and female mice. $^{\#}p < 0.05$, significantly different from wildtype mice. (B, D) $^{\#}p < 0.05$, significantly different from male wildtype mice; $^{*}p < 0.05$, significantly different from female wildtype mice. Data are expressed as mean ± SEM.

interaction, $F_{8,76}$ = 38.29, p < 0.001; Figure 2). In contrast, these effects in the hind-paw pressure test were not significantly different between male and female mice (no significant difference between sexes, $F_{1,19}$ = 0.58, p = 0.45; no sex × dose interaction, $F_{4,124}$ = 0.78, p = 0.54; Figure 2B). Two-way, mixed-design ANOVA revealed that the mechanical antinociceptive effects of (-)-pentazocine were significantly different among genotypes ($F_{2,22}$ = 257.5, p < 0.001; Figure 2A). The mechanical antinociceptive effects of (-)-pentazocine in both heterozygous and homozygous MOP-KO mice were significantly lower than in wildtype mice (p < 0.05, Bonferroni *post hoc* test).

Visceral chemical antinociceptive effects

The visceral chemical antinociceptive effects of (-)-pentazocine (10 mg/kg, s.c.) were analyzed in wildtype, heterozygous, and homozygous MOP-KO mice using the writhing test. Interestingly, (-)-pentazocine induced visceral chemical antinociceptive effects not only in wildtype and heterozygous MOP-KO mice, but also in homozygous MOP-KO mice. One-way factorial ANOVA revealed that (-)-pentazocine significantly decreased writhing (Figure 3A) in wildtype mice ($F_{1,17}$ = 128.1, p < 0.001), heterozygous MOP-KO mice ($F_{1,16}$ = 125.4, p < 0.001), and homozygous MOP-KO mice ($F_{1,18}$ = 87.40, p < 0.001). Although no significant differences in writhing

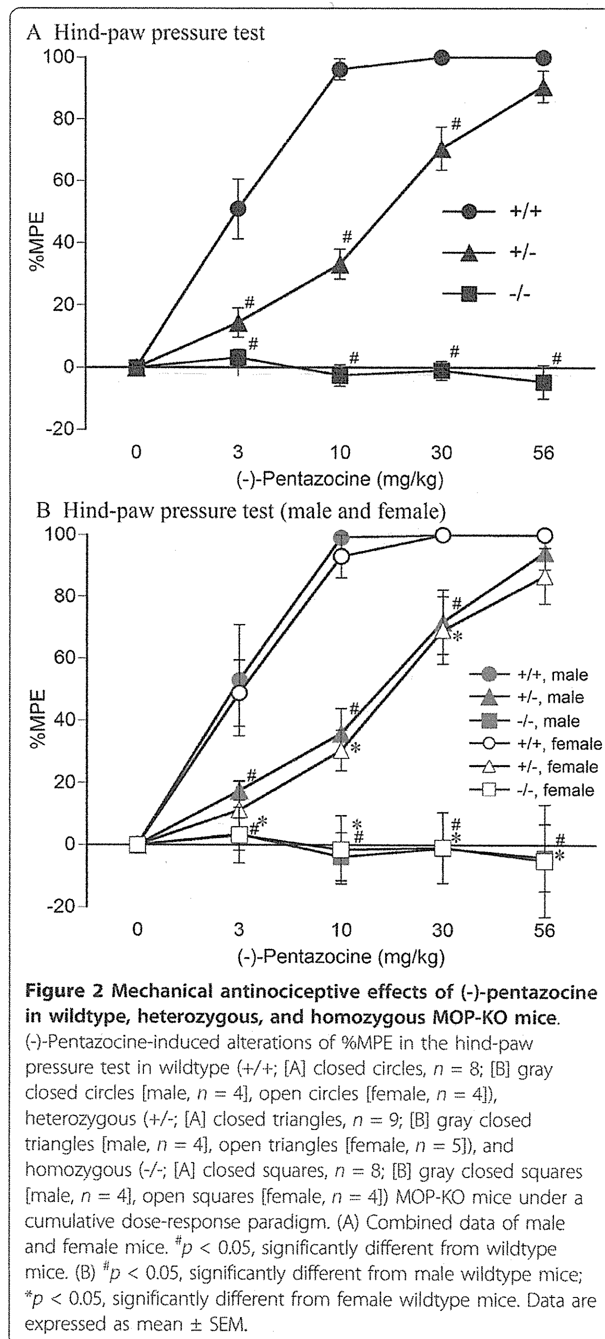

Figure 2 Mechanical antinociceptive effects of (-)-pentazocine in wildtype, heterozygous, and homozygous MOP-KO mice. (-)-Pentazocine-induced alterations of %MPE in the hind-paw pressure test in wildtype (+/+; [A] closed circles, $n = 8$; [B] gray closed circles [male, $n = 4$], open circles [female, $n = 4$]), heterozygous (+/-; [A] closed triangles, $n = 9$; [B] gray closed triangles [male, $n = 4$], open triangles [female, $n = 5$]), and homozygous (-/-; [A] closed squares, $n = 8$; [B] gray closed squares [male, $n = 4$], open squares [female, $n = 4$]) MOP-KO mice under a cumulative dose-response paradigm. (A) Combined data of male and female mice. $^{\#}p < 0.05$, significantly different from wildtype mice. (B) $^{\#}p < 0.05$, significantly different from male wildtype mice; $^{*}p < 0.05$, significantly different from female wildtype mice. Data are expressed as mean ± SEM.

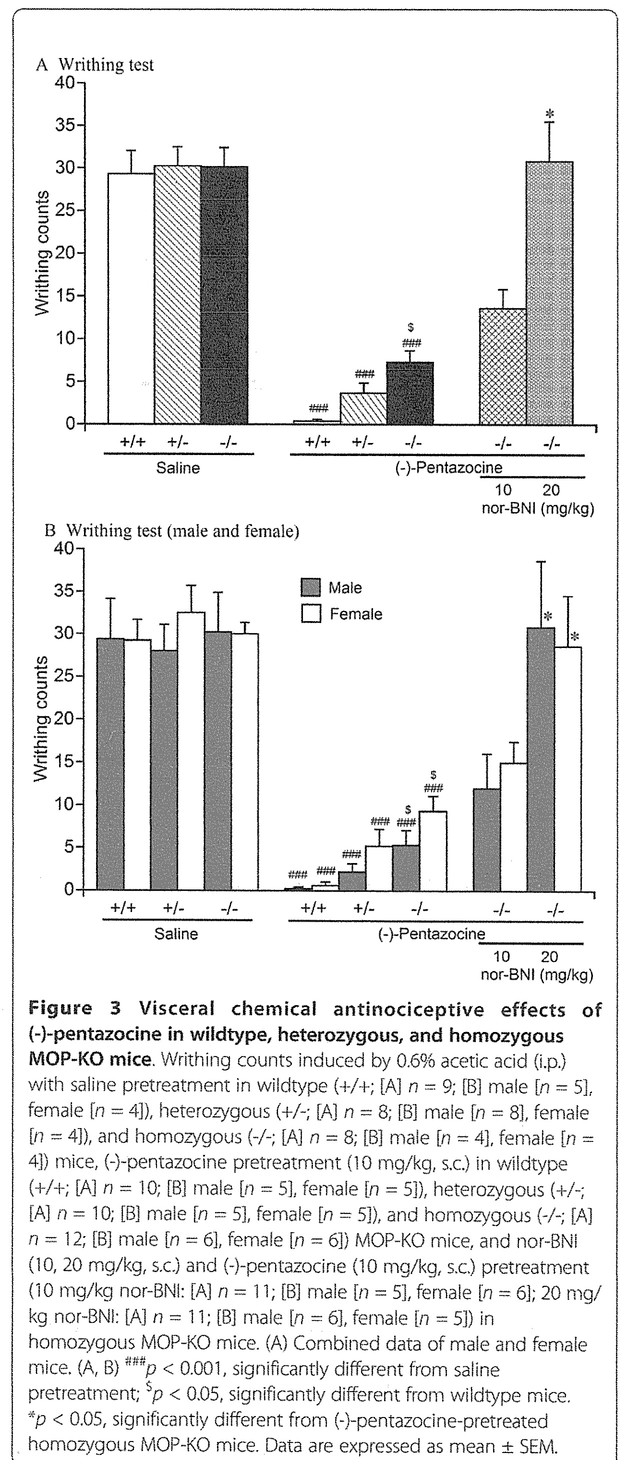

Figure 3 Visceral chemical antinociceptive effects of (-)-pentazocine in wildtype, heterozygous, and homozygous MOP-KO mice. Writhing counts induced by 0.6% acetic acid (i.p.) with saline pretreatment in wildtype (+/+; [A] $n = 9$; [B] male [$n = 5$], female [$n = 4$]), heterozygous (+/-; [A] $n = 8$; [B] male [$n = 8$], female [$n = 4$]), and homozygous (-/-; [A] $n = 8$; [B] male [$n = 4$], female [$n = 4$]) mice, (-)-pentazocine pretreatment (10 mg/kg, s.c.) in wildtype (+/+; [A] $n = 10$; [B] male [$n = 5$], female [$n = 5$]), heterozygous (+/-; [A] $n = 10$; [B] male [$n = 5$], female [$n = 5$]), and homozygous (-/-; [A] $n = 12$; [B] male [$n = 6$], female [$n = 6$]) MOP-KO mice, and nor-BNI (10, 20 mg/kg, s.c.) and (-)-pentazocine (10 mg/kg, s.c.) pretreatment (10 mg/kg nor-BNI: [A] $n = 11$; [B] male [$n = 5$], female [$n = 6$]; 20 mg/kg nor-BNI: [A] $n = 11$; [B] male [$n = 6$], female [$n = 5$]) in homozygous MOP-KO mice. (A) Combined data of male and female mice. (A, B) $^{\#\#\#}p < 0.001$, significantly different from saline pretreatment; $^{\$}p < 0.05$, significantly different from wildtype mice. $^{*}p < 0.05$, significantly different from (-)-pentazocine-pretreated homozygous MOP-KO mice. Data are expressed as mean ± SEM.

counts were observed in the saline-treated groups, two-way factorial ANOVA with two between-subjects factors (genotype and sex) showed significant differences in writhing counts between genotypes in the (-)-pentazocine-treated group ($F_{2,26} = 12.06$, $p < 0.001$). Furthermore, writhing counts in female mice in the (-)-pentazocine-treated group were higher than in male mice ($F_{1,26} = 4.42$, $p < 0.05$; Figure 3B). Writhing counts in (-)-pentazocine-treated homozygous MOP-KO mice were significantly higher than in both male and female wild-type mice treated with (-)-pentazocine ($p < 0.05$, Bonferroni *post hoc* test).

The remaining visceral chemical antinociceptive effects of (-)-pentazocine in homozygous MOP-KO mice

were dose-dependently antagonized by pretreatment with nor-BNI (s.c.). Two-way factorial ANOVA with two between-subjects factors (sex and nor-BNI dose) in MOP-KO mice revealed a significant difference in writhing counts between nor-BNI doses ($F_{2,28} = 48.07$, $p < 0.05$) but no difference between males and females. Treatment with 20 mg/kg nor-BNI in MOP-KO mice significantly antagonized the remaining visceral chemical antinociceptive effects of (-)-pentazocine ($p < 0.05$, Bonferroni *post hoc* test).

Somatic chemical antinociceptive effects

The somatic chemical antinociceptive effects of (-)-pentazocine (10 mg/kg, s.c.) were analyzed in wildtype, heterozygous, and homozygous MOP-KO mice using the formalin test. (-)-Pentazocine exerted somatic chemical antinociceptive effects in both wildtype and heterozygous MOP-KO mice but not in homozygous MOP-KO mice (Figure 4). Four-way, mixed-design ANOVA with three between-subjects factors (sex, genotype, and drug treatment) and one within-subjects factor (time) revealed that the nociceptive scores in the formalin test were significantly different among these genotypes (significant difference between genotypes, $F_{2,51} = 8.26$, $p < 0.005$; significant genotype × drug treatment interaction, $F_{2,51} = 11.45$, $p < 0.001$; significant genotype × time interaction, $F_{22,561} = 2.82$, $p < 0.001$; significant genotype × time × drug treatment interaction, $F_{22,561} = 2.20$, $p < 0.005$; Figure 4A). Moreover, we found a significant difference between sexes ($F_{1,51} = 7.57$, $p < 0.01$) and a significant sex × time interaction ($F_{11,561} = 2.97$, $p < 0.005$) but no sex × drug treatment interaction.

Two phases of spontaneous nociceptive behavior were analyzed (phase 1 beginning at 0 min and lasting for 15 min, and phase 2 beginning at 15 min). Therefore, the effects of (-)-pentazocine were based on the cumulative number of nociceptive scores for each phase for each mouse (Figure 4B, C). One-way factorial ANOVA revealed that (-)-pentazocine significantly decreased nociceptive scores during both phases (Figure 4B) in wildtype mice (Phase 1, $F_{1,19} = 16.79$, $p < 0.005$; Phase 2, $F_{1,19} = 99.92$, $p < 0.001$) and heterozygous MOP-KO mice (Phase 1, $F_{1,18} = 11.46$, $p < 0.005$; Phase 2, $F_{1,18} = 13.97$, $p < 0.005$) but not in homozygous MOP-KO mice. Although no significant differences in nociceptive scores were observed in the saline-treated groups, two-way factorial ANOVA with two between-subjects factors (genotype and sex) showed significant differences in nociceptive scores between the genotypes in the (-)-pentazocine-treated groups (Phase 1, $F_{2,26} = 16.33$, $p < 0.001$; Phase 2, $F_{2,26} = 21.81$, $p < 0.001$). The nociceptive scores of both the heterozygous and homozygous MOP-KO mice in the (-)-pentazocine-treated groups were significantly higher than in wildtype mice during both

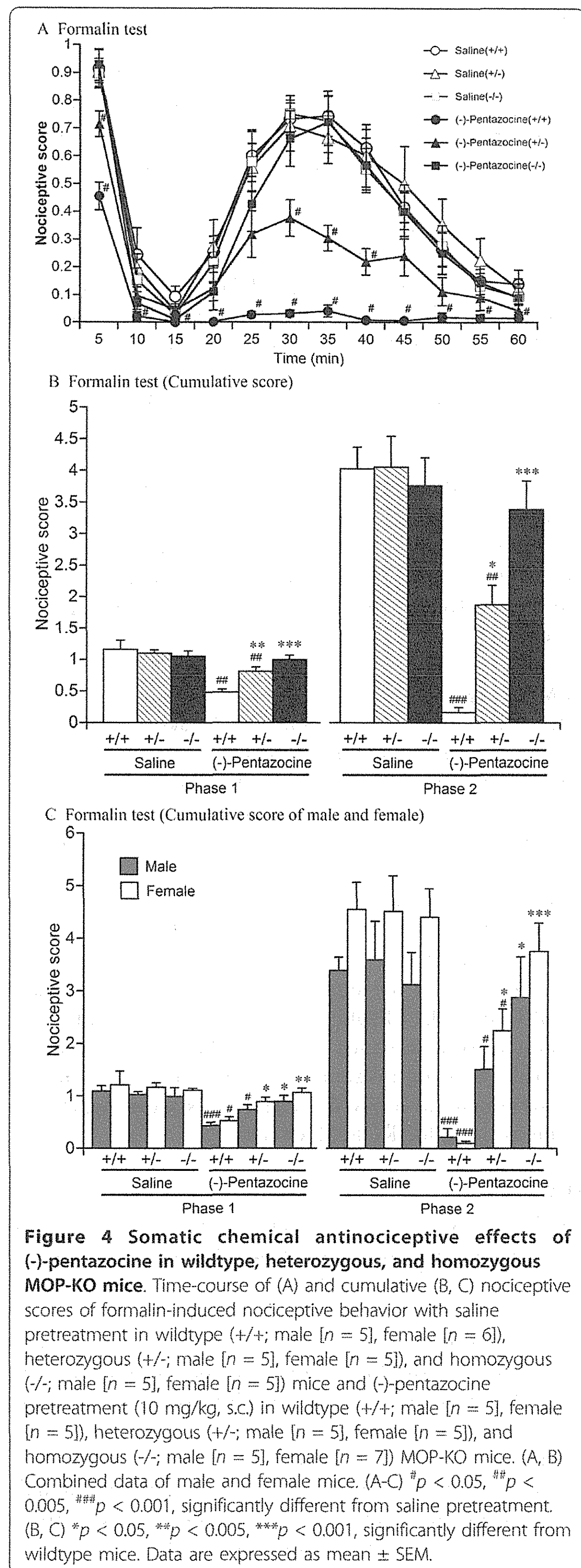

Figure 4 Somatic chemical antinociceptive effects of (-)-pentazocine in wildtype, heterozygous, and homozygous MOP-KO mice. Time-course of (A) and cumulative (B, C) nociceptive scores of formalin-induced nociceptive behavior with saline pretreatment in wildtype (+/+; male [*n* = 5], female [*n* = 6]), heterozygous (+/-; male [*n* = 5], female [*n* = 5]), and homozygous (-/-; male [*n* = 5], female [*n* = 5]) mice and (-)-pentazocine pretreatment (10 mg/kg, s.c.) in wildtype (+/+; male [*n* = 5], female [*n* = 5]), heterozygous (+/-; male [*n* = 5], female [*n* = 5]), and homozygous (-/-; male [*n* = 5], female [*n* = 7]) MOP-KO mice. (A, B) Combined data of male and female mice. (A-C) $^{\#}p < 0.05$, $^{\#\#}p < 0.005$, $^{\#\#\#}p < 0.001$, significantly different from saline pretreatment. (B, C) $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$, significantly different from wildtype mice. Data are expressed as mean ± SEM.

phases ($p < 0.05$, Bonferroni *post hoc* test). By contrast, although the nociceptive scores of female mice in the (-)-pentazocine-treated groups tended to be higher than those of male mice, no significant differences were observed between sexes (Phase 1, $F_{1,26} = 3.50$, $p = 0.073$; Phase 2, $F_{1,26} = 1.59$, $p = 0.219$; Figure 4C).

Binding characteristics

(-)-Pentazocine competition experiments using membranes prepared from MOP/CHO, DOP/CHO, and KOP/CHO cells revealed apparent binding affinities for each opioid receptor subtype (Figure 5A, Table 1). (-)-Pentazocine bound with higher affinity than morphine to membranes prepared from KOP/CHO cells. The morphine results were obtained from previous data [17] that were reanalyzed according to the present methods. Although the affinity of (-)-pentazocine for the KOP receptor was slightly higher than for the MOP receptor, (-)-pentazocine showed moderate affinity for the MOP receptor. The affinities of (-)-pentazocine for MOP and KOP receptors were higher than for DOP receptors.

cAMP assay

The effects of (-)-pentazocine on forskolin-stimulated cAMP accumulation in MOP/CHO, DOP/CHO, and KOP/CHO cells were also tested. (-)-Pentazocine concentration-dependently suppressed forskolin-stimulated cAMP accumulation in all three cell types (Figure 5B). The I_{max} values of (-)-pentazocine were lower than those of morphine for MOP/CHO cells and were the same as those of morphine for DOP/CHO and KOP/CHO cells (Table 1). The IC_{50} values of (-)-pentazocine were lower than those of morphine for DOP/CHO and KOP/CHO cells (Table 1). The morphine results were obtained from previous data [17] that were reanalyzed according to the present methods. The IC_{50} values of (-)-pentazocine for MOP/CHO cells were nearly the same as those for KOP/CHO cells.

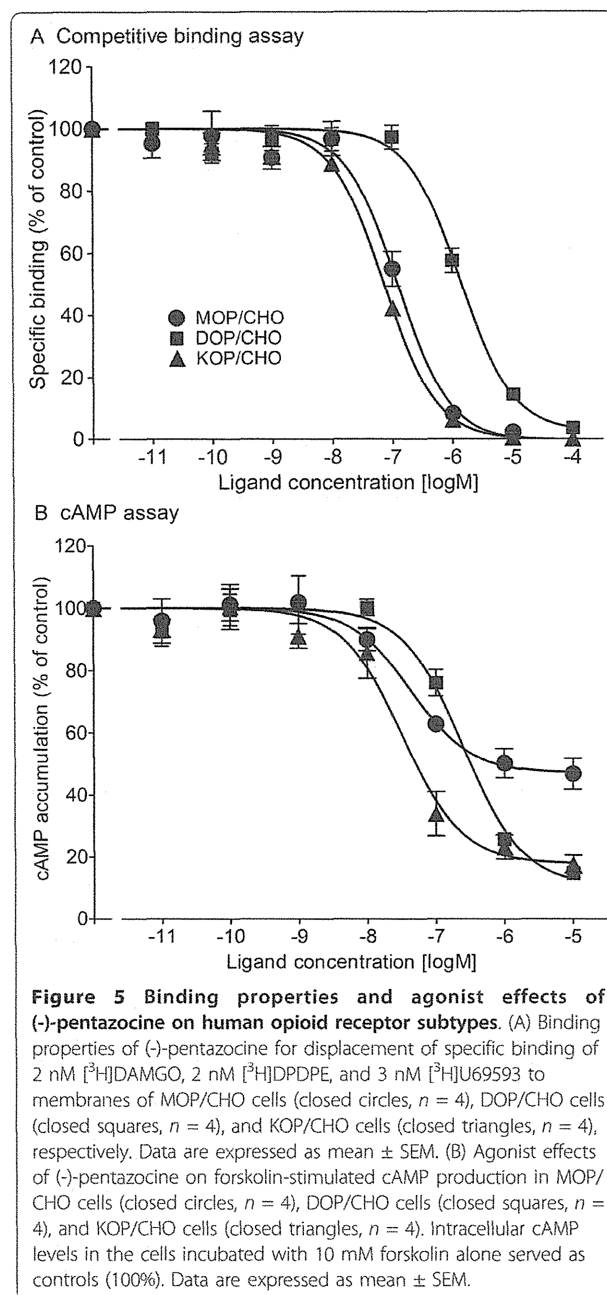

Figure 5 Binding properties and agonist effects of (-)-pentazocine on human opioid receptor subtypes. (A) Binding properties of (-)-pentazocine for displacement of specific binding of 2 nM [^{3}H]DAMGO, 2 nM [^{3}H]DPDPE, and 3 nM [^{3}H]U69593 to membranes of MOP/CHO cells (closed circles, $n = 4$), DOP/CHO cells (closed squares, $n = 4$), and KOP/CHO cells (closed triangles, $n = 4$), respectively. Data are expressed as mean ± SEM. (B) Agonist effects of (-)-pentazocine on forskolin-stimulated cAMP production in MOP/CHO cells (closed circles, $n = 4$), DOP/CHO cells (closed squares, $n = 4$), and KOP/CHO cells (closed triangles, $n = 4$). Intracellular cAMP levels in the cells incubated with 10 mM forskolin alone served as controls (100%). Data are expressed as mean ± SEM.

Discussion

In the present study, the antinociceptive effects of (-)-pentazocine on various types of nociceptive stimuli were significantly reduced in heterozygous and homozygous MOP-KO mice compared with wildtype mice. The antinociceptive effects of (-)-pentazocine in these tests increased in a MOP receptor gene dose-dependent fashion. The copy numbers of the MOP receptor gene are zero in homozygous MOP-KO mice, one in heterozygous MOP-KO mice, and two in wildtype mice. These results were obtained in not only male but also female mice, although female mice may respond differently in pain tests during different phases of their estrous cycle [18]. These results suggest that the MOP receptor is the main opioid receptor involved in (-)-pentazocine-induced antinociception. The antinociceptive effects of (-)-pentazocine were previously hypothesized to be mediated by its agonist action at KOP receptors [13]. A previous report showed that the antinociceptive effects of (-)-pentazocine were antagonized by nor-BNI, a selective KOP receptor antagonist, but not by β-funaltrexamine, a selective MOP receptor antagonist, in the mouse tail-flick test [3]. However, other groups reported that the antinociceptive effects of (-)-pentazocine were

Table 1 Binding properties and agonist effects of (-)-pentazocine and morphine on human opioid receptor subtypes

	MOP/CHO	DOP/CHO	KOP/CHO
Competitive binding assay			
K_i value (nM)			
(-)-Pentazocine	85.6 ± 13.3	641 ± 88	35.2 ± 2.6
Morphine	21.0 ± 3.7	524 ± 83	247 ± 13
cAMP assay			
IC_{50} (nM)			
(-)-Pentazocine	42.8 ± 12.9	255 ± 46	39.6 ± 14.8
Morphine	25.0 ± 9.0	610 ± 220	340 ± 160
I_{max} (%)			
(-)-Pentazocine	52.8 ± 3.0	89.3 ± 4.3	82.1 ± 3.7
Morphine	88.0 ± 3.1	83.7 ± 2.7	84.3 ± 3.3

Morphine data were obtained from a reanalysis of the data in [10].

antagonized by β-funaltrexamine in the mouse hot-plate test [3] and writhing test [4]. The discrepancy between these studies might be attributable to differences in the type of nociceptive test, strain of mice, or injection route. Furthermore, the most selective ligands for a specific subtype of opioid receptors possess certain affinities for other opioid receptor subtypes [5]. Some *in vivo* studies also demonstrated antagonist effects of nor-BNI at other opioid receptor subtypes [19,20]. Thus, the role of the MOP receptor in the antinociceptive effects of (-)-pentazocine has not been clearly evaluated by traditional pharmacological studies that only used selective ligands. The results of our *in vitro* experiments that used human MOP, DOP, and KOP receptor cDNA suggest that (-)-pentazocine induces its antinociceptive effects via the MOP receptor in humans. Although (-)-pentazocine bound to human MOP receptor with moderate affinity and showed moderate I_{max} values for the MOP receptor in the cAMP assays, (-)-pentazocine had high IC_{50} values for the MOP receptor in the cAMP assays, which were nearly the same as the IC_{50} values for the KOP receptor and the IC_{50} values of morphine for the MOP receptor. These results suggest that the MOP receptor could be involved in the antinociceptive effects of (-)-pentazocine in humans and rodents.

The antinociceptive effects of morphine, a MOP receptor agonist with low affinity for DOP and KOP receptors, are reduced in several strains of heterozygous MOP-KO mice and completely abolished in homozygous MOP-KO mice [7-9]. Furthermore, the thermal and mechanical antinociception induced by buprenorphine and butorphanol, nonselective opioid receptor partial agonists, are abolished in MOP-KO mice [10,11]. In contrast, the antinociceptive effects of morphine are not altered in mice that lack the DOP receptor [21] or in mice that lack the KOP receptor [22]. The present results, together with these previous reports, suggest that the MOP receptor may play a critical role in the analgesia induced by opioid partial agonists. MOP receptor tolerance and inactivation or individual differences in the number of MOP receptors are thus important for most of the variations in the degree of analgesia induced by opioids. Still unclear, however, is whether DOP and KOP receptors modulate the antinociceptive effects of not only (-)-pentazocine but also other opioid partial agonists. Further studies of DOP-KO, KOP-KO, and double DOP/KOP-KO mice will reveal the mechanisms that underlie these antinociceptive effects.

In contrast to thermal, mechanical, and somatic chemical antinociception, (-)-pentazocine exerted significant visceral chemical antinociception in homozygous MOP-KO mice, although the visceral chemical antinociceptive effects of (-)-pentazocine increased in a MOP receptor gene dose-dependent fashion. The residual visceral chemical antinociception induced by (-)-pentazocine was abolished by pretreatment with nor-BNI. These results indicate that both MOP and KOP receptors play dominant roles in (-)-pentazocine-induced visceral chemical antinociception, which is consistent with previous reports. The enhanced response of KOP-KO mice in the acetic acid writhing test has been previously demonstrated [22]. Furthermore, butorphanol has been shown to abolish thermal and mechanical antinociception and the nor-BNI-sensitive retention of visceral chemical antinociception in MOP-KO mice [11]. The present results, together with previous studies, suggest that both MOP and KOP receptors play important roles in visceral chemical analgesia mediated by opioid partial agonists. Furthermore, both MOP and KOP receptor-selective agonists reportedly exert significant antinociceptive effects in mice in a visceral mechanical pain model that utilizes colorectal distension [23], and peripheral KOP receptor agonists reportedly reduce visceral pain in humans [24]. The pain pathways that mediate

visceral and somatic pain have several differences [25]. Notably, treatments with KOP but not MOP or DOP receptor agonists have been shown to attenuate the responses of afferent fibers to colorectal distension [26]. The KOP receptor may play a primary role in the antinociceptive effect of opioid agonists on visceral pain via peripheral mechanisms, and MOP and KOP receptors may play a role via central mechanisms. The present results, together with previous studies, suggest that pain induced by various visceral stimuli can be better controlled by a nonselective opioid that acts at both MOP and KOP receptors. Further studies on the receptor mechanisms that underlie the analgesic effects of opioids will lead to the development of better clinical treatments of various types of pain.

Sex differences in the antinociceptive effects of (-)-pentazocine were also demonstrated in the present study. The antinociceptive effects of (-)-pentazocine were significantly higher in male than in female mice in both the hot-plate and writhing tests and tended to be high in the tail-flick and formalin tests. These sex differences appear to be pronounced in heterozygous MOP-KO mice, although sex differences in the antinociceptive effects of (-)-pentazocine in wildtype mice might not be noticeable because of a possible ceiling effect in the present nociceptive tests. The present results are consistent with previous reports. Pentazocine has been shown to exert more potent antinociception in males than in females in both mice [27] and rats [28]. These reports also showed that U50488H, a selective KOP receptor agonist, and other opioids (e.g., U69593, bremazocine, and butorphanol) are more effective in males than in females. Furthermore, with regard to MOP receptor-selective agonists, morphine exerted greater antinociceptive effects in male than in female mice [29], rats [30,31], and monkeys [32]. Additionally, female mice may differentially respond in pain tests during different phases of their estrous cycle [18]. In humans, males required less morphine or fentanyl than females for postoperative pain relief [33,34]. In contrast, some inconsistent human studies have reported that the antinociceptive effects of pentazocine on postoperative pain were higher in females than in males [35-37]. The discrepancy between these studies might be attributable to differences in the body weight-adjusted dose of pentazocine, although other factors (e.g., type of nociceptive stimulus, type of clinical surgery, estrous cycle phase, patient race, and ethnicity) might affect these results. Thus, the present results, together with previous reports, suggest that not only MOP receptor-selective opioids, but also other subtype-nonselective opioids such as pentazocine, are more effective in males than in females.

Conclusions

The present study demonstrated the abolition of the thermal, mechanical, and somatic chemical antinociceptive effects of (-)-pentazocine in male and female MOP-KO mice, suggesting that thermal, mechanical, and somatic chemical antinociception induced by (-)-pentazocine is completely mediated by the MOP receptor partial agonist effects of (-)-pentazocine. We also demonstrated the retention of (-)-pentazocine-induced visceral chemical antinociception in MOP-KO mice and abolition of (-)-pentazocine-induced visceral chemical antinociception by pretreatment with nor-BNI. Our *in vitro* data showed that (-)-pentazocine more strongly acted at KOP and MOP receptors than DOP receptors, suggesting that (-)-pentazocine-induced visceral chemical antinociception is mediated by its MOP receptor partial agonist effects and full KOP receptor agonist effects. In the clinic, (-)-pentazocine may effectively control visceral pain. Future studies will elucidate the precise molecular mechanisms that underlie the antinociceptive effects of (-)-pentazocine and will contribute to the better use of opioid drugs for pain management.

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Authors' contributions

The study was conceived and the experiments were designed by SI, MM, MS, and KI. SI performed the experiments, performed the statistical analyses, and wrote the manuscript. MOP-KO mice were developed by IS and GRU. KI supervised the experiments and finalized the manuscript. All authors contributed to writing the manuscript, and all authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Inhibitory Role of Inducible cAMP Early Repressor (ICER) in Methamphetamine-Induced Locomotor Sensitization

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Abstract

Background: The inducible cyclic adenosine monophosphate (cAMP) early repressor (ICER) is highly expressed in the central nervous system and functions as a repressor of cAMP response element-binding protein (CREB) transcription. The present study sought to clarify the role of ICER in the effects of methamphetamine (METH).

Methods and Findings: We tested METH-induced locomotor sensitization in wildtype mice, ICER knockout mice, and ICER I-overexpressing mice. Both ICER wildtype mice and knockout mice displayed increased locomotor activity after continuous injections of METH. However, ICER knockout mice displayed a tendency toward higher locomotor activity compared with wildtype mice, although no significant difference was observed between the two genotypes. Moreover, compared with wildtype mice, ICER I-overexpressing mice displayed a significant decrease in METH-induced locomotor sensitization. Furthermore, Western blot analysis and quantitative real-time reverse transcription polymerase chain reaction demonstrated that ICER overexpression abolished the METH-induced increase in CREB expression and repressed cocaine- and amphetamine-regulated transcript (CART) and prodynorphin (Pdyn) expression in mice. The decreased CART and Pdyn mRNA expression levels *in vivo* may underlie the inhibitory role of ICER in METH-induced locomotor sensitization.

Conclusions: Our data suggest that ICER plays an inhibitory role in METH-induced locomotor sensitization.

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Introduction

The inducible cyclic adenosine monophosphate (cAMP) early repressor (ICER) is the collective name for a group of proteins produced from the cAMP response element modulator (CREM)/ ICER gene driven by the P2 internal promoter located in an intron of the CREM gene [1]. Lacking the CREM *N*-terminus, ICER only contains two DNA binding domains (DBD I and DBD II) and lacks the activation and kinase-inducible domains. Consequently, ICER functions as an endogenous repressor of transcription of several cAMP response element (CRE)-containing genes [1–3]. The P2 promoter of the ICER gene contains two pairs of CRE sequences. The phosphorylated CRE-binding protein (CREB) can induce transcription of the ICER gene from the P2 promoter. The increased ICER competes with CREB in binding with the CRE sequence, blocking transcription from CRE-containing promoters, including ICER's own promoter, and functioning as a potent endogenous CREB antagonist [1,4].

Four ICER isoforms have been identified: ICER I, ICER Iγ, ICER II, and ICER IIγ. ICER I mRNA contains DBD I and DBD II, but DBD II is absent in the ICER I protein because a stop codon exists at the end of DBD I. The ICER II isoform contains only DBD II. ICER Iγ and ICER IIγ are characterized by a deficiency of exon γ from ICER I and ICER II, respectively [4].

Numerous reports have shown that CREB in the nucleus accumbens (NAc) is associated with responses to drugs of abuse and emotional responses. Chronic drug administration increases levels of CREB immunoreactivity and CRE-binding activity [5–6]. Overexpression of CREB by introducing herpes simplex virus-CREB into the NAc decreases behavioral responses to drug administration, whereas blockade of CREB transcription via introducing a dominant-negative CREB mutant or via genetic knockout increases behavioral responses to drug administration [7–10]. However, other studies showed that genetic ablation of CREB did not affect the rewarding effects of psychostimulants [11–13], indicating that the role of CREB in drug-induced responses is debatable. Recent findings suggest that ICER mRNA expression was threefold higher in the striatum after amphetamine injection [14], suggesting that the endogenous functional CREB antagonist ICER may participate in the mechanisms that underlie the effects of drugs of abuse.

The prodynorphin (Pdyn) peptide is an endogenous ligand of the κ opioid receptor. Cocaine- and amphetamine-regulated transcript (CART) was first sequenced as a peptide with unknown function [15], and previous studies revealed that the CART

peptide is co-localized with Pdyn in brain regions associated with drug reward, including the NAc and ventral tegmental area (VTA) [16–17]. Both CART and Pdyn play roles as psychostimulant neuromodulators [8,18–20]. CART and Pdyn mRNA are suggested to be CRE-mediated transcripts regulated by CREB *in vitro* and *in vivo* [8,21–23].

Kojima *et al.* [24] generated two types of ICER mutant mice—ICER knockout mice and ICER-overexpressing mice—and suggested a negative role for ICER in regulating long-term fear memory and kindling epileptogenesis. The present study used two types of transgenic mice with opposite genetic alterations of ICER gene expression (i.e., ICER knockout and ICER I-overexpressing mice) and investigated the role of ICER in methamphetamine (METH)-induced locomotor sensitization. Locomotor sensitization is characterized by the progressive enhancement of locomotor activity after repeated psychostimulant exposure [25–26]. The augmentation of this behavioral response can be maintained for several months after the cessation of drug treatment [27]. We observed an inhibitory effect of ICER on METH-induced locomotor sensitization. To identify the downstream components of ICER-mediated gene transcription *in vivo* and provide a possible mechanism that contributes to the inhibitory role of ICER in METH-induced locomotor sensitization, we determined METH-induced CREB and phosphorylated CREB (pCREB) levels using Western blot analysis and further determined CART and Pdyn mRNA expression levels in the striatum (caudate putamen [CPu], which mediates locomotor activity) but not in the NAc (which mainly mediates the rewarding effects of drugs of abuse) in ICER I-overexpressing mice and their littermates using real-time reverse transcription polymerase chain reaction (RT-PCR).

Results

METH-induced locomotor sensitization in ICER I-overexpressing mice

Consistent with a previous study [28], on Day 1, the initially elevated levels of locomotor activity in wildtype mice were reduced to near-zero levels after 180 min habituation. ICER I-overexpressing mice displayed a similar pattern of locomotor activity as wildtype mice (Fig. 1*a*). No significant difference in baseline locomotion was observed between genotypes ($n=7$ for wildtype mice; $n=9$ for ICER I-overexpressing mice; $F_{1,16}=0.49$, $p=0.49$; Fig. 1*a*). On Day 20, ICER I-overexpressing mice displayed decreased levels of spontaneous locomotor activity during the 180 min habituation period compared with wildtype mice ($F_{1,14}=9.934$, $p=0.007$; Fig. 1*b*). After a METH injection (1 mg/kg), a significant difference was observed between the two genotypes ($F_{1,14}=14.566$, $p=0.0019$; Fig. 1*b*). Repeated administration of METH (1 mg/kg) on Days 1, 3, 5, 7, 9, 11, 13, and 20 significantly increased locomotor activity in both wildtype and ICER I-overexpressing mice (Fig. 1*c*). A two-way, mixed-design analysis of variance (ANOVA; Genotype×Day) revealed a significant effect of Day ($F_{7,98}=19.13$, $p<0.0001$), indicating the presence of METH-induced locomotor sensitization. METH-induced locomotor sensitization in ICER I-overexpressing mice significantly decreased compared with wildtype mice ($F_{1,14}=12.54$, $p=0.0033$; Fig. 1*c*), and a significant Genotype×Day interaction was observed ($F_{7,98}=6.52$, $p<0.0001$; Fig. 1*c*). From Day 5, locomotor activity in ICER I-overexpressing mice was significantly lower than in wildtype mice (Student's *t*-test).

METH-induced locomotor sensitization in ICER knockout mice

On Day 1, the levels of locomotor activity in wildtype and ICER knockout mice were reduced to near-zero after 180 min habitua-

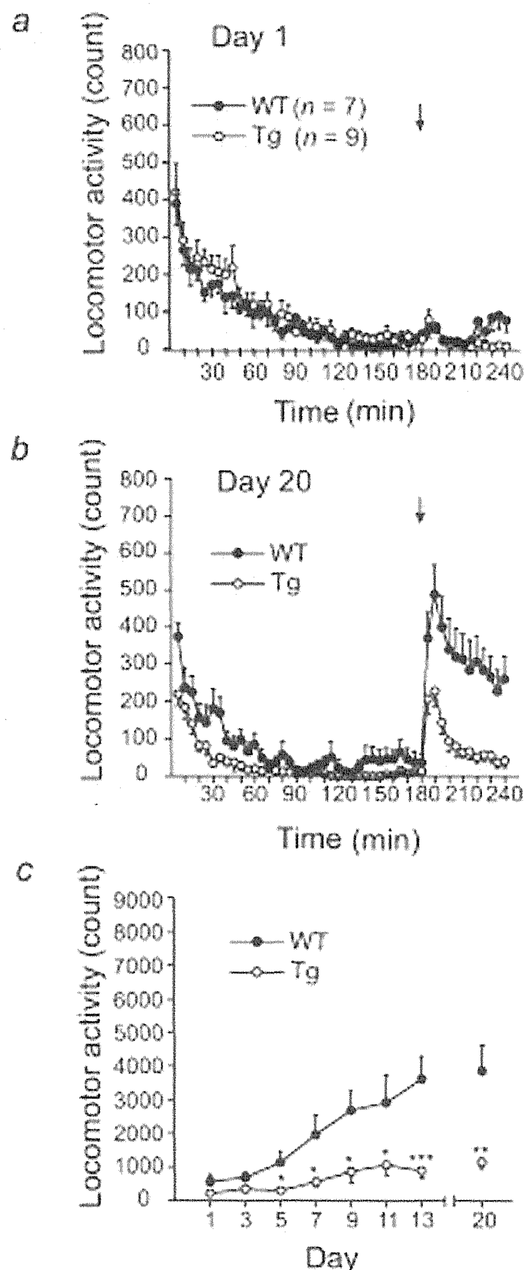

Figure 1. Spontaneous and METH-stimulated locomotor activity in wildtype mice (WT) and ICER I-overexpressing mice (Tg). METH (1 mg/kg) was administered once per day on Days 1, 3, 5, 7, 9, 13, and 20 in WT ($n=7$) and Tg ($n=9$) mice. *a*. Time-course of spontaneous locomotor activity before and after METH administration on Day 1. The data are expressed as mean ± SEM beam breaks in 5 min bins. The arrow indicates the start of a METH injection. *b*. Time-course of spontaneous locomotor activity before and after METH administration on Day 20. The data are expressed as mean ± SEM beam breaks in 5 min bins. The arrow indicates the start of a METH injection. *c*. METH-induced locomotor sensitization. The data are expressed as mean ± SEM beam breaks during the 60 min period after METH injection (1 mg/kg) on Days 1, 3, 5, 7, 9, 13, and 20. *$p<0.05$, **$p<0.01$, ***$p<0.001$, significant difference in locomotor activity scores between WT and Tg mice.
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tion. No significant difference in baseline locomotion was observed between genotypes ($n = 13$ for both wildtype and knockout mice; $F_{1,24} = 0.27, p = 0.61$; Fig. 2*a*). After repeated procedures on Days 1, 3, 5, 7, 9, 11, and 13 and a 7 day drug-free period, on Day 20, the levels of locomotor activity in the two genotypes were reduced but did not reach near-zero levels after 180 min habituation, which might have been caused by the repeated METH administration. No significant difference was detected between the two genotypes during the habituation period ($F_{1,24} = 2.731, p = 0.12$; Fig. 2*b*). After a METH injection (1 mg/kg), locomotor activity in both genotypes increased significantly. No significant difference was observed between the two genotypes ($F_{1,24} = 2.071$, $p = 0.16$; Fig. 2*b*). Repeated administration of METH (1 mg/kg) significantly increased locomotor activity in both wildtype and ICER knockout mice (Fig. 2*c*). A two-way, mixed-design ANOVA (Genotype×Day) revealed a significant effect of Day ($F_{7,168} = 25.88$, $p<0.0001$), indicating the presence of METH-induced locomotor sensitization. ICER knockout mice showed a tendency toward higher locomotor activity compared with their wildtype littermates ($F_{1,24} = 2.96$, $p = 0.098$). ICER knockout mice displayed greater locomotor activity on Day 3 and Day 11 compared with wildtype mice ($p<0.05$; Tukey-Kramer *post hoc* test). No significant Genotype× Day interaction was observed ($F_{7,168} = 0.62, p = 0.74$).

METH-induced CREB expression and phosphorylation in the CPu was abolished in ICER I-overexpressing mice

Two-way ANOVA revealed marginal differences between genotypes in CREB and pCREB protein levels in the CPu after repeated METH treatment (CREB: $F_{1,40} = 3.76, p = 0.06$; pCREB: $F_{1,40} = 3.51$, $p = 0.07$). No significant difference in the effect of METH was found (CREB: $F_{3,40} = 1.28, p = 0.29$; pCREB: $F_{3,40} = 1.38, p = 0.26$), and no Genotype×METH interaction was observed (CREB: $F_{3,40} = 1.90$, $p = 0.15$; pCREB: $F_{3,40} = 1.79$, $p = 0.16$). The Dunnett *post hoc* test revealed that repeated METH/saline challenge significantly increased CREB protein levels in wildtype mice compared with the saline group ($n = 6$ per group, $p<0.05$; Fig. 3*a*). The level of activated CREB protein (pCREB) in the repeated METH/saline challenge group also significantly increased in wildtype mice ($n=6$ per group, $p<0.05$, Dunnett *post hoc* test; Fig. 3*b*). However, the levels of CREB and pCREB protein were not significantly altered after repeated METH injection in ICER I-overexpressing mice (Fig. 3).

ICER overexpression significantly reduced CART and Pdyn mRNA expression in the CPu

To identify the downstream components of CRE-mediated gene transcription that contribute to reduced METH-induced locomotor sensitization in ICER I-overexpressing mice, real-time RT-PCR was conducted. First, ICER mRNA levels were evaluated using ICER-specific primers. Significant effects were found for Genotype ($F_{1,24} = 1850.5$, $p<0.001$, two-way ANOVA; Fig. 4*a*). However, METH injection did not significantly affect ICER mRNA levels in wildtype mice ($n=4$ per group, $p>0.05$, Dunnett *post hoc* test). Furthermore, we evaluated CART and Pdyn mRNA levels because they are suggested to be CRE-mediated transcripts and psychostimulant neuromodulators. Although METH did not alter CART or Pdyn mRNA expression in ICER I-overexpressing mice and their littermates (CART: $F_{3,24} = 0.31$, $p = 0.81$; Pdyn: $F_{3,24} = 0.38$, $p = 0.77$; two-way ANOVA), CART and Pdyn mRNA expression levels were significantly reduced in ICER I-overexpressing mice compared with their littermates (CART: $F_{1,24} = 17.25$, $p<0.01$; Pdyn: $F_{1,24} = 12.21$, $p<0.01$; two-way ANOVA; Fig. 4*b*, *c*). No significant Genotype×METH interaction was observed (CART: $F_{3,24} = 0.21, p = 0.89$; Pdyn: $F_{3,24} = 0.17, p = 0.92$).

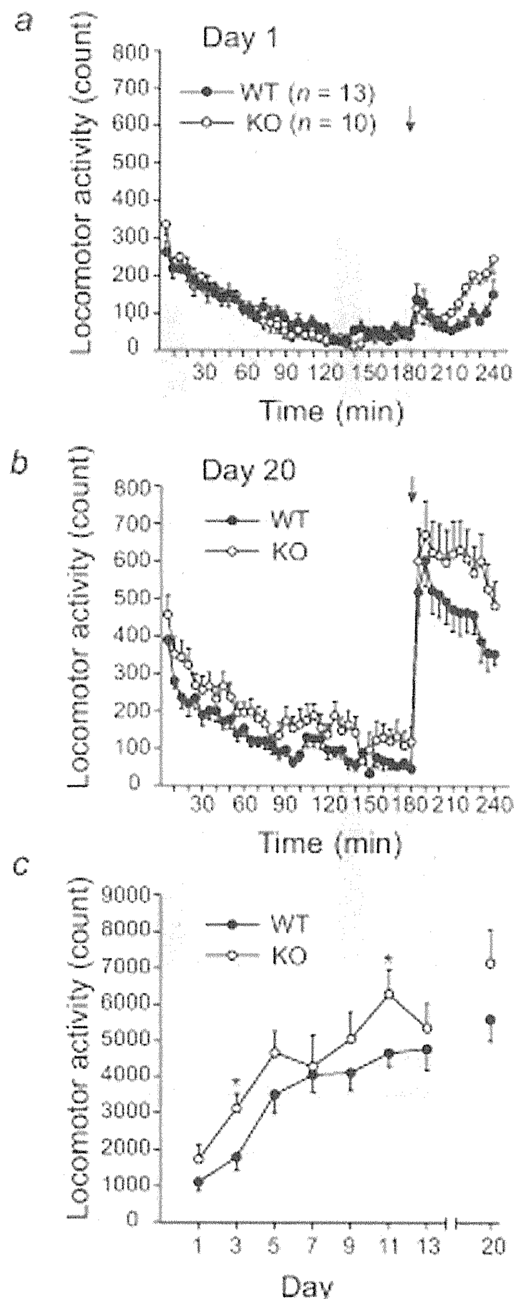

Figure 2. Spontaneous and METH-stimulated locomotor activity in wildtype (WT) and ICER knockout (KO) mice. METH (1 mg/kg) was administered once per day on Days 1, 3, 5, 7, 9, 13, and 20 in WT ($n=13$) and ICER-KO ($n=13$) mice. *a*. ime-course of spontaneous locomotor activity before and after METH administration on Day 1. The data are expressed as mean ± SEM beam breaks in 5 min bins. The arrow indicates the start of a METH injection. *b*. Time-course of spontaneous locomotor activity before and after METH administration on Day 20. The data are expressed as mean ± SEM beam breaks in 5 min bins. The arrow indicates the start of a METH injection. *c*. METH-induced locomotor sensitization. The data are expressed as mean ± SEM beam breaks during the 60 min period after METH injection (1 mg/kg). *$p<0.05$, significant difference in locomotor activity scores between WT and KO mice.
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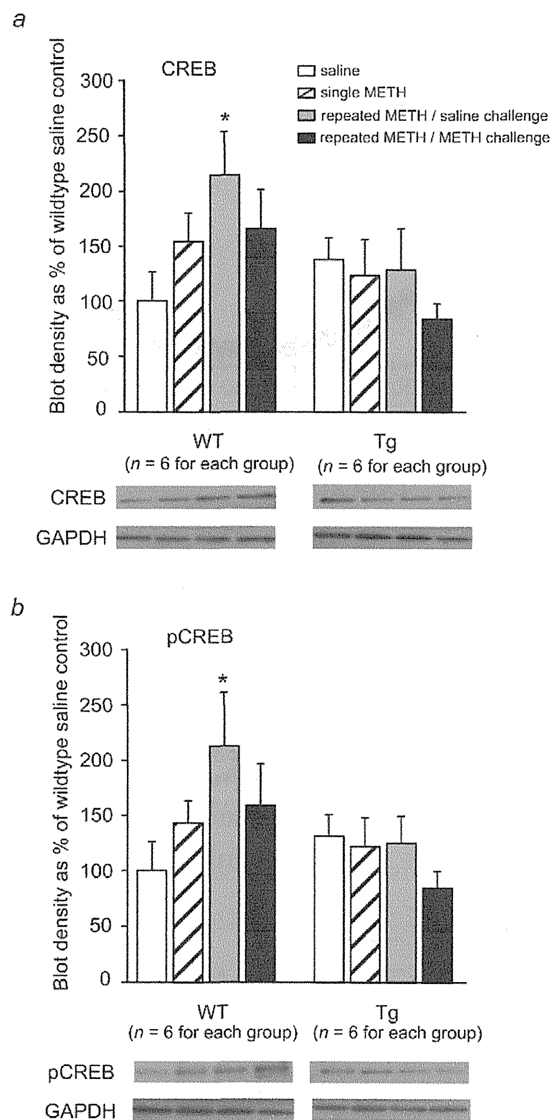

Figure 3. CREB expression and phosphorylation in the CPu after single and repeated METH treatment. The mice were administered METH (1 mg/kg, i.p.) or saline once or received METH (1 mg/kg, i.p.) once every other day from Day 1 to Day 13 and challenged with saline or METH (1 mg/kg, i.p.) on Day 20 after a 7 day drug-free period. The mice were decapitated 1 h after the last METH or saline treatment. The blot density of each group was normalized to that of the wildtype saline group and is expressed as mean ± SEM ($n = 6$). *a*. METH-induced CREB expression in the CPu in wildtype mice (WT) and ICER I-overexpressing mice (Tg). *$p<0.05$, significant difference in normalized CREB blot density compared with wildtype saline group. *b*. METH-induced CREB phosphorylation in the CPu in wildtype mice (WT) and ICER I-overexpressing mice (Tg). *$p<0.05$, significant difference in normalized pCREB blot density compared with wildtype saline group.
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Discussion

The present study investigated the role of ICER in long-lasting METH-induced behavioral alterations by evaluating METH-induced locomotor sensitization in ICER knockout and ICER-overexpressing mice. The major findings of the present study were that ICER I overexpression significantly inhibited METH-induced locomotor sensitization and blocked METH-induced increases in CREB and pCREB protein levels. Additionally, CART and Pdyn mRNA expression levels in the CPu were significantly reduced in ICER-overexpressing mice. ICER knockout mice displayed a tendency toward higher activity after repeated METH administration compared with their wildtype littermates, although no significant difference was detected between ICER knockout mice and their wildtype littermates. Considering the negative regulatory role of CREB in the effects of psychostimulants [18,29–30], the reduction in METH-induced locomotor sensitization in ICER-overexpressing mice may be attributable to reduced CART and Pdyn mRNA expression, rather than attributable to increased CREB and pCREB protein levels.

Inhibitory role of ICER in METH-induced locomotor sensitization

Although the mechanisms that underlie locomotor sensitization are not fully understood, it is hypothesized to reflect neuronal adaptations in several brain regions, including in dopamine neurons and the CPu [25]. In the present study, ICER I-overexpressing mice exhibited a significant reduction in METH-induced locomotor sensitization compared with wildtype mice (Fig. 1*c*), whereas ICER knockout mice showed a minimal enhancement of METH-induced locomotor sensitization compared with wildtype mice (Fig. 2*c*). Altogether, these results suggest that ICER plays an inhibitory role in METH-induced locomotor sensitization.

CREB overexpression in the NAc reportedly decreased cocaine- and morphine-induced conditioned place preference (CPP), and decreased CREB in the NAc increased cocaine- and morphine-induced CPP [7–8], suggesting that increased CREB in the NAc has an inhibitory effect on the induction of CPP. However, recent studies have reported conflicting results, in which genetic ablation of CREB did not affect the rewarding properties of psychostimulants [11–13]. Similarly, some studies demonstrated an inhibitory role of CREB in cocaine-induced sensitization [31–32], whereas other studies with CREB mutant mice suggested either minor effects [33] or no effects [11] of CREB on cocaine-induced sensitization. In the present study, overexpression of the endogenous CREB repressor ICER inhibited METH-induced locomotor sensitization. Thus, the inhibitory effect of CREB on the psychostimulant-induced response is debatable. A possible explanation for these discrepant results may include the different gene manipulations (i.e., forebrain- or NAc-specific gene manipulation), different drug types (i.e., METH or cocaine/morphine), and different targeted genes (i.e., ICER or CREB).

Enhanced pCREB in the striatum is a molecular marker of neuroadaptations to chronic psychostimulant-induced plasticity [8,21,29,34–35]. In the present study, both CREB and pCREB levels increased in wildtype mice after repeated METH injection. The increased CREB and subsequent pCREB induced by repeated METH might homeostatically oppose the effect of METH [29]. However, the repeated METH-induced increases in CREB levels were blocked by ICER I overexpression, suggesting that the negative regulation of the CREB pathway was absent in ICER I-overexpressing mice. Therefore, the CREB pathway may not be involved in the reduced locomotor sensitization observed in ICER I-overexpressing mice. Additionally, ICER expression was 60-fold greater in ICER overexpressing mice than in wildtype mice, which may not occur under physiological conditions. The 60-fold increase in expression may interfere with the CREB signaling pathway and homeostatic regulation of CREB.

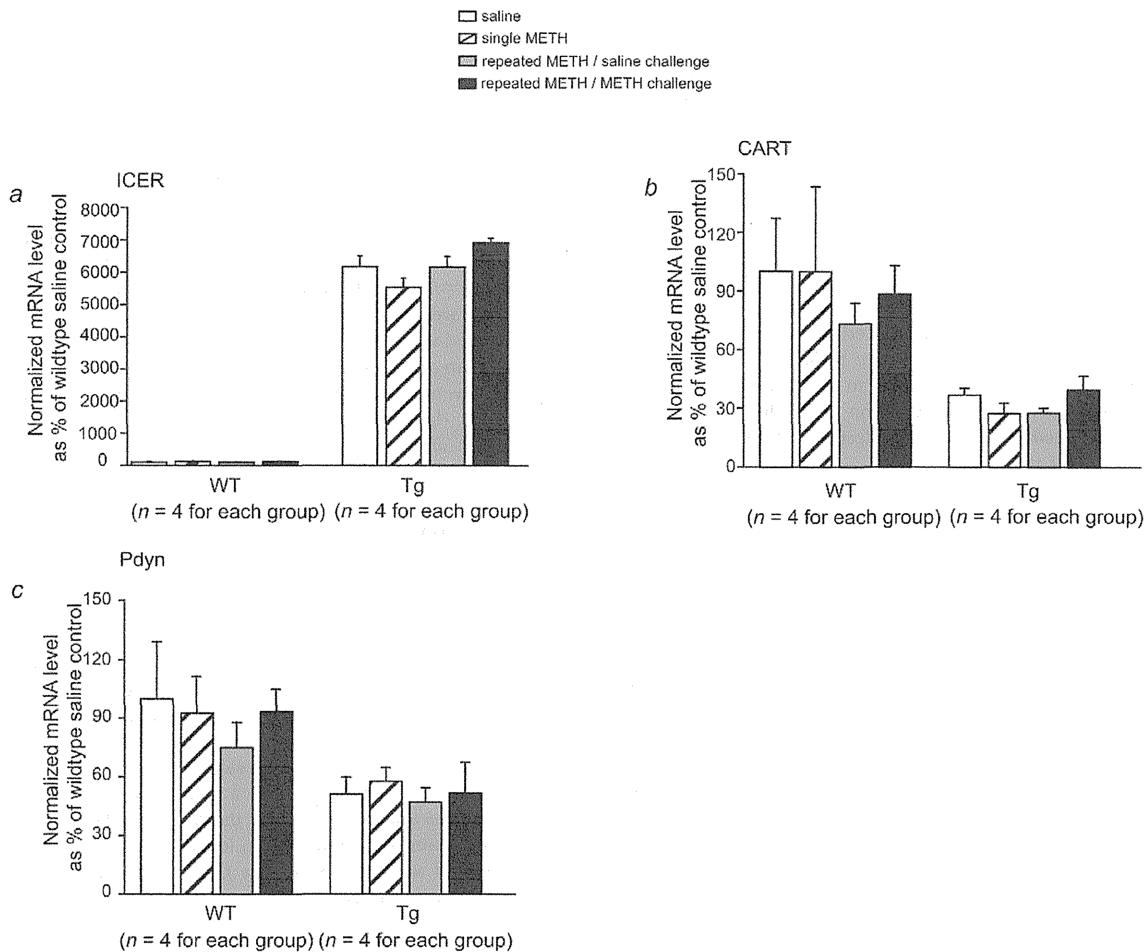

Figure 4. ICER, CART, and Pdyn mRNA levels in the CPu after single and repeated METH treatment. The mice were administered METH (1 mg/kg, i.p.) or saline once or received METH (1 mg/kg, i.p.) once every other day from Day 1 to Day 13 and challenged with saline or METH (1 mg/kg, i.p.) on Day 20 after a 7 day drug-free period. The mice were decapitated 1 h after the last METH or saline treatment. *a*. ICER mRNA expression in the CPu in wildtype (WT) and ICER I-overexpressing (Tg) mice. The data are expressed as mean ± SEM ($n = 4$). *b*. CART mRNA expression in the CPu after single and repeated METH treatment. The data are expressed as mean ± SEM ($n = 4$). *c*. Pdyn mRNA expression in the CPu after single and repeated METH treatment. The data are expressed as mean ± SEM ($n = 4$).
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Inhibitory role of ICER in regulating CART and Pdyn mRNA expression

CART and dynorphin are peptidergic neurotransmitters expressed in the CPu and other brain regions and modulate the rewarding effects of drugs of abuse [17,26,36]. CART's involvement in the actions of psychostimulants was first noted in a study that demonstrate that acute cocaine and amphetamine upregulated CART mRNA in the rat brain [37]. However, this report has been controversial because this finding has been difficult to replicate [38–40]. Other studies found that binge cocaine exposure, rather than acute administration, reliably increases CART expression [38,41]. Additionally, Pdyn mRNA has been reported to increase or not change in response to binge cocaine administration [42,43]. In the present study, neither acute nor repeated administration of METH (1 mg/kg) altered CART and Pdyn mRNA expression in wildtype mice. Furthermore, METH administration (1 mg/kg) did not alter ICER mRNA expression in wildtype mice. A possible reason for this might be that the 1 mg/kg dose of METH may not have been sufficient to induce detectable alterations of ICER, CART, and Pdyn mRNA. However, CART and Pdyn mRNA expression levels significantly decreased as ICER mRNA levels significantly increased, suggesting an inhibitory role of ICER in CART and Pdyn expression. Both the CART and Pdyn genes contain a CRE site in their promoter regions [21–22], and CART and Pdyn mRNA levels are regulated by CREB *in vitro* [21,44] and *in vivo* [8,23]. Therefore, as a CRE-mediated gene transcription repressor, ICER may inhibit the expression of CART and Pdyn *in vivo*. Our studies using ICER I-overexpressing mice support this hypothesis.

CART and Pdyn as neuromodulators of the behavioral effects of psychostimulants

The CART and Pdyn peptides are neurotransmitters expressed in brain regions associated with drug reward, including the NAc and VTA [16–17]. Numerous studies have suggested that CART and Pdyn play a homeostatic role in the NAc to oppose the effects of cocaine. For example, pretreatment with Dyn A (1–17) is effective at decreasing striatal dopamine levels and attenuating

cocaine-induced CPP in mice [45]. Overexpression of CREB, with resulting increases in Pdyn gene expression, in the NAc has been shown to decrease the rewarding effects of cocaine [8]. Microinjection of CART peptide 55–102 into the NAc blocked the rewarding effects of cocaine and amphetamine [46–48]. CREB overexpression increases CART mRNA levels in the NAc and decreases the rewarding effects of drugs [23]. However, studies in knockout mice have reported conflicting results. CART knockout mice exhibited attenuated locomotor sensitization induced by amphetamine [18], and Pdyn knockout mice showed decreased locomotor activity evoked by cocaine [30]. ICER I-overexpressing mice with decreased CART and Pdyn expression levels displayed attenuated METH-induced locomotor sensitization in the present study. These discrepant results among CART and Pdyn studies may be attributable to differences between systemic and NAc-specific downregulation of CART or Pdyn. Further studies are needed to clarify the effects of CART and Pdyn in brain regions other than the NAc.

Conclusion

The present study suggests that ICER plays an inhibitory role in METH-induced locomotor sensitization. Our results support the modulatory effects of the ICER pathway in regulating the effects of drugs of abuse and provide an incentive for exploring the therapeutic potential of stimulating the ICER pathway in the treatment of drug abuse.

Materials and Methods

Ethics statement

The experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committee (Animal Experimentation Ethics Committee of Tokyo Metropolitan Institute of Medical Science, Approval ID: 11-029), and all animals were cared for and treated humanely in accordance with our institutional animal experimentation guidelines.

Animals

Wildtype, ICER knockout, and ICER I-overexpressing mice were produced by conventional gene targeting and transgenic methods [24]. Briefly, the P2 exon encoding the 5′ coding sequence of ICER was deleted to generate ICER-specific knockout mice. To generate ICER I-overexpressing mice, the entire coding sequence of cDNA was subcloned into a pNN265 vector, and the promoter for Ca^{2+}/calmodulin-dependent protein kinase II α (CaMKIIα) was used to express ICER I in the forebrain. The expression patterns of other CREB/CREM family members are not altered in either ICER knockout mice or ICER I-overexpressing mice. ICER knockout mice and their littermates were produced by heterozygote-heterozygote mating. ICER I-overexpressing mice and their wildtype littermates were produced by mating ICER I-overexpressing mice (line I-19) and C57BL/6 mice (CLEA Japan Inc., Shizuoka, Japan) because C57BL/6 is the genetic background strain of ICER I-overexpressing mice. Only naive male mice were used for the experiments. The mice were housed five per cage in a temperature- (22±2°C) and humidity-controlled (55±5%) environment on a 12 h/12 h light/dark cycle (lights on 8:00 a.m. to 8:00 p.m.). The mice had *ad libitum* access to a standard laboratory diet and water. All animal experiments were conducted during the light phase of the cycle, between 9:00 a.m. and 5:00 p.m.

Drugs

Methamphetamine hydrochloride (Dainippon-Sumitomo Pharmaceuticals, Osaka, Japan) was dissolved in saline (0.9% sodium chloride) and administered intraperitoneally (i.p.) in a volume of 10 ml/kg.

Locomotor activity

Locomotor activity corresponding to distance travelled was evaluated in a test chamber (25 cm diameter, 27 cm height) and measured in 5 min bins using digital counters with passive infrared sensors (Supermex system, Muromachi Kikai, Tokyo, Japan). Wildtype littermates of ICER knockout mice ($n = 13$), ICER knockout mice ($n = 13$), wildtype littermates of ICER I-overexpressing mice ($n = 7$), and ICER I-overexpressing mice ($n = 9$) were used. The mice were first habituated to the apparatus for 180 min and then injected with METH (1 mg/kg, i.p.). Locomotor activity was then measured for 60 min after the injection. The procedure was repeated seven times, once every other day from Day 1 to Day 13. After a 7 day drug-free period, locomotor activity was measured again after an injection of METH (1 mg/kg, i.p.) on Day 20.

Western blot analysis

The experiment involved four groups of ICER I-overexpressing mice and wildtype mice: Saline, Single METH, Repeated METH/Saline Challenge, and Repeated METH/METH Challenge. Saline and METH (1 mg/kg, i.p.) were administered once to Saline and Single METH mice, respectively, and the mice were decapitated 1 h after the injection. The Repeated METH/Saline Challenge and Repeated METH/METH Challenge groups received METH (1 mg/kg, i.p.) once every other day from Day 1 to Day 13 and were challenged with saline and METH (1 mg/kg, i.p.), respectively, on Day 20 after a 7 day drug-free period. The mice were decapitated 1 h after the last METH or saline treatment. The brains were removed in less than 45 s and cooled rapidly in ice-cold saline for 30 s. The CPu was then dissected. The tissue was quickly frozen on dry ice, stored at −80°C, and homogenized in 100 µl phosphate-buffered saline containing protease inhibitors (Roche Applied Science, Mannheim, Germany) and PhosStop phosphatase inhibitors (Roche Applied Science, Mannheim, Germany). The homogenate was diluted to 4 µg/µl with 2× Laemmli buffer, heated to 95°C for 2 min, and loaded (20 µg of protein) onto 5–20% gradient polyacrylamide gels. The proteins from eight groups were loaded onto the same gel and separated at 50 mA for approximately 1 h and then transferred onto polyvinylidene membranes in a semi-dry blotter. Nonspecific protein binding sites were blocked by incubating in Blocking One Solution (Nakalai Tesque Inc., Kyoto, Japan). The membranes were incubated overnight at 4°C with phosphor (Ser133) CREB (pCREB) antibody (1:2000; Millipore, Billerica, MA, USA). After incubation in secondary antibody (horseradish peroxidase-conjugated goat antibody to rabbit, 1:50,000; Zymed Labs, South San Francisco, CA, USA) for 1 h, the membrane was treated with chemiluminescent substrate (Millipore, Billerica, MA, USA) and visualized by exposure to Hyperfilm electrochemiluminescence film (GE Healthcare Bio-Sciences, Tokyo, Japan). pCREB blots were stripped with 10% acetic acid solution for 15 min at room temperature. The membranes were reprobed for CREB antibody (1:2000; Cell Signaling Technology, Tokyo, Japan). Finally, the blots were stripped and reprobed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA), and the sizes were compared with prestained molecular-weight standards. Individual CREB and pCREB values were divided by their respective sample GAPDH values to obtain CREB/GAPDH and pCREB/GAPDH ratio

values for each sample. The CREB/GAPDH and pCREB/GAPDH ratio values from the wildtype saline group were averaged, and the mean was used as a control value. Therefore, the CREB/GAPDH and pCREB/GAPDH ratio values of each sample were calculated as a percentage of this control.

Quantitative real-time reverse transcription polymerase chain reaction

The experiment involved four groups of ICER I-overexpressing mice and wildtype mice: Saline, Single METH, Repeated METH/Saline Challenge, and Repeated METH/METH Challenge. The saline and METH treatments, euthanasia, brain dissection, and storage of brain tissues were the same as described above for Western blot. Total RNA was isolated using Trizol reagent (Invitrogen Life Technology, Tokyo, Japan) and converted into cDNA using a SuperScript VILO cDNA Synthesis Kit (Invitrogen Life Technology, Tokyo, Japan). The real-time RT-PCR reaction was conducted using a LightCycler 480 Instrument (Roche Applied Science, Mannheim, Germany). The ICER, CART, Pdyn, and β-actin primers for real-time RT-PCR were the following: ICER (5′-GCTGAGGCTGATGAAAAACA-3′ and 5′-GCCACACGATTTTCAAGACA-3′), CART (5′-CGAGAAGAAGTACGGCCAAG-3′ and 5′-CACACAGCTTCCCGATCC-3′), Pdyn (5′-TTATGGCGGACTGCCTGT-3′ and 5′-CACTCCAGGGAGCAAATCAG3′), and β-actin (5′-CTAAGGCCAACCGTGAAAAG-3′ and 5′-ACCAGAGGCATACAGGACA-3′). Universal Probes #4, #108, #99, and #64 (Roche Applied Science, Mannheim, Germany) were used for ICER, CART, Pdyn, and β-actin, respectively. Amplification consisted of a preincubation step (95°C for 10 min), 45 cycles of denaturation for 10 s at 95°C, and annealing for 30 s at 60°C. Amplification curves were produced to calculate the crossing point at which the fluorescence of a sample rises above the initial lag phase. Absolute quantification analysis was performed using LightCycler 480 software (Roche Applied Science, Mannheim, Germany). Serial dilutions of an external standard with a predefined, known concentration were used to create a standard curve. The standard dilutions were amplified in separate wells but within the same instrument as the target samples. The crossing points of standards and unknown samples were then used to determine the concentration of the target mRNA. ICER, CART, and Pdyn mRNA levels were normalized according to β-actin mRNA levels. The ICER/β-actin, CART/β-actin, and Pdyn/β-actin values from the wildtype saline group were averaged, and the mean was used as a control value. Therefore, the relative expression levels of CART and Pdyn were calculated as a percentage of this control.

Statistical analysis

The data are expressed as mean ± SEM. The data for the Western blot, real-time RT-PCR, and locomotor sensitization experiments were analyzed by two-way, mixed-design ANOVA and repeated-measures ANOVA followed by the Dunnett *post hoc* test (for the Western blot analysis and real-time RT-PCR experiments) or Tukey-Kramer *post hoc* test (for the locomotor sensitization experiment). Values of $p<0.05$ were considered statistically significant.

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Author Contributions

Conceived and designed the experiments: NK KI. Performed the experiments: WH YT HY SK NK. Analyzed the data: WH YT KI. Contributed reagents/materials/analysis tools: SE TS NK KI. Wrote the paper: WH NK KI.

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Diversity of Opioid Requirements for Postoperative Pain Control Following Oral Surgery—Is It Affected by Polymorphism of the μ-Opioid Receptor?

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We experience individual differences in pain and sensitivity to analgesics clinically. Genetic factors are known to influence individual difference. Polymorphisms in the human *OPRM1* gene, which encodes the μ-opioid receptors, may be associated with the clinical effects of opioid analgesics. The purpose of this study was to determine whether any of the 5 common single-nucleotide polymorphisms (SNPs) of the *OPRM1* gene could affect the antinociceptive effect of fentanyl. Fentanyl was less effective in subjects with the G allele of the *OPRM1* A118G SNP than in those with the A allele, and subjects with the G allele required more fentanyl for adequate postoperative pain control than those with the A allele. In the future, identifying SNPs might give us information to modulate the analgesic dosage of opioid individually for better pain control. Factors underlying individual differences in sensitivity to pain other than genetic factors may include environmental and psychological factors. We therefore examined the effects of preoperative anxiety on the analgesic efficacy of fentanyl in patients undergoing sagittal split mandibular osteotomy (SSMO). From among the patients enrolled in the study, 60 patients (male/female: 18/42, age: 24.6 ± 6.7 years) who gave informed consent were examined for correlations between preoperative trait/state anxiety, as measured by the state-trait anxiety inventory (STAI) on the day before surgery, and postoperative consumption of patient-controlled analgesia (PCA) fentanyl and visual analog scale (VAS) assessment by patients. Levels of trait and state anxieties measured by the STAI were correlated with neither the consumption of PCA fentanyl nor postoperative VAS assessment. These findings suggest that psychological factors are unlikely to affect postoperative pain or the use of analgesics.

Key Words: Polymorphism; μ-Opioid receptor; Postoperative pain; Patient-controlled analgesia; Preoperative anxiety.

Individual differences in sensitivity to pain and to analgesics are known to vary among patients in daily clinical practice. The amount of analgesics, such as opioids, used for postoperative pain control, even following the same surgery, also varies substantially among patients. Factors possibly underlying these individual differences include environmental, psychological, and genetic factors. We conducted a study involving patients undergoing oral surgery to identify genetic and psychological factors responsible for the diversity of opioid requirements by examining, respec-

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