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Reduced emotional and corticosterone responses to stress in μ-opioid receptor knockout mice

Soichiro Ide [a,b], Ichiro Sora [c], Kazutaka Ikeda [d], Masabumi Minami [a], George R. Uhl [e], Kumatoshi Ishihara [b,*]

[a] Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan
[b] Laboratory of Neuropharmacology, Faculty of Pharmaceutical Sciences, Hiroshima International University, Hirokoshingai, Kure 737-0112, Japan
[c] Department of Biological Psychiatry, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan
[d] Division of Psychobiology, Tokyo Institute of Psychiatry, Tokyo 156-8585, Japan
[e] Molecular Neurobiology, National Institute on Drug Abuse, Baltimore, MD 21224, USA

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ABSTRACT

The detailed mechanisms of emotional modulation in the nervous system by opioids remain to be elucidated, although the opioid system is well known to play important roles in the mechanisms of analgesia and drug dependence. In the present study, we conducted behavioral tests of anxiety and depression and measured corticosterone concentrations in both male and female μ-opioid receptor knockout (MOP-KO) mice to reveal the involvement of μ-opioid receptors in stress-induced emotional responses. MOP-KO mice entered more and spent more time in the open arms of the elevated plus maze compared with wild-type mice. MOP-KO mice also displayed significantly decreased immobility in a 15 min tail-suspension test compared with wild-type mice. Similarly, MOP-KO mice exhibited significantly decreased immobility on days 2, 3, and 4 in a 6 min forced swim test conducted for 5 consecutive days. The increase in plasma corticosterone concentration induced by tail-suspension, repeated forced swim, or restraint stress was reduced in MOP-KO mice compared with wild-type mice. Corticosterone levels were not different between wild-type and MOP-KO mice before stress exposure. In contrast, although female mice tended to exhibit fewer anxiety-like responses in the tail-suspension test in both genotypes, no significant gender differences were observed in stress-induced emotional responses. These results suggest that MOPs play an important facilitatory role in emotional responses to stress, including anxiety- and depression-like behavior and corticosterone levels.

1. Introduction

Stress is hypothesized to be one of the triggering factors that causes mental illness, including anxiety and depression. Several brain areas are hypothesized to be involved in stress-induced emotional responses via corticosterone release by the hypothalamic-pituitary-adrenal (HPA) axis. Although several neurotransmitter systems, such as serotonin and catecholamines, have been hypothesized to be involved in these mechanisms, the precise molecular mechanisms are still unclear. Endogenous opioid peptides, such as endorphins, have been shown to modulate serotonergic and catecholaminergic neurotransmission (Chen et al., 2001; Hung et al., 2003; Ukai and Lin, 2002). Furthermore, pretreatment with naloxone, a nonselective opioid receptor antagonist, decreased immobility time in mice in a forced swim test (Amir, 1982). Chronic morphine facilitated immobility in a forced swim test (Molina et al., 1994). Opioids have also been reported to increase stress-related hormone levels (Mellon and Bayer, 1998). These previous reports indicate that the endogenous opioid system impacts behavioral responses to stress.

Opioid receptors have been classified into at least three subtypes, μ, δ, and κ (MOP, DOP, and KOP, respectively). Endomorphin-1 and -2, endogenous peptides that are selective for MOP, reportedly decreased immobility time in both the forced swim and tail-suspension tests (Fichna et al., 2007). A DOP selective agonist, SNC80, also decreased immobility time in a forced swim test (Broom et al., 2002). Furthermore, the KOP selective agonist U69593 increased, and the KOP selective antagonist nor-binaltorphimine decreased, immobility time in a forced swim test (Mague et al., 2003). Although three opioid receptor subtypes may be involved in stress-induced emotional responses, even the most selective ligands for a specific subtype (i.e., β-funaltrexamine for

* Corresponding author. Tel.: +81 823 73 8980; fax: +81 823 73 8981.
E-mail address: ishihara@ps.hirokoku-u.ac.jp (K. Ishihara).

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MOP, naltrindole for DOP, and nor-binaltorphimine for KOP) possess certain affinities for other subtypes (Newman et al., 2002) which may contribute to the discrepant findings about the role of opioid receptor subtypes in stress responses. Therefore, the precise molecular mechanisms underlying stress-induced emotional responses have not yet been clearly delineated by traditional pharmacological studies that use only selective ligands.

Recent success in developing knockout (KO) mice with MOP gene deletion has revealed the central role of MOPs, rather than other opioid receptor subtypes, in various opioid effects, including analgesia, reward, and tolerance (Ide et al., 2004; Kieffer, 1999; Loh et al., 1998; Sora et al., 2001, 1997). Although several compensatory changes might occur in KO animals, these animals have potential utility in investigating the *in vivo* roles of specific proteins. Opioid receptors have been shown to modulate responses to stress, including depression-like behavior (Filliol et al., 2000; McLaughlin et al., 2003). Thus, the use of MOP-KO mice has provided novel theories on the molecular mechanisms underlying stress-induced emotional responses. Both the forced swim test (Porsolt et al., 1977) and tail-suspension test (Steru et al., 1985) have been widely used to assess depression-like behavior, with several modifications. Many reports using these two tests have shown that the inescapable stress of swimming or suspending a mouse by its tail can provide valuable information about emotional responses in stressful situations. The present study investigated the contributory role of the MOP in emotional responses to height, tail-suspension, repeated forced swim, and restraint stress using MOP-KO mice.

2. Materials and methods

2.1. Animals

The present study used wild-type and homozygous MOP-KO mouse littermates on a C57BL/6J genetic background (backcrossed at least 10 generations) as previously described (Sora et al., 2001). 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*. All behavioral tests and blood sample collections were conducted between 1:00 pm and 6:00 pm.

2.2. Elevated plus maze

The testing apparatus was a white plastic plus-shaped maze, elevated 80 cm from the floor. The maze consisted of two open arms (50 × 10 cm) and two closed arms (50 × 10 × 50 cm) without a roof. During testing, the time spent in the open arms and the number of entries into the open arms were recorded for 5 min. A mouse was considered to have entered an arm only if all four paws entered that arm.

2.3. Locomotor activity

Locomotor activity was assessed with an animal activity-monitoring apparatus equipped with an infrared detector (SUPERMEX, CompACT FSS, Muromachi Kikai Co., Tokyo, Japan). Mice were placed individually in 30 × 45 × 30 cm plastic cages, to which they had not been previously exposed, under dim light and sound-attenuated conditions. Locomotor activity was monitored for 3 h.

2.4. Tail-suspension test

For tail-suspension testing, mice were suspended by their tail which was taped on a metal hook in test chambers (20 × 20 × 25 cm) constructed of white plastic walls and floor. Each hook was connected to a computerized strain gauge that was adjusted to detect animal movements (Tail-suspension System, Neuroscience Inc., Osaka, Japan). The total duration of immobility was measured for 15 min per day for 2 consecutive days.

2.5. Forced swim test

For forced swim testing, animals were forced to swim in a cylindrical Plexiglas tank (30 cm height × 30 cm diameter) containing 20 cm deep water for 6 min per day for 5 consecutive days. The water temperature was maintained at approximately 25 °C. Immobility time was recorded with an animal activity-monitoring apparatus equipped with an infrared detector (SUPERMEX, CompACT FSS, Muromachi Kikai Co., Tokyo, Japan). After each session, the mice were immediately removed from the cylinder, dried with a towel, and kept under a heating lamp until completely dry, before being returned to their home cages.

2.6. Stress procedures and corticosterone enzyme immunoassay

After the 2 day tail-suspension test or 5 day forced swim test, blood samples (50 μl) were obtained from the tail vein. For restraint stress, mice were placed in a 50 ml conical centrifuge tube with multiple ventilation holes. Mice were restrained vertically in the tube for 12 h, followed by a 12 h rest with food and water available *ad libitum*. Mice were restrained again for 12 h, and then blood samples were obtained. All blood samples were immediately centrifuged for 20 min at 1000 × *g*. Plasma samples were stored at −80 °C until analysis. Plasma corticosterone levels were determined with a Corticosterone Enzyme Immunoassay Kit (Assay Design Inc., Ann Arbor, MI, USA).

2.7. Statistical analysis

Entry counts and time spent on the open arms of the elevated plus maze and stress-induced changes in plasma corticosterone concentrations were analyzed with Student's *t*-test. The results of other analyses were statistically evaluated with analysis of variance (ANOVA) followed by the Tukey–Kramer test. Values of $p < 0.05$ were considered statistically significant.

3. Results

We first assessed basal anxiety-like behavior of both mouse genotypes in the elevated plus maze (Fig. 1). Compared with wild-type mice, MOP-KO mice had significantly higher entry counts ($p < 0.05$, Student's *t*-test) and a longer time spent on the open arms ($p < 0.01$, Student's *t*-test) in both male and female mice. Although female mice tended to have more entry counts and more time spent in the open arms than male mice in both genotypes, no significant differences were observed.

When spontaneous locomotor activity of both wild-type and MOP-KO mice was analyzed (Fig. 2), MOP-KO mice displayed normal locomotor activity, similar to wild-types, during the 3 h test. A three-way, mixed-design ANOVA of spontaneous locomotor activity with two within-subjects factors (genotype and gender) showed no significant interactions (genotype: $F_{1,30} = 1.56$, $p = 0.221$; gender: $F_{1,30} = 0.08$, $p = 0.784$).

To test the influence of MOP-KO in stress-induced responses, immobility time in a 15 min tail-suspension test was analyzed every minute in wild-type and MOP-KO mice (Fig. 3). A three-way, mixed-design ANOVA of immobility time with two within-subjects factors (genotype and gender) revealed that immobility time was significantly different between genotypes in the tail-suspension test ($F_{1,22} = 6.92$, $p < 0.05$), although both genotypes showed time-dependent increases (Fig. 3a). The ANOVA also revealed that immobility time was not significantly different between male and female mice ($F_{1,22} = 3.01$, $p = 0.097$), although female mice tended to show less immobility than males. When the data of male and female mice were combined (Fig. 3b), significant differences were found in immobility time between genotypes ($F_{1,24} = 5.45$, $p < 0.05$, two-way, repeated-measures ANOVA). *Post hoc* tests revealed that MOP-KO mice had significantly less immobility time compared with wild-type mice from 7 to 9, 12 and 13 min after the tail-suspension test commenced. These differences in immobility time between wild-type and MOP-KO mice were not found during the second trial of the tail-suspension test on the next day (data not shown).

To test another type of stress stimulus, immobility time during the 6 min, 5-consecutive-day forced swim test was also analyzed in wild-type and MOP-KO mice (Fig. 4). Both genotypes and both male and female mice showed time-dependent increases in immobility time (Fig. 4a–d). Furthermore, immobility time during the 6 min forced swim test significantly increased, or tended to increase, in

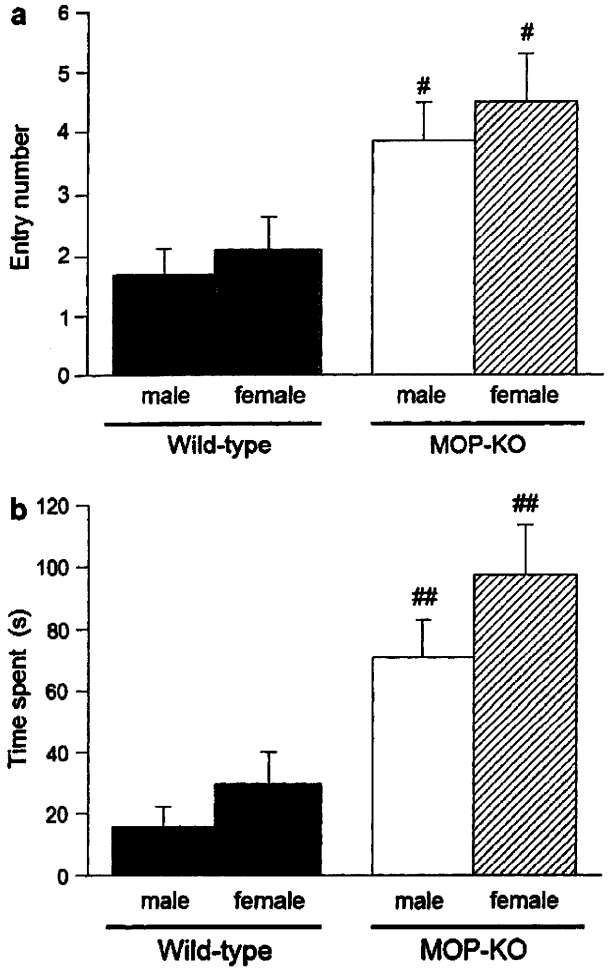

Fig. 1. Anxiety-like behavior in wild-type and MOP-KO mice in the elevated plus maze. The (a) number of entries and (b) time spent in the open arms of the elevated plus maze were measured for 5 min in wild-type mice (male, $n = 10$; female, $n = 9$) and MOP-KO mice (male, $n = 12$; female, $n = 13$). $^{*}p < 0.05$, $^{**}p < 0.01$, significant difference from corresponding value in wild-type mice. Data are expressed as mean ± SEM.

a day-dependent manner (wild-type male mice: $F_{4,45} = 8.07$, $p < 0.001$; wild-type female mice: $F_{4,40} = 11.9$, $p < 0.001$; MOP-KO male mice: $F_{4,30} = 2.35$, $p = 0.077$; MOP-KO female mice: $F_{4,30} = 7.00$, $p < 0.001$; two-way, repeated-measures ANOVA). *Post hoc* comparisons revealed that immobility time on days 2–5 significantly increased compared with day 1 in both wild-type male and female mice ($p < 0.05$). Immobility time significantly increased on day 5 compared with day 1 in MOP-KO male mice and on days 4 and 5 compared with day 1 in MOP-KO female mice ($p < 0.05$). A three-way, mixed-design ANOVA of total immobility time during the 6 min tests on each of the 5 days with two within-subjects factors (genotype and gender) revealed that immobility time was significantly different between genotypes ($F_{1,29} = 10.9$, $p < 0.005$) but was not significantly different between genders ($F_{1,29} = 1.39$, $p = 0.248$) (Fig. 4e). Thus, when the male and female data were combined (Fig. 4f), MOP-KO mice showed significantly less immobility time compared with wild-type mice on days 2, 3, and 4.

We then analyzed stress-induced changes in plasma corticosterone concentrations in wild-type and MOP-KO mice (Fig. 5). The three types of stress significantly increased plasma corticosterone

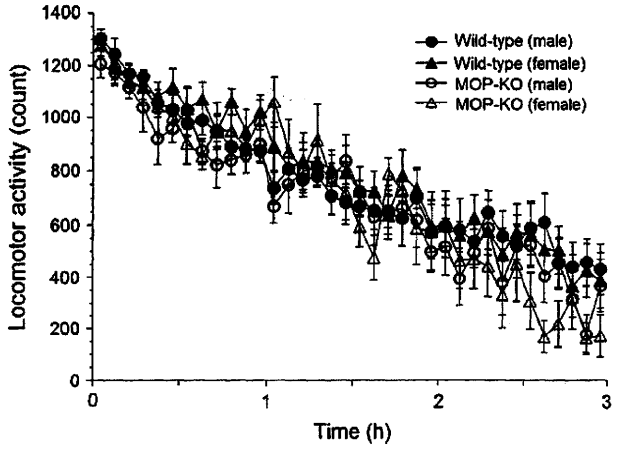

Fig. 2. Spontaneous locomotion in wild-type and MOP-KO mice. Spontaneous locomotion during 3 h habituation to a novel environment in wild-type mice (male, $n = 12$; female, $n = 9$) and MOP-KO mice (male, $n = 6$; female, $n = 7$). Each point represents the sum of 5 min locomotor activity. Data are expressed as mean ± SEM.

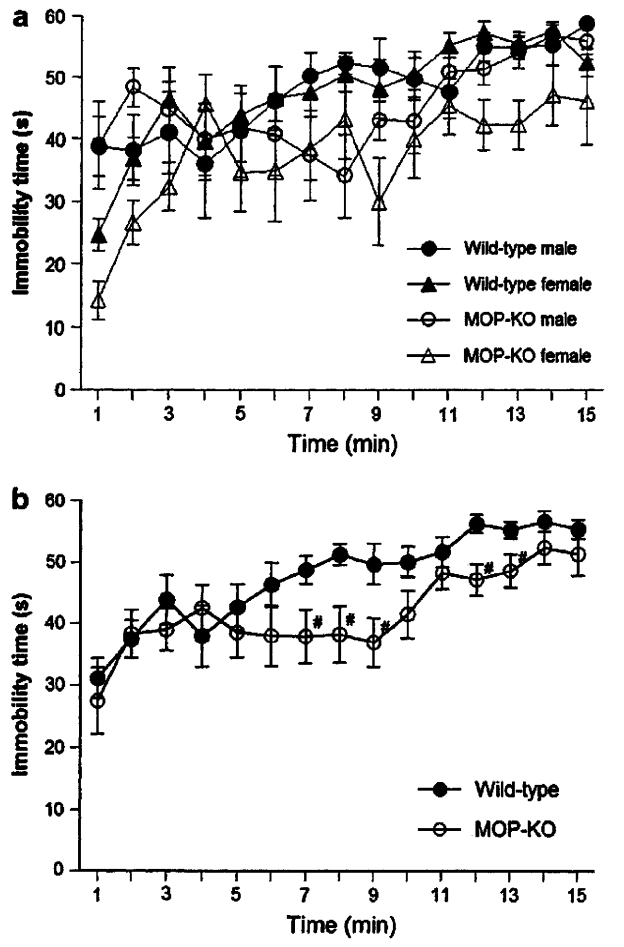

Fig. 3. Immobility in wild-type and MOP-KO mice in the 15 min tail-suspension test. (a) Immobility time was measured in wild-type mice (male, $n = 6$; female, $n = 7$) and MOP-KO mice (male, $n = 7$; female, $n = 6$). (b) Combined data of male and female mice in the 15 min tail-suspension test. $^{*}p < 0.05$, significant difference from corresponding value in wild-type mice. Data are expressed as mean ± SEM.

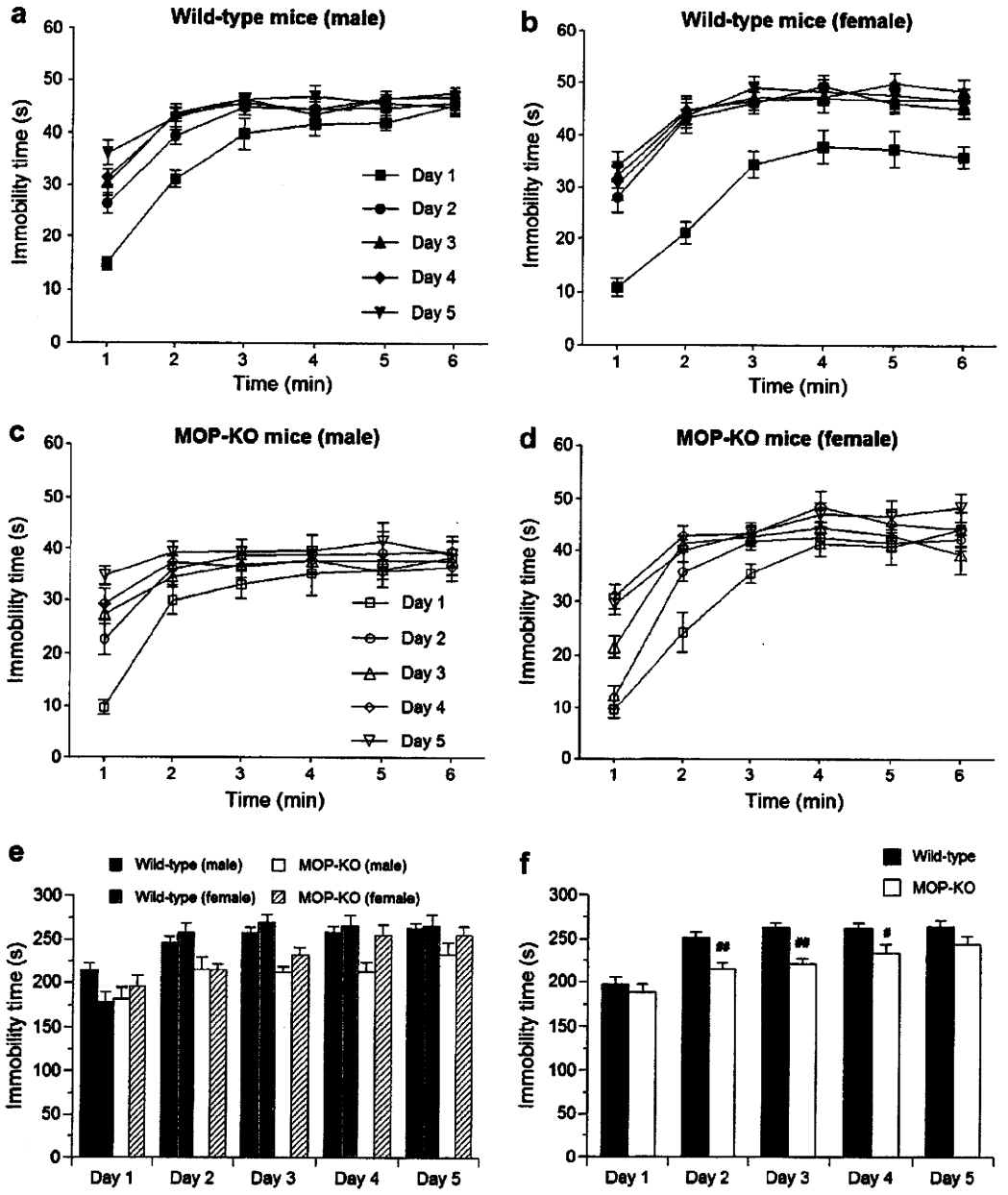

Fig. 4. Immobility in wild-type and MOP-KO mice in the 6 min, 5-consecutive-day forced swim test. Immobility time was measured in (a) wild-type male mice ($n = 10$), (b) wild-type female mice ($n = 9$), (c) MOP-KO male mice ($n = 7$), and (d) MOP-KO female mice ($n = 7$). (e) Sum of 6 min immobility time over 5 days. (f) Combined data of male and female mice. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, significant difference from corresponding value in wild-type mice. Data are expressed as mean ± SEM.

concentrations in both genotypes and in both male and female mice ($p < 0.05$, Student's t-test). Although no significant differences were observed in basal plasma corticosterone concentrations in naive mice, the stress-induced increases in plasma corticosterone concentrations were significantly different ($p < 0.05$, Student's t-test), or tended to be significantly different (restraint stress in female mice: $p = 0.065$, Student's t-test), between genotypes in both male and female mice. Both male and female MOP-KO mice had significantly lower plasma corticosterone concentrations compared with wild-type mice after the stress procedures. Although female mice tended to have slightly higher corticosterone concentrations than male mice (i.e., naive or after tail-suspension or restraint stress), no significant differences were observed (Student's t-test). Contrary to these findings, female mice tended to exhibit lower corticosterone concentrations than male mice after forced swim stress in both genotypes, although no significant differences were observed (Student's t-test).

4. Discussion

In the present study, MOP-KO mice displayed significantly decreased immobility time in both the tail-suspension and repeated forced swim tests and significantly reduced stress-induced increases in plasma corticosterone concentrations compared with wild-type mice. Moreover, MOP-KO mice also entered more, and spent more time in, the open arms of the

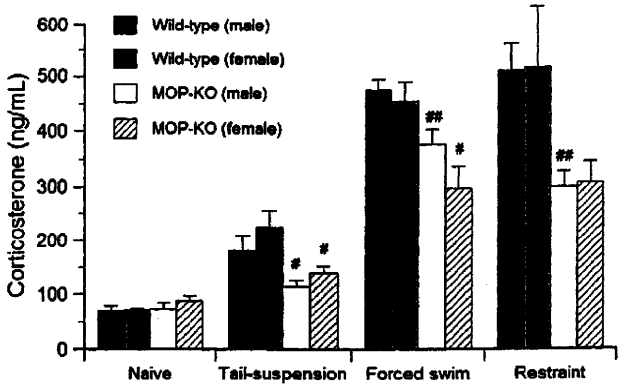

Fig. 5. Stress-induced increase in plasma corticosterone concentrations in wild-type and MOP-KO mice. Plasma corticosterone levels were analyzed (i) in naive wild-type mice (male, $n = 6$; female, $n = 5$) and MOP-KO mice (male, $n = 9$; female, $n = 8$), (ii) after the 2 day tail-suspension test in wild-type mice (male, $n = 6$; female, $n = 5$) and MOP-KO mice (male, $n = 9$; female, $n = 8$), (iii) after the 5 day forced swim test in wild-type mice (male, $n = 10$; female, $n = 8$) and MOP-KO mice (male, $n = 7$; female, $n = 7$), and (iv) after restraint stress in wild-type mice (male, $n = 6$; female, $n = 5$) and MOP-KO mice (male, $n = 9$; female, $n = 8$). $^{*}p < 0.05$, $^{**}p < 0.01$, significant difference from corresponding value in wild-type mice. Data are expressed as mean ± SEM.

elevated plus maze. These results suggest that MOP-KO mice are resistant to stress exposure and exhibit fewer stress-induced emotional responses (i.e., anxiety- and depression-like behaviors) compared with wild-type mice, although the influences of other factors (e.g., response to novelty) should be considered in future studies.

No significant differences were observed in locomotor activity between wild-type and MOP-KO mice, although MOP-KO mice exhibited a slight tendency toward decreased locomotion. These results indicate that the present behavioral effects in MOP-KO mice were not attributable to variations in locomotor activity. MOP-KO mice entered more, and spent more time in, the open arms of the elevated plus maze in the present study. Similar results have been reported with another MOP-KO mouse strain in both the elevated plus maze test and light–dark box test (Filliol et al., 2000). This anxiolytic-like state of MOP-KO mice is consistent with a previous report in which the MOP-selective agonist DAMGO ([D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin) induced anxiogenic-like activity in the elevated plus maze (Calenco-Choukroun et al., 1991). In contrast, several contradictory studies have reported an anxiolytic-like effect of morphine and MOP agonists (Asakawa et al., 1998; Koks et al., 1999). One of the reasons for this discrepancy using MOP-selective ligands might involve other opioid receptor subtypes. The most selective ligands for a specific opioid receptor subtype possess certain affinities for other subtypes (Newman et al., 2002). Although further studies using our and other MOP-KO mouse strains in various paradigms to assess anxiety-like responses (e.g., open field test) might be needed, the present results suggest that MOPs are involved in anxiety-like responses to height stress.

The decrease in immobility time in MOP-KO mice compared with wild-type mice in both the tail-suspension and repeated forced swim tests is consistent with previous reports. The decrease in immobility time in the forced swim test has been reported using another MOP-KO mouse strain (Filliol et al., 2000). These results suggest that MOP activation facilitates stress-induced, depression-like behavioral responses. Additionally, Fichna et al. (2007) reported contradictory findings in which intracerebroventricular treatment with endomorphin-1 and -2, endogenous MOP-selective peptides, decreased immobility time in both the forced swim and tail-suspension tests. Codeine, a relatively weak MOP agonist, also decreased immobility time in tail-suspension tests in mice (Berrocoso and Mico, in press). Although these reports might suggest that the MOP modulates depression-like behavior in contrast to our present results, other reports are consistent with our results. Chronic morphine facilitated immobility time in a rat forced swim test (Molina et al., 1994). Pretreatment with naloxone, a nonselective opioid receptor antagonist, decreased immobility time in a forced swim test in mice (Amir, 1982). Furthermore, intraperitoneal treatment of morphine enhanced immobility time in rats in a naloxone-sensitive manner (Zurita and Molina, 1999). These discrepant results might be attributable to differences in animals, mouse strains, time course, injection route, or other experimental conditions. Notably, different mouse strains have exhibited differential responses in forced swim tests (David et al., 2003). Further studies may reveal the reasons for these discrepant results.

To study the involvement of the MOP in emotional responses to repeated stress, the present study used both the 6 min forced swim test conducted for 5 consecutive days and the 15 min tail-suspension test conducted for 2 consecutive days, two regimens which were modified from typically used procedures in mice (Porsolt et al., 1977; Steru et al., 1985). When we analyzed immobility time from day 1 at 3–6 min in the forced swim test (excluding the data from the first 2 min), no significant differences were found between wild-type and MOP-KO mice. Additionally, no significant differences in immobility time were observed from day 1 for the first 6 min between wild-type and MOP-KO mice in the tail-suspension test. Although standard procedures for the analysis of depression-like behavior did not reveal significant differences, MOP-KO mice showed significant differences in depression-like behavior after repeated or longer stress exposure in the forced swim and tail-suspension tests. Our present results might suggest that MOPs facilitate emotional responses to repeated or longer stress exposure. In the present procedures, MOP-KO mice exhibited significantly decreased immobility time in the repeated forced swim test only on days 2, 3, and 4, and they only showed a tendency toward decreased immobility on day 5. In the tail-suspension test, MOP-KO mice had significantly decreased immobility time only after the first 5 min from the beginning of the test during the first trial, and no significant differences were observed during the second trial. Interestingly, the increase in plasma corticosterone concentrations in MOP-KO mice was still significantly lower than wild-type mice after the differences in behaviors between wild-type and MOP-KO mice in both tests disappeared. MOPs may facilitate the early behavioral responses to stress but are not necessary to fully express the behavioral responses after chronic stress procedures. Other neuronal systems might regulate the expression of stress-induced behavioral responses, and MOPs might facilitate this regulation.

At the hormonal level, one of the major responses to stress is an increase in corticosterone secretion caused by stimulation of the HPA axis. In the present study, plasma corticosterone concentration significantly increased after stress exposure in both wild-type and MOP-KO mice. The increased corticosterone levels after both forced swim and restraint stress were higher than after the tail-suspension test. This finding might be attributable to differences in the intensity of the stressors, although variations in the duration and frequency of these stressors might modify these levels. Additionally, the stress-induced increases in plasma corticosterone concentration were less in MOP-KO mice compared with wild-type mice. Our present results are consistent with previous reports. Endogenous opioids have been reported to have facilitatory effects on the HPA axis (Douglas et al., 1998). The increase in plasma corticosterone levels by morphine indicated activation of the HPA axis by MOP (Coventry et al., 2001; Ignar and Kuhn, 1990). In a different MOP-KO mouse strain, morphine- and restraint

stress-induced increases in plasma corticosterone levels were also reduced (Roy et al., 2001; Wang et al., 2002). Stress is well known to activate the HPA axis and increase norepinephrine release in the locus coeruleus. Moreover, stress-induced norepinephrine release in the locus coeruleus is partially regulated by both opioid and noradrenergic mechanisms (Nakai et al., 2002; Nestler et al., 1999; Valentino and Van Bockstaele, 2001), suggesting that MOPs may be involved in the activation of the HPA axis and locus coeruleus.

Knockout animals may be hypothesized to have potential utility in investigating the *in vivo* roles of specific proteins. Previous reports using gene mutant mice suggest that MOPs play an important role in various effects of opioids, such as antinociception, tolerance, reward, and locomotion (Ide et al., 2004; Matthes et al., 1996; Sora et al., 2001, 1997). Our present results also demonstrated the involvement of MOPs in stress-induced emotional responses. However, although no differences in DOP and KOP expression were evident in MOP-KO mice in the present study (Sora et al., 1997), several compensatory changes might occur in MOP-KO mice. These possible compensatory changes, especially with regard to neurotransmitter release and hormonal valence, could elicit changes in stress-induced emotional responses. Future studies, such as behavioral analyses using MOP-KO mice with viral expression of MOPs, may reveal the influences of compensatory changes in stress-induced emotional responses.

Gender differences in emotional responses may also exist (Toufexis, 2007; Toufexis et al., 2006). In the present study, several differences were found between male and female mice in stress-induced emotional responses, although these differences were not significant. In the elevated plus maze, female mice showed less anxiety-like behavior than male mice of both genotypes. These results are consistent with previous reports using rodents (Fernandes et al., 1999; Steenbergen et al., 1990) and suggest the presence of gender differences in anxiety-like behavior. However, no differences in immobility time were found between male and female wild-type mice in either the tail-suspension or forced swim tests. A previous report found that male and female C57BL/6J mice, the genetic background strain used in the present study, exhibited no differences in immobility time in either the tail-suspension or forced swim tests (Caldarone et al., 2003). Interestingly, female MOP-KO mice tended to exhibit less immobility in the tail-suspension test and more immobility in the forced swim test compared with male MOP-KO mice. Although the present study found no significant differences between genders, and additional studies may be required, MOPs may differentially modulate depression-like responses in both tests, especially in female mice.

In conclusion, we found decreased anxiety-like behavior in the elevated plus maze, decreased immobility in both the tail-suspension and forced swim tests, and reduced stress-induced plasma corticosterone concentrations in MOP-KO mice compared with wild-type mice. These results suggest that MOPs play an important facilitatory role in stress sensitivity and/or stress-induced emotional responses, including anxiety- and depression-like responses.

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Chapter 29

Genetic Polymorphisms and Human Sensitivity to Opioid Analgesics

Daisuke Nishizawa, Masakazu Hayashida, Makoto Nagashima, Hisashi Koga, and Kazutaka Ikeda

Abstract

Opioid analgesics are commonly used for the treatment of acute as well as chronic, moderate to severe pain. Well-known, however, is the wide interindividual variability in sensitivity to opioids that exists, which has often been a critical problem in pain treatment. To date, only a limited number of studies have addressed the relationship between human genetic variations and sensitivity to opioids, and such studies are still in their early stages. Therefore, revealing the relationship between genetic variations in many candidate genes and individual differences in sensitivity to opioids will provide valuable information for appropriate individualization of opioid doses required for adequate pain control. Although the methodologies for such association studies can be diverse, here we summarize protocols for investigating the association between genetic polymorphisms and sensitivity to opioids in human volunteers and patients undergoing painful surgery.

Key words: Analgesics, Genetic polymorphisms, Single nucleotide polymorphism (SNP), Genotype–phenotype association, Haplotype, Opioids, Opiates, Pain relief, Personalized medicine, Pharmacogenomics

1. Introduction

Opioid analgesics are commonly used for the treatment of acute or chronic, moderate to severe pain. However, wide interindividual variability exists in sensitivity to opioid analgesics (1). Because of this variability, a dose of an opioid that can produce satisfactory pain relief without significant adverse effects in some patients might cause underdosing or overdosing in others. Individual differences can be attributed to both genetic and environmental factors,

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although the relative influence of each of these factors is diverse (2). Genetic variations in many genes involved in opioid pharmacokinetics and pharmacodynamics might be responsible, at least partially, for the individual differences in phenotypes related to analgesic efficacy of opioids.

Numerous molecules are known to be involved in the pharmacological effects of opioids. The genes encoding these molecules are candidates for exploring the relationships between genetic variations and individual differences in phenotype traits related to opioid actions. Recently, several studies in humans have investigated the relationships between the individual genetic variations in the μ-opioid receptor gene (*OPRM1*) and sensitivity to opioids (2–8). However, such studies are still in their early stages and await future meta-analyses for clarification of the precise phenotype-to-genotype relationships.

Therefore, revealing the relationships between genetic variations in many candidate genes and interindividual differences in sensitivity to opioids will facilitate a better understanding of how human genetic polymorphisms can cause differences in sensitivity to opioids. Data from such studies will provide valuable information for appropriate individualization of opioid doses to achieve adequate pain control and open new avenues for personalized pain treatment.

2. Materials

2.1. Collecting Clinical Data

1. A form describing the design of the study approved by each Institutional Review Board (IRB) at the respective institutions.
2. Letters to the candidate subjects explaining the outline or details of the study and reply cards on which the candidates indicate whether they are interested in participating in the study.
3. Packages to be sent to participating subjects that include explanatory leaflets describing the detailed study protocol, written informed consent forms, instructions for collecting oral mucosa samples (see section 3.2.1.), cotton swabs to collect oral mucosa samples, test tubes to enclose the samples, and stamped return-mail envelopes.
4. In the case of retrospective studies, lists of ex-patient or patient candidates who had previously undergone surgery (e.g., major open abdominal surgery) and received opioids (e.g., fentanyl or morphine) postoperatively during a specific period (e.g., the first 24 h postoperative period) at the hospitals where clinical data are collected.

5. Several references of papers or books describing the method of properly converting the dose of an opioid analgesic to the equivalent dose of another opioid analgesic. For example, to allow for intersubject comparisons of opioid doses required during the specific period, doses of opioid analgesics used during this period were converted to an equivalent dose of systemic fentanyl in our study.

2.2. Preparation of Genomic DNA

1. Four cotton swabs for each subject (see Note 1).
2. Screw-cap centrifuge tube for each subject in which to enclose swabs (e.g., Corning® 15 mL PP Centrifuge Tubes; Corning Inc., Corning, NY).
3. Cup of water to rinse out subject's mouth.
4. DNA extraction kit (e.g., QIAamp DNA Mini Kit; QIAGEN, Hilden, Germany).
5. Phosphate buffered saline (PBS): 1 tablet of Phosphate Buffered Salts Tablets (Takara Bio, Otsu, Japan) is dissolved in 100 mL of distilled water.
6. Whole genomic DNA amplification kit (e.g., illustra GenomiPhi V2 Kit, 100rxns; GE Healthcare UK, Buckinghamshire, United Kingdom).
7. TE buffer: 300 μL of 1 M tris base, 60 μL of 0.5 M EDTA (pH 8.0), and distilled water to a total volume of 30 mL.
8. Spectrophotometer for measurement of the concentration of genomic DNA (e.g., NanoDrop ND-1000 Spectrophotometer; NanoDrop Technologies, Wilmington, DE).

2.3. Genotyping

2.3.1. Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR–RFLP)

1. Purified 5–50 ng genomic DNA.
2. Forward and reverse oligonucleotide primer set encompassing the specific region, including the polymorphic site for PCR amplification.
3. Reaction buffer for PCR including DNA polymerase, dioxyribonucleoside triphosphate (dNTP), and $MgCl_2$ (e.g., GoTaq® Master Mix; Promega, Madison, WI).
4. Thermal cycler (e.g., PROGRAM TEMP CONTROL SYSTEM PC-818-02; Astec, Fukuoka, Japan).
5. Agarose (e.g., Agarose ME, Classic Type; Nacalai Tesque, Kyoto, Japan), stored at room temperature.
6. TAE buffer (50×): 242 g of tris base, 57.1 mL of glacial acetic acid, 100 mL of 0.5 M EDTA (pH 8.0), and water to a total volume of 1,000 mL, stored at room temperature.
7. DNA size marker (e.g., Loading Quick® 100 bp DNA Ladder; Toyobo, Osaka, Japan), stored at −20°C.

8. Ethidium bromide solution (Sigma-Aldrich, St. Louis, MO), stored at 4°C.
9. Appropriate restriction enzymes for digestion of PCR products.

2.3.2. Allele-Specific PCR (AS-PCR)

1. Purified 5–50 ng genomic DNA.
2. Two forward oligonucleotide primers, whose 3′ ends are specific for detecting each of the two alleles at the polymorphic site, and a reverse oligonucleotide primer.
3. DNA polymerase attached with reaction buffer, dNTP, and $MgCl_2$ (e.g., GoTaq® Master Mix; Promega) (see Note 2).
4. Thermal cycler (e.g., PROGRAM TEMP CONTROL SYSTEM PC-818-02; Astec, Fukuoka, Japan).
5. Agarose (e.g., Agarose ME, Classic Type; nacalai tesque), stored at room temperature.
6. TAE buffer (50×): 242 g of tris base, 57.1 mL of glacial acetic acid, 100 mL of 0.5 M EDTA (pH 8.0), and water to a total volume of 1,000 mL, stored at room temperature.
7. DNA size marker (e.g., Loading Quick® 100 bp DNA Ladder; Toyobo), stored at −20°C.
8. Ethidium bromide solution (Sigma-Aldrich), stored at 4°C.

2.3.3. TaqMan® SNP Genotyping Assays

1. Purified 5–50 ng genomic DNA.
2. 40× (or 20× or 80×) SNP Genotyping Assay containing sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest and two TaqMan® MGB probes labeled with VIC® dye to detect the sequence of one allele and with FAM™ dye to detect the sequence of another allele.
3. TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA).
4. Real-time PCR system (e.g., 7300 Real-Time PCR System; Applied Biosystems).

2.3.4. Multiple Primer Extension (MPEX)

2.3.4.1. Oligonucleotide Module Fabrication

1. S-Bio® PrimeSurface® (BS-11608) consisting of COC grafted with an original biocompatible phospholipid polymer, poly [2-methacryloyloxyethyl phosphorylcholine (MPC)-co-n-butyl methacrylate (BMA)-co-p-nitrophenyloxycarbonyl polyethyleneglycol methacrylate (MEONP)] (PMBN) hydrophilic polymer (Sumitomo Bakelite, Tokyo, Japan).
2. Oligonucleotide probes (see Note 3) designed to hybridize allele-specific PCR products of the arbitrary gene (e.g., *OPRM1*).

3. Spotting solution (250 mM sodium carbonate buffer, pH 9.0), stored at room temperature.
4. BioChip Arrayer® spotting robot (Filgen, Nagoya, Japan).
5. Oligonucleotide modules (gasket-type hybridization cassettes; one module consisting of 16 [8×2 lanes] hybridization wells; Sumitomo Bakelite).
6. Blocking buffer solution (0.5 N NaOH), stored at room temperature.

2.3.4.2. Preparation of Template Multiplex PCR Products and Their Confirmation

1. Multiplex PCR Mix® (TaKaRa Bio), stored at −30°C.
2. Primer pairs designed to amplify allele-specific PCR products of the arbitrary gene (e.g., *OPRM1*).
3. Thermal cycler (e.g., TaKaRa PCR Thermal Cycler Dice® Model TP600; TaKaRa Bio).
4. Wizard® SV 96 PCR Clean-Up System (Promega), stored at room temperature (22–25°C).
5. 80% ethanol, stored at room temperature.
6. Agarose (e.g., Agarose S; Nippon Gene, Tokyo, Japan), stored at room temperature.
7. TAE buffer (50×): 242 g of tris base, 57.1 mL of glacial acetic acid, 100 mL of 0.5 M EDTA (pH 8.0), and water to a total volume of 1,000 mL, stored at room temperature.
8. DNA size marker (e.g., 100 bp DNA Ladder; New England Biolabs, Ipswich, MA), stored at −20°C.
9. Ethidium bromide (Nippon Gene), stored at 4°C.

2.3.4.3. Modified MPEX Reaction

1. dNTP Set: 100 mM Solutions (GE Healthcare UK; working solution 1 mM for each dNTP), stored at −30°C.
2. HotStar Taq™ DNA polymerase (QIAGEN), stored at −30°C.
3. Biotin-11-dUTP (PerkinElmer, Wellesley, MA; working solution 1 mM), stored at −30°C and protected from prolonged exposure to light, with minimal freeze-thaw cycles.
4. 10× MPEX Buffer A: 1% TritonX100, stored at room temperature.
5. 2× MPEX Buffer B: 0.1 M phosphate buffer, pH 7.0, stored at room temperature.
6. Hybridization oven (e.g., Hybaid Midi Dual-14; Hybaid, Middlesex, United Kingdom).

2.3.4.4. Visualization by Colorimetric Reaction

1. Washing Buffer A: 10 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20, stored at room temperature.
2. Washing Buffer B: 0.1% Tween 20, stored at room temperature.

3. Streptavidin-AP (PerkinElmer), stored at −20°C, and then stored at 4°C after thawing, without refreezing.
4. BCIP/NBT substrate solution (PerkinElmer), stored at 2–8°C.
5. Scanner (e.g., GT-9700F personal image scanner; Epson, Tokyo, Japan).

2.4. Statistical Analyses

1. Suitable commonly used statistical software such as SPSS (SPSS Inc., Chicago, IL), SAS (SAS Institute, Cary, NC), JMP (SAS Institute, Cary, NC), R (freely available; http://www.r-project.org/), and/or software programs for genetic analyses (Table 1) to perform tests of Hardy–Weinberg equilibrium and linkage disequilibrium (LD) and association analyses.
2. Computer environment capable of accessing any websites of the databases of interest.

3. Methods

There are many ways of designing studies to explore the relationship between polymorphisms in some candidate genes and human sensitivity to opiates. In study designs, research subjects can be human volunteers undergoing standardized pain tests before and after administration of a given opioid, or patients undergoing standardized surgery and receiving opioids for postoperative pain control. Endpoint data that may represent the phenotypic traits related to analgesic efficacy of opioids can include the analgesic effect of the opioid evaluated by a standardized pain test or opioid requirements during and/or after standardized surgery as well as postoperative pain scores. Below are examples of an experimental study enrolling human volunteers, a prospective clinical study enrolling patients who are scheduled to have elective surgery of a given type, and a retrospective clinical study enrolling patients or ex-patients who previously underwent surgery of a given type. Research subjects are human volunteers or patients with American Society of Anesthesiologists Physical Status I or II ((9); Table 2) who do not have serious coexisting disease or a history of using opioids or other psychoneurotic agents.

3.1. Clinical Data Collection

The quality of clinical data is critical for accurately detecting polymorphisms associated with human sensitivity to opiates. Researchers may design many ways of collecting clinical data. We describe examples of our procedures and some points or issues that should be noted.

Table 1
Useful software programs for genetic analyses

Name	Application (main characteristics)	Platform	URL	Reference
Haplotype/Linkage disequilibrium analysis				
GENEPOP	A population genetics software package	–	http://genepop.curtin.edu.au	(33)
PHASE	Reconstructing haplotypes from population genotype data	–	http://www.stat.washington.edu/stephens/software.html	(34)
HAPLOVIEW	Haplotype analysis; single SNP haplotype association tests	Win/Mac/Unix	http://www.broad.mit.edu/mpg/haploview/index.php	(35)
LDSELECT	Analyses on patterns of linkage LD between polymorphic sites in a locus	–	http://droog.gs.washington.edu/ldSelect.html	(25)
GOLD	A software package that provides a graphical summary of LD in human genetic data	–	http://www.sph.umich.edu/csg/abecasis/GOLD	(36)
HAPLOTYPER	Estimation of haplotypes by MCMC	–	http://www.people.fas.harvard.edu/~junliu/Haplo/docMain.htm	(37)
Tagger	A tool for the selection and evaluation of tag SNPs from genotype data	web-based	http://www.broad.mit.edu/mpg/tagger	(38)
HAPLOBLOCKFINDER	A package for haplotype block identification, visualization and htSNP selection	UNIX/Win	http://cgi.uc.edu/cgi-bin/kzbang/haploBlockFinder.cgi	(39)
GENECOUNTING	Haplotype analysis with permutation tests for global association and specific haplotypes	Win/UNIX (Solaris)/Linux	http://www.mrc-epid.cam.ac.uk/Personal/jinghua.zhao/software.htm http://www.mrc-epid.cam.ac.uk/Personal/jinghua.zhao/software/	(40)

(continued)

Table 1 (continued)

Name	Application (main characteristics)	Platform	URL	Reference
HAPBLOCK	Dynamic programming algorithms for haplotype block partitioning and tag SNPs selection	Win/Linux/ UNIX(Solaris)	http://www-hto.usc.edu/msms/ HapBlock/	(41)
HAPLOBLOCK	Haplotype block identification, haplotype resolution and linkage disequilibrium mapping	UNIX(Solaris)/Linux/ MacOS X	http://bioinfo.cs.technion.ac.il/ haploblock/	(42)
SNPAlyze	A SNP and disease association analysis software	Win(98Me/NT4.0/ 2000/XP)	http://www.dynacom.co.jp/e/ products/package/snpalyze/index. html	(43)
EH (EHPLUS, EH+)	Estimation of haplotypes and case–control study based on estimated haplotypes	MS-DOS/UNIX	http://www.genemapping.cn/eh.htm http://linkage.rockefeller.edu/ software/eh	(44)
SNPHAP	A program for estimating frequencies of haplotypes of large numbers of diallelic markers	–	http://www-gene.cimr.cam.ac.uk/ clayton/software	–
Arlequin	An exploratory population genetics software environment able to handle large samples of molecular data	Win	http://cmpg.unibe.ch/software/ arlequin3/ http://lgb.unige.ch/arlequin/	(45)
HelixTree	Comprehensive toolset for population-based association studies	Win/Linux/MacOS X	http://www.goldenhelix.com/SNP_ Variation/HelixTree/index.html	(46)
Association study				
HARDY	MCMC program for association in two-dimensional contingency tables	UNIX (DEC-UNIX/..)	http://www.stat.washington.edu/ thompson/Genepi/Hardy.shtml	(47)

haplo.stats (formerly haplo.score)	A suite of routines for the analysis of indirectly measured haplotypes	UNIX	http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm	(48)
UNPHASED	A suite of programs for association analysis of multilocus haplotypes from unphased genotype data	UNIX(Solaris)/Linux/Win	http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/	(49)
HTR	Haplotype association mapping using unrelated individuals; "fixed" and "sliding" window analysis	Win/UNIX(Solaris)	http://statgen.ncsu.edu/zaykin/htr.html http://statgen.ncsu.edu/pub/zaykin/htr/	(46)
GENETIC POWER CALCULATOR	Automated power analysis for VC QTL linkage and other common tests	–	http://pngu.mgh.harvard.edu/~purcell/gpc/	(50)
CHAPLIN	Identifying specific haplotypes or haplotype features that are associated with disease	Win(2000/XP)	http://www.genetics.emory.edu/labs/epstein/software/chaplin/index.html	(51)
PAWE	Power and sample size calculations for genetic case–control association studies allowing for errors	–	http://linkage.rockefeller.edu/pawe/	(52)
Quanto	A program that computes sample size or power for association studies	Win (98/NT 2000/..)	http://hydra.usc.edu/GxE/	(53)
Hplus	Performing haplotype estimation on genetic markers and handling datasets that include case–control status	MS-Windows/Linux	http://cougar.fhcrc.org/hplus/	(54)
PLINK	A whole-genome association analysis toolset focusing purely on analysis of genotype/phenotype data	–	http://pngu.mgh.harvard.edu/purcell/plink/	(55)

SNP single nucleotide polymorphism, *LD* linkage disequilibrium, *MCMC* Markov chain Monte Carlo, *htSNP* haplotype tagging SNP, *VC* variance components, *QTL* quantitative trait locus

Table 2
American Society of Anesthesiologists Physical Status Classification System

P1:	A normal healthy patient
P2:	A patient with mild systemic disease
P3:	A patient with severe systemic disease
P4:	A patient with severe systemic disease that is a constant threat to life
P5:	A moribund patient who is not expected to survive without the operation
P6:	A declared brain-dead patient whose organs are being removed for donor purposes

3.1.1. Ethical Issues and Study Designs

1. Plan study protocol such that it meets all requirements imposed by the laws and guidelines regarding studies that handle human genomes (see Note 4).
2. Obtain approval from each respective institutional IRB for the study protocol.
3. Obtain written informed consent from each human subject after appropriately explaining, in written form, the clinical data sampling and DNA analysis.
4. A personal information manager responsible for managing personal information of the research subjects and making such information unidentifiable based on instructions from the head of the respective research institution should anonymize the collected samples before and after researchers handle the data to ensure the protection of personal information. Fig. 1 shows an example of the procedure in which personal information is protected.

3.1.2. Collecting Data in an Experimental Study Enrolling Human Volunteers

1. A given dose of a given opioid analgesic (e.g., intravenous [i.v.] injection of fentanyl, 2 μg/kg; i.v. injection of morphine, 0.2 mg/kg; i.v. infusion of remifentanil, 0.2 μg/kg/min over hours) is administered to human volunteers.
2. A standardized pain test, such as for thermal, mechanical, or electrical pain, is performed before, during, and after opioid administration (10).
3. An appropriate cutoff point is set to avoid tissue damage.
4. The pain test can be performed repeatedly, at a given interval, during and/or after opioid administration (e.g., every hour during and after i.v. infusion of remifentanil) over 4 h (10).
5. End-points can be latency to pain perception and/or pain tolerance, or pain perception and/or pain tolerance thresholds.

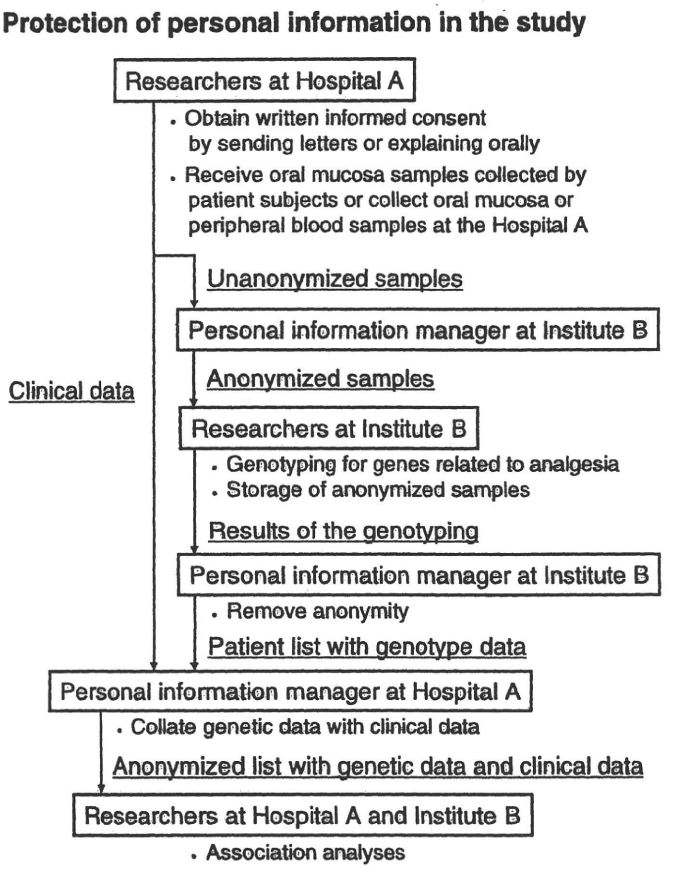

Fig. 1. Example of the procedure in which the personal information of research subjects is properly preserved. In this case, clinical data are collected at Hospital A, and genetic data are collected at Institute B.

6. The percent maximum possible effect (%MPE) can be calculated as an easy-to-interpret indicator of analgesic efficacy of the opioid: *%MPE = (cutoff value − maximum value during or after opioid administration)/(cutoff value − value before opioid administration) × 100.*
7. Whole blood (10 ml) or oral mucosa is sampled for genomic DNA analysis.

3.1.3. Collecting Clinical Data in a Prospective Study

1. Subjects are patients who are scheduled to have surgery of a given type that involves well-standardized procedures (e.g., distal gastrectomy for gastric cancer) under standardized anesthesia (e.g., sevoflurane–remifentanil anesthesia with or without epidural anesthesia).
2. Postoperative pain is managed with a single opioid analgesic according to a standardized protocol using a patient-controlled analgesia (PCA) pump (e.g., fentanyl, 20 μg per

demand dose with a lockout interval of 5 min; morphine, 2 mg per demand dose with a lockout interval of 10 min) (11).

3. Rescue analgesics (e.g., nonsteroidal antiinflammatory drugs) should be prescribed whenever the analgesic effect of the opioid is inadequate or the use of the opioid is discontinued because of significant adverse effects.
4. Postoperative pain scores are recorded at given postoperative time-points (e.g., 3, 6, 12, and 24 h after surgery) using an appropriate pain scale (e.g., visual analog scale, verbal pain rating scale, numerical pain rating scale) (11).
5. Presence/absence and severity (if present) of adverse effects of the opioid (e.g., nausea/vomiting and respiratory depression) are recorded.
6. Clinical data that may relate to analgesic efficacy of the opioid are recorded, including age, gender, type of surgery, duration of surgery, type of anesthesia, intraoperative opioid requirements, postoperative opioid requirements, rescue analgesic requirements during a given postoperative period (e.g., during the first 24 h after surgery), and postoperative pain scores.
7. Whole blood (10 ml) or oral mucosa is sampled for genomic DNA analysis.

3.1.4. Collecting Clinical Data in a Retrospective Study

1. A researcher in charge of clinical data collection (Researcher C) lists ex-patient or patient candidates who previously underwent surgery of a given type and received opioids for postoperative pain control at a particular hospital.
2. Researcher C mails letters to these candidates explaining the outline of the study protocol and reply cards on which the candidates can indicate their interest in participating in the study.
3. A researcher in charge of genomic DNA analysis (Researcher D) receives the reply cards from the candidates who are willing to participate in the study.
4. Researcher D sends packages to these candidates that include explanatory leaflets describing the detailed study protocol, written informed consent forms, instructions to collect oral mucosa samples, cotton swabs to collect oral mucosa samples, test tubes to enclose the samples, and stamped return-mail envelopes.
5. Researcher D receives signed informed consent forms and oral mucosa samples from the candidates who have been determined to be research subjects.
6. Researcher C collects clinical data from the hospital records of the research subjects that may relate to analgesic efficacy of opioids (see Subheading 3.1.3).