Additionally, if two SNPs are in strong LD, and almost no recombination is assumed between them, genotyping only one of the SNPs is sufficient. The strength of LD between two SNPs is often measured by the values D' and r^2, both of which are commonly used together but calculated by different formulas. Furthermore, a subset of the SNPs that is selected based on the information of LD relationships and tags the representative haplotypes at the region appropriately (25) are termed "haplotype tagging SNPs" or "tag SNPs" and might promote the efficiency of genotyping and further analyses without reducing the power to detect the expected association.

1. Estimate the strength of LD between the SNPs by calculating the values D' and r^2 based on the genotype data of the subjects using the suitable genetic analysis software programs (Table 1).
2. Select the tag SNPs that best represent the haplotypes at the region by using the suitable genetic analysis software programs, such as "Tagger" ((36); Table 1).

3.4.3. Association Study for Distinct SNPs

To explore the association between SNPs and some quantitative traits with normal distribution, several types of statistical analysis are possible, such as analysis of variance (ANOVA) and linear regression, which assumes a linear relationship between the mean value of the trait and the genotype.

1. Perform the statistical tests by running a software program to investigate the association between each distinct SNP and the phenotypic traits of interest. In the case of ANOVA, phenotypic traits and genotypes of a SNP should be treated as dependent and independent variables, respectively. Most software programs can also accept covariable data as well as genotype and phenotype data such as age, sex, and other characteristics of the subjects.
2. Check whether the output P-value achieves the level of significance for detecting a positive association.
3. In many cases, corrections of multiple testing might be required for the number of SNPs tested to avoid a type I error. However, corrections such as Bonferroni might not be required in some cases to avoid a type II error (26, 27).

3.4.4. Association Study for Haplotypes

Relatively few software programs are available for analyzing association between haplotypes and quantitative traits such as analgesic requirements compared with software programs that are available for case–control studies. Furthermore, estimated haplotypes of individuals or haplotype frequencies of the population tend to differ among the software used, depending on the algorithms employed by the programs. Minor differences

in haplotype frequency estimates can produce very large differences in statistical tests (28). Therefore, comparing the outcomes of similar analyses carried out by different software programs is advisable to confirm the results.

1. Perform the statistical tests by running a software program to investigate the association between one or more haplotypes and the phenotypic traits of interest. In many cases, haplotype-based association studies might be completed concurrently with haplotype estimations of the populations involved.
2. Check whether the output *P*-value achieves the level of significance for detecting a positive association.
3. In some cases, corrections of multiple testing might be required for the number of haplotypes tested to avoid a type I error. However, corrections such as Bonferroni might not be required in some cases to avoid a type II error (26, 27).

3.4.5. Utilization of Databases

Numerous databases have become openly available that are helpful for designing a study and for analyzing the results. Below are a few databases that are useful for surveying genes and genetic polymorphisms and downloading data that could expedite study analyses. A recent review article discusses bioinformatics approaches for SNP analyses (29).

1. To obtain overall information of any SNP, consult the dbSNP database ((30); http://www.ncbi.nlm.nih.gov/SNP/), which contains the largest amount of data on genetic variations, including SNPs.
2. To obtain Genotype-to-Phenotype information, consult HGVbase (Human Genome Variation database; http://hgvbase.cgb.ki.se; formerly known as HGBASE), which provides a high-quality, nonredundant database of available genomic variation data of all types (31).
3. To obtain information of SNPs related to drug response, consult the PharmGED database (http://bidd.cz3.nus.edu.sg/phg/), which provides information about the effects of a particular protein polymorphism, noncoding region mutation, splicing alteration, or expression variation on the response of a particular drug.
4. To obtain overall information about the haplotype map of the human genome or to download genotype data for linkage disequilibrium analysis or other purposes, consult the HapMap database ((32); http://www.hapmap.org/index.html.en), which helps researchers find genes associated with human disease and pharmaceutical response.

4. Notes

1. Any form of clean cotton swab can be used, such as the commercially available Sterile Omni Swab (Whatman plc, Kent, United Kingdom) or a commonly used small wad of cotton wrapped around the end of a small rod made of wood, rolled paper, or plastic. To maintain cleanliness, each swab should be packaged before use.
2. The DNA polymerase used in the AS-PCR should be the one that lacks proofreading activity because the polymerase with proofreading activity could substitute a mismatched base for the correct base at the polymorphic site, owing to its exonuclease activity from 3′ to 5′.
3. The oligonucleotides used here were single-stranded 19–27 mer 5′-C6-amino-oligonucleotides. The oligonucleotides were designed and synthesized by NovusGene (Tokyo, Japan).
4. All researchers handling human genomes must observe the laws and guidelines set forth in their respective countries. Such laws and guidelines may be enacted based on the Declaration of Helsinki, World Medical Association (WMA), and Universal Declaration on the Human Genome and Human Rights adopted by the UNESCO Bioethics Programme General Conference in 1997. For example, researchers in Japan must observe the "Ethical Guidelines for Analytical Research on the Human Genome/Genes" issued by the Japanese Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labour and Welfare, and the Ministry of Economy, Trade and Industry.
5. Clean the scissors used to separate the swab from the sticks with ethanol each time to avoid genomic DNA contamination from the previous sample.
6. The concentration of the purified DNA should be similar among samples because heterogeneity in the concentration of genomic DNA could increase the generation of false products in the AS-PCR, leading to misjudging the genotype of the sample.

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Chapter 27

Combination of Cell Culture Assays and Knockout Mouse Analyses for the Study of Opioid Partial Agonism

Soichiro Ide, Masabumi Minami, Ichiro Sora, and Kazutaka Ikeda

Abstract

Nonselective opioid partial agonists, such as buprenorphine, butorphanol, and pentazocine, have been widely used as analgesics and for anti-addiction therapy. However, the precise molecular mechanisms underlying the therapeutic and rewarding effects of these drugs have not been clearly delineated. Recent success in developing μ-opioid receptor knockout (MOP-KO) mice has elucidated the molecular mechanisms underlying the effects of morphine and other opioids. We have revealed the in vivo roles of MOPs in the effects of opioid partial agonists by using MOP-KO mice for behavioral tests (e.g., several kinds of antinociceptive tests for analgesic effects, conditioned place preference test for dependence). The combination of the cell culture assays using cDNA for μ, δ, and κ opioid receptors and the behavioral tests using MOP-KO mice has provided novel theories on the molecular mechanisms underlying the effects of opioid ligands, especially opioid partial agonists.

Key words: Pain, Knockout mouse, Behavior, Conditioned place preference, Binding assay

1. Introduction

Nonselective opioid partial agonists, such as buprenorphine, butorphanol, and pentazocine, have been widely used as analgesics and for anti-addiction therapy. However, the precise molecular mechanisms underlying the therapeutic and rewarding effects of these drugs have not been clearly delineated, although investigators have estimated their antinociceptive and rewarding effects using selective opioid receptor agonists and antagonists.

Cell culture assays using cDNA for μ (MOP), δ (DOP), and κ (KOP) opioid receptors are useful for investigating the molecular mechanisms underlying the effects of opioid partial agonists. Because the most selective ligands for a specific subtype of opioid

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receptor (e.g., β-funaltrexamine for MOP, naltrindole for DOP, and norbinaltorphimine [nor-BNI] for KOP) possess certain affinities for other subtypes (1), the true affinity, activity, and selectivity of opioid ligands for opioid receptor subtypes should be analyzed using cell lines expressing only one specific opioid receptor subtype.

Recent success in developing MOP knockout (KO) mice has elucidated the molecular mechanisms underlying the effects of opioids (2–5). The analgesic effects of morphine in both the tail-flick and hot-plate tests and the rewarding effects of morphine in self-administration tests are abolished in MOP-KO mice (3–5). By contrast, buprenorphine, a nonselective opioid receptor partial agonist, has no analgesic effect in the tail-flick and hot-plate tests but a significant rewarding effect in the conditioned place preference test in homozygous MOP-KO mice (6). These observations are especially interesting because the distributions of DOP and KOP are not apparently altered in MOP-KO mice (2, 3, 5). Furthermore, using MOP-KO mice in tail-flick and hot-plate tests, the antinociceptive effects of tramadol, an analgesic possessing of both opioid and nonopioid activities, were shown to be mediated mainly by MOPs and adrenergic α_2 receptors (7). Although several compensatory changes might occur in KO animals, they have potential utility in investigating the in vivo roles of specific proteins. Thus, the use of MOP-KO mice has provided novel theories on the molecular mechanisms underlying the effects of opioid ligands, especially opioid partial agonists.

2. Materials

2.1. Cell Culture Assays

1. Cell lines stably expressing MOPs, DOPs, and KOPs.
2. Binding buffer (10×): 500 mM Tris-HCl, pH 7.4, 100 mM $MgCl_2$, 10 mM EDTA. Store at 4°C.
3. Cold and hot [^{3}H]opioid receptor subtype selective ligands (e.g., DAMGO for MOP, DPDPE for DOP, and U69593 for KOP).
4. Whatman GF/C glass fiber filters pretreated with 0.1% polyethyleneimine. Store at 4°C.
5. HEPES-buffered saline (1×): 15 mM HEPES, pH 7.4, 140 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 11 mM glucose. Store at 4°C.
6. 100 μM forskolin. Store at 4°C.
7. 1 mM 3-isobutyl-1-methylxanthine. Store at 4°C.
8. 10% trichloroacetic acid. Store at −20°C.

9. 3′,5′-cyclic adenosine monophosphate (cAMP) assay kit (e.g., Amersham, Buckinghamshire, UK).
10. Homogenizer.
11. Crushed ice.
12. Scintillation cocktail.
13. Liquid scintillation counter.
14. Computer software for nonlinear regression analysis (e.g., GraphPad Prism, GraphPad, San Diego, CA, USA).

2.2. Mice and Genotyping

1. DNA polymerase (e.g., KOD Dash polymerase, Toyobo Co., Ltd., Tokyo, Japan).
2. Buffer for tissue lysis (1×): 70 mM Tris-HCl, pH 8.2, 20 mM EDTA, 100 mM NaCl, 30 mM *N*-lauroylsarcosine sodium salt. Store at 4°C.
3. Proteinase K solution: 20 mg/ml. Store at −20°C.
4. MOP-KO mice (2, 3, 5, 8).
5. Selective primer pairs.
6. Apparatus for polymerase chain reaction (PCR).
7. Apparatus for gel electrophoresis.

2.3. Behavioral Analyses

1. Tail-flick apparatus (MK-330B, Muromachi Kikai Co., Tokyo, Japan).
2. Hot-plate apparatus (MK-350D, Muromachi Kikai Co., Tokyo, Japan).
3. Pressure Analgesy-Meter (Model MK-201D, Muromachi Kikai Co., Tokyo, Japan).
4. Black felt towel.
5. Locomotor activity test apparatus (SUPERMEX, CompACT AMS v. 3, Muromachi Kikai Co., Tokyo, Japan).
6. Conditioned place preference apparatus (SUPERMEX, CompACT CPP, Muromachi Kikai Co., Tokyo, Japan).
7. Morphine hydrochloride (Sankyo Co., Tokyo, Japan).
8. Naloxone hydrochloride (Sigma Chemical Co., St. Louis, MO, USA).
9. Naltrindole hydrochloride (Sigma Chemical Co., St. Louis, MO, USA).
10. norBNI dihydrochloride (Sigma Chemical Co., St. Louis, MO, USA).
11. 0.9% Sterile saline.
12. Acetic acid.

3. Methods

3.1. Cell Culture Assays

In vitro assays using cell lines stably expressing MOPs, DOPs, and KOPs are useful for revealing the characteristics of opioid ligands. Although our previous data in in vitro analyses using cell lines stably expressing human MOPs, DOPs, and KOPs indicated that the most partial opioid ligands show partial agonism for MOP (6, 9), these affinities and efficacies for each subtype would change slightly when the different species of cDNA are used (i.e., mouse, rat). The methods of the representative cell culture assays are specified below and shown in Fig. 1.

3.1.1. Radioligand Binding Assay

1. Harvest cells stably expressing MOPs, DOPs, and KOPs after 65 h in culture.
2. Homogenize harvested cells in ice-cold binding buffer.
3. Pellet by centrifugation for 20 min at 30,000×*g*, and resuspend in binding buffer.
4. For saturation binding assays, incubate the cell membrane suspensions for 60 min at 25°C with various concentrations of [^{3}H]DAMGO for human MOP, [^{3}H]DPDPE for human DOP, or [^{3}H]U69593 for human KOP. Nonspecific binding is determined in the presence of 10 μM unlabeled ligands.
5. For competitive binding assays, incubate the cell membrane suspensions for 60 min at 25°C with 2 nM [^{3}H]DAMGO for human MOP, 2 nM [^{3}H]DADLE for human DOP, or 3 nM [^{3}H]U69593 for human KOP in the presence of various concentrations of ligands (see Note 1).
6. Filtrate membrane suspensions rapidly using glass fiber filters, and wash each filter with binding buffer four times.
7. Add scintillation cocktail for each filter and stay overnight at room temperature.
8. Measure the radioactivity on each filter by liquid scintillation counting.
9. Obtain K_d values of the radiolabeled ligands by Scatchard analysis of the data from the saturation binding assay.
10. Calculate K_i values from the IC_{50} values obtained from the competitive binding assay in accordance with the equation $K_i = IC_{50}/(1 + [\text{radiolabeled ligand}]/K_d)$, where IC_{50} is the concentration of unlabeled ligand producing 50% inhibition of the specific binding of radiolabeled ligand (see Note 2).

3.1.2. cAMP Assay

1. Place 10^5 cells stably expressing MOPs, DOPs, and KOPs into each well of a 24-well plate and grow for 24 h.
2. Wash and incubate with 0.45 ml of HEPES-buffered saline for 10 min at 37°C (see Note 3).

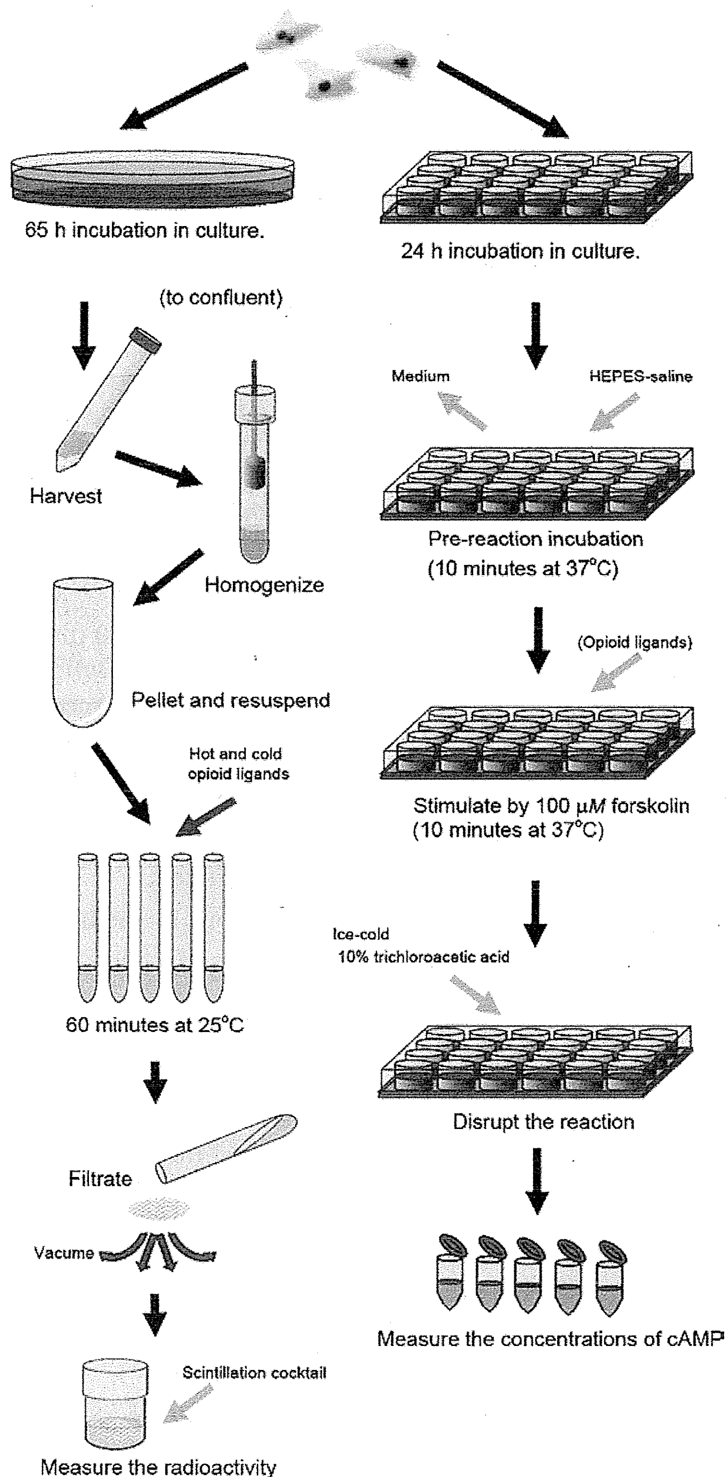

Fig. 1. Experimental procedure for representative in vitro analyses using cell lines stably expressing MOPs, DOPs, and KOPs. *Left flow* is for a binding assay. *Right flow* is for a cAMP accumulation assay

3. Stimulate for 10 min by the addition of 50 μl of HEPES-buffered saline containing 100 μM forskolin and 1 mM 3-isobutyl-1-methylxanthine to each well in the presence or absence of various concentrations of opioid ligands.
4. Disrupt the reaction by adding 0.5 ml of ice-cold 10% trichloroacetic acid to each well.
5. Measure the concentrations of cAMP by immunoassay (Amersham, Buckinghamshire, UK).
6. Calculate IC_{50} values as the concentration of ligand producing 50% of the maximal inhibition of cAMP accumulation.

3.2. Mice and Genotyping

Wildtype, heterozygous, and homozygous MOP-KO mouse littermates are generated from crosses of heterozygous/heterozygous MOP-KO mice. Ideally, mice with a C57BL/6J genetic background are preferable (4) (see Note 4). The method of genotyping is specified below.

1. Cut the tip (approximately 5 mm^2) of the mouse ear (see Note 5).
2. Prepare genomic DNA: Add the 50 μl buffer for tissue lysis (1×) and 0.25 μl proteinase K solution to the mouse ear fragment. Heat at 56°C for 3 h or overnight with continuous shaking or vortexing once per hour. Then heat at 95°C for 15 min and centrifuge at 3,000×*g* for 15 min. The prepared genomic DNA can be stored at −20°C (see Note 6).
3. Amplify the DNA fragment using polymerase chain reaction (PCR) with the pairs of primers.
4. Analyze the DNA fragments by the usual gel electrophoresis method.

3.3. Behavioral Analyses

To evaluate partial agonism of opioid ligands using MOP-KO mice, the analgesic effects should be tested not with just one type of noxious stimulus, but rather with several types (e.g., thermal, mechanical, and chemical) because analgesic efficacy is substantially different among different types of noxious stimuli. Furthermore, examination of locomotor activity and drug preference is also useful. The methods for several representative behavioral tests are described below. Additionally, investigations of opioid partial agonists in MOP-KO mice with selective opioid antagonists (e.g., β-funaltrexamine for MOP, naltrindole for DOP, and nor-BNI for KOP) in these tests are powerful methods (Fig. 2).

3.3.1. Antinociceptive Tests

The hot-plate test is used to evaluate supraspinal thermal antinociception. A commercially available apparatus consisting of an acrylic resin cage and a thermo-controlled aluminum plate (Model MK-350A, Muromachi Kikai Co., Tokyo, Japan) can be used for this test following the methods of Woolfe and MacDonald (10) with slight modifications.

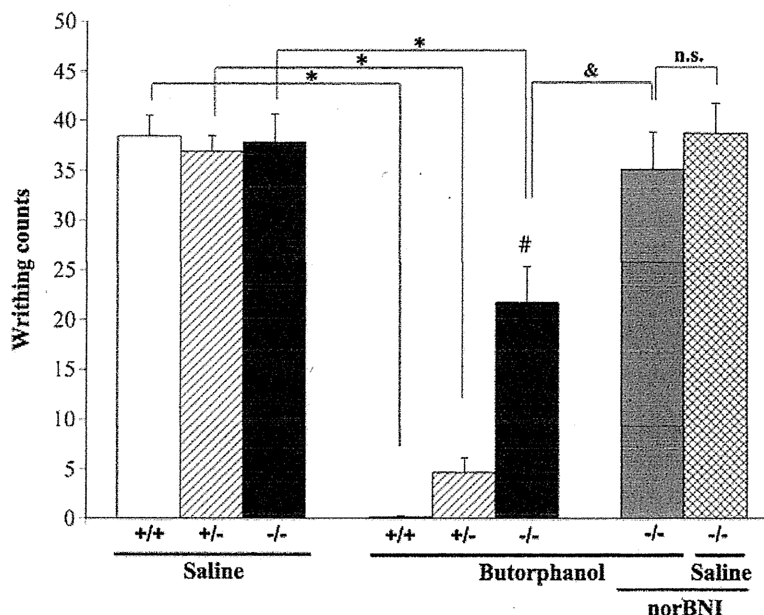

Fig. 2. Visceral chemical antinociceptive effects of butorphanol in wildtype, heterozygous, and homozygous MOP-KO mice. Writhing counts induced by 0.6% acetic acid (i.p.) with saline pretreatment in wildtype [+/+, $n=9$ (male, 5; female, 4)], heterozygous [+/–, $n=10$ (male, 5; female, 5)], and homozygous [–/–, $n=7$ (male, 4; female, 3)] mice, butorphanol pretreatment (3 mg/kg, s.c.) in wildtype [+/+, $n=8$ (male, 5; female, 3)], heterozygous [+/–, $n=8$ (male, 4; female, 4)], and homozygous [–/–, $n=12$ (male, 5; female, 7)] MOP-KO mice, and nor-BNI (5 mg/kg, s.c.), butorphanol (3 mg/kg, s.c.) [–/–, $n=13$ (male, 7; female, 6)], or saline pretreatment [–/–, $n=13$ (male, 4; female, 5)] in homozygous MOP-KO mice. $^{\#}p<0.05$ Significantly different from wildtype mice. $^{*}p<0.05$ Significantly different from saline pretreatment. $^{\&}p<0.05$ Significantly different from nor-BNI pretreatment. *n.s.* Not significant. Data are expressed as mean ± SEM. (Reproduced from ref. 13 with permission from Elsevier Science.)

1. Habituate mouse to the hot-plate apparatus before heating the floor plate (see Notes 7).
2. Place a mouse on a 52 ± 0.2°C hot-plate, and record latency to lick the hind-paw, lift the hind-paw, or jump with a cut-off time of 60 s (see Notes 8).

The tail-flick test is used to evaluate spinal thermal antinociception. A commercially available apparatus consisting of an irradiator for heat stimulation and a photosensor for detecting tail-flick behavior (Model MK-330A, Muromachi Kikai Co., Tokyo, Japan) can be used for this test following the method of D'Amour and Smith (11) with slight modifications.

1. Hold a mouse on the tail-flick apparatus and set the mouse's tail on an irradiator (see Note 9).
2. Heat the mouse's tail, and automatically record tail-flick latencies with a cut-off time of 15 s.

The hind-paw pressure test is used to evaluate mechanical antinociception. A commercially available apparatus (Pressure Analgesy-Meter, Model MK-201D, Muromachi Kikai Co., Tokyo, Japan) can be used following the method of Randall and Selitto (12) with slight modifications.

1. Hold a mouse on the hind-paw pressure apparatus and set the mouse's hind paws on a stage (see Note 9).
2. Gradually press the mouse's hind paws, and automatically record hind-paw withdrawing or struggle latencies with a cut-off pressure at 250 mmHg (see Note 9).

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

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

The writhing test is used to evaluate visceral chemical antinociception.

1. Inject acetic acid (0.6% v/v, 10 ml/kg) intraperitoneally (i.p.).
2. Place the mouse in a large plastic cage.
3. Count the total number of writhes occurring between 0 and 15 min after acetic acid injection. (The writhing response consists of contraction of the abdominal muscles.)
4. Nociception is expressed as writhing scores during the 15 min period.

A large number of KO mice are needed for testing the antinociceptive effects of drugs, and preparation may be troublesome for researchers. Using cumulative dose-response analyses can dramatically reduce the number of mice used in the hot-plate, tail-flick, and hind-paw pressure tests. For example, in the cumulative dose-response analyses, butorphanol is administered subcutaneously (s.c.) at doses of 0.3, 0.7, 2.0, and 7.0 mg/kg, yielding cumulative doses of 0.3, 1.0, 3.0, and 10 mg/kg, respectively. Antinociceptive tests are conducted 20 min after each drug injection. Drug is injected immediately after the previous test (see Note 10). However, although cumulative dose-response analyses have the great advantage of reducing the number of mice required for testing, researchers should pay attention to several points, such as the attenuation of antinociceptive effects by acute tolerance to the test drugs (see Fig. 3). Furthermore, the time-course of drug efficacy should be determined (see Note 11).

3.3.2. Conditioned Place Preference (CPP) Test

The CPP test is used to evaluate the rewarding effect of drugs. Two- or three-compartment Plexiglas chambers are used, in which one compartment is black with a smooth floor, one

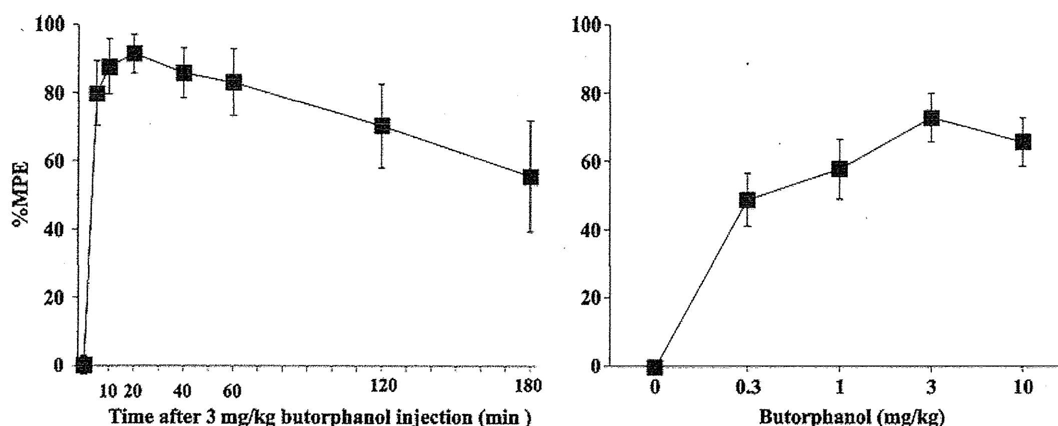

Fig. 3. Thermal antinociceptive effects of butorphanol in wildtype mice in the tail-flick test. (*Left*) Butorphanol (3 mg/kg, s.c.)-induced alterations of %MPE in the tail-flick test in wildtype mice [n= 8 (male, 4; female, 4)] under the time-course paradigm. (*Right*) Butorphanol-induced alterations of %MPE in the tail-flick test in wildtype mice [n= 11 (male, 6; female, 5)] under the cumulative dose-response paradigm. (Reproduced from ref. 13 with permission from Elsevier Science.)

compartment is white with a textured floor, and the middle compartment has a gray floor (when a three-compartment model is used). For pre- and post-conditioning test phases, a division with openings allows access to both compartments. During the conditioning phases, the openings are closed to restrict mice to a single compartment. Locomotion and time spent in each compartment are recorded using an animal activity monitoring apparatus (SUPERMEX, CompACT CPP, Muromachi Kikai Co., Tokyo, Japan). The apparatus should be sound- and light-attenuated (see Note 12). Conditioned place preference is assessed by a protocol consisting of three phases, preconditioning, conditioning, and test.

1. Allow mice to freely explore each compartment through the openings for 15 min and acclimatize to the apparatus on days 1 and 2.
2. Perform the same trial on day 3 (preconditioning phase). Measure the time spent in each compartment for 15 min. Determine whether the animal has a preference for a particular compartment before conditioning (see Note 13). When planning a test schedule after the preconditioning phase, a counterbalanced protocol is preferable to nullify each mouse's initial preference, as previously discussed (13).
3. Conditioning is conducted once daily for 4 consecutive days (days 4–7). Perform the assignment of the conditioned compartment randomly, and counterbalance across subjects. Administer drug or saline to the mouse, and immediately confine the mouse to the black or white compartment for 50 min on day 4.

4. Administer the alternate saline or drug to the mouse, and immediately confine the mouse to the opposite compartment for 50 min on day 5.
5. Repeat the same conditioning procedure from days 4 and 5 on days 6 and 7.
6. Measure the time spent in each compartment for 15 min without drug injection during the test phase on day 8.
7. The CPP score is designated as the time spent in the drug-paired compartment on day 8 minus the time spent in the same compartment during the preconditioning phase on day 3.

4. Notes

1. The concentration of radiolabeled ligands used for competitive binding assays is determined from K_d values of the radiolabeled ligands obtained by Scatchard analysis of the data from the saturation binding assay.
2. For the estimation of the inhibitory concentration at 50% (IC_{50}) in the competitive binding assay, a binding model for one-site or two-site competition binding is preferable. Nonlinear regression analysis using computer software (GraphPad Prism, GraphPad, San Diego, CA, USA) can estimate the binding model and the IC_{50} value.
3. All buffers for wash and reaction should be warmed at 37°C before use.
4. To study specific genetic factors for screening behavioral changes and/or drug actions using KO mice, littermate mice are preferable to minimize other genetic effects and nongenetic factors.
5. Although the usual method is cutting the tip of the mouse tail (approximately 5 mm), this should be avoided when mice are tested with noxious stimuli applied to their tails (e.g., tail-immersion test, tail-flick test).
6. After the heat shock at 95°C for 15 min, the genomic DNA solution can be used for PCR without usual phenol extraction. In this procedure, the volume of the genomic DNA solution should be less than 5% of the total volume of the PCR reaction.
7. Mice should be habituated to the hot-plate apparatus before heating the floor plate because mice show exploratory behavior that can cause unstable results when tested without habituation.

8. To evaluate the thermal antinociceptive effects of opioid partial agonists, the temperature of the hot-plate should be set at 52°C or lower (48–52°C) because thermal antinociceptive effects of opioid partial agonists are relatively weak and cannot be clearly analyzed when using a 54°C hot-plate (a temperature usually reserved for rats). If the higher temperature is used to test a full opioid agonist (e.g., morphine, fentanyl), the cut-off time should be 45 s or less.
9. If the mouse struggles and cannot be set on the apparatus, it can be loosely wrapped in a black felt towel.
10. In the cumulative dose-response analyses, the number of drug doses for the test should be less than five. Excessive, repeated testing can have a noxious influence on the mice.
11. The effective duration of tested drugs should be determined before using the cumulative dose-response analyses.
12. Although the compartment usually has dim illumination (about 40 lux), the intensity of light should be adjusted to the condition in which mice show nearly the same time spent in both compartments before the conditioning phase.
13. Biased mice that spend more than 80% of the time (i.e., 12 min) on one side on day 3 or more than 10 min on one side on day 2 and more than 10 min on the other side on day 3 are not recommended to be used for further experiments.

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Chapter IV

Advantage of Using Wild-Derived Mouse Strains for a Variety of Pain-Related Studies: Genetic Diversity and New Genetic Tools

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Abstract

Pain sensitivity has proved to be extremely variable among human individuals. One of the most important factors for such variations in pain-related phenomena is genetic diversity. A variety of mouse strains are reportedly suitable animal models for investigating the genetic basis of large individual differences in pain sensitivity. Laboratory strains have been reported to exhibit different behavioral traits due to variations in their genetic background. However, they show low genetic polymorphism because the original colony bred to produce the strains comprises a relatively low number of mice belonging to the subspecies *Mus musculus domesticus*. The low heterogeneity of laboratory strains makes their behavioral phenotype less variable. Therefore, the use of inbred strains derived from different mouse subspecies for pain-related phenotype studies is a great advantage. Several research groups have been involved in the long-term process of establishing a variety of wild-derived inbred strains

from wild mice captured all over the world after at least 20 generations of brother-sister mating. The genetic diversity of wild-derived strains is advantageous for the analysis of phenotypic differences among strains. We previously identified a marked variety in pain and morphine sensitivity in a series of wild-derived inbred strains. In particular, we found that the MSM strain established from the Japanese wild mouse, *Mus musculus molossinus*, one of the subspecies of the musculus subspecies group, exhibits significant differences in pain-related phenotypes compared to C57BL/6 (B6). In order to study genetic factors associated with these differences, we used a panel of consomic strains, established by replacing each B6 chromosome by that of MSM on a B6 background. Our study identified multiple chromosomes related to reduced pain sensitivity in both hot-plate and tail-flick tests. Further mapping using subconsomic strains carrying a shorter segment of the chromosome allowed the successful characterization of a locus related with reduced pain sensitivity using the hot-plate test. Thus, this chapter reports on the usefulness of consomic strains for genetic analyses of pain-related phenotypes in mice.

Introduction

Pain is an important stimulus for animals to recognize tissue injury, environmental danger and disease, but surplus pain degrades quality of life. Although many analgesics have been developed to reduce human pain, dependence, tolerance, and sensitivity to them vary significantly among individuals, and no ideal drugs have yet been developed. One of the most important factors causing individual differences in pain-related phenomena is genetic diversity (Lariviere et al., 2002). Laboratory strains have been reported to show different behavioral traits due to differences in genetic background. In this regard, selecting the best genetic strain for objective behavioral study is recommended (Crawley et al., 1997). However, laboratory mice have been domesticated during the process of establishing laboratory strains, causing them to lose their characteristic behavior due to domestication. In addition to the obedient phenotype, most laboratory strains show low genetic polymorphism since the original colony of the laboratory strains comprises a relatively small number of mice mostly belonging to *M. m. domesticus* subspecies (Bonhomme and Guénet, 1996; Ferris et al., 1982; Yonekawa et al., 1980). Low heterogeneity in laboratory strains makes the behavioral phenotype less variable among them. Therefore, behavioral tests may show non significant values as a consequence of their low genetic variability. On the other hand, results using inbred strains derived from different mouse subspecies are valuable for behavioral and brain research in the field of neurological science.

Wild Mice and Wild Strains

The *Mus musculus* species is divided into four major subspecies groups: domesticus, musculus, castaneus, and bactrianus (Figure 1) (Bonhomme and Guénet, 1996; Moriwaki et al., 1994; Silver, 1995). These four subspecies groups are genetically different according to their diversity of biochemical markers and mtDNA patterns. Further taxonomic subdivisions have been applied using their geographical distribution and morphological characteristics, although these local subspecies have been classed as heterogeneous with the major