GIRK knockout mice show decreased cocaine self-administration (Morgan *et al*, 2003), selective GIRK channel inhibitors might be potential agents for the treatment of abusers of cocaine. Therefore, as GIRK channels are considered candidates for clinically relevant targets, it may be important to clarify the pharmacological and physiological effects of various agents interacting with GIRK channels.

ACKNOWLEDGEMENTS

We thank Dr Kansaku Baba for cooperation, and Tomio Ichikawa, Kazuo Kobayashi, and Kazuyo Sekikawa for their assistance. We also thank Dr Steven C Hebert for providing the Kir1.1 cDNA, and Dr Lily Y Jan for providing the Kir2.1 cDNA. This work was supported by research grants from the Ministry of Education, Science, Sports and Culture of Japan, the Ministry of Health, Labour and Welfare of Japan, and the RIKEN Brain Science Institute.

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Neuropsychopharmacology (2004), 1–8

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Buprenorphine Antinociception is Abolished, but Naloxone-Sensitive Reward is Retained, in μ-Opioid Receptor Knockout Mice

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Buprenorphine is a relatively nonselective opioid receptor partial agonist that is used in the management of both pain and addiction. To improve understanding of the opioid receptor subtypes important for buprenorphine effects, we now report the results of our investigation on the roles of μ-, δ-, and κ-opioid receptors in antinociceptive responses and place preferences induced by buprenorphine. Buprenorphine antinociception, assessed by hot-plate and tail-flick tests, was significantly reduced in heterozygous μ-opioid receptor knockout (MOR-KO) mice and abolished in homozygous MOR-KO mice. In contrast, buprenorphine retained its ability to establish a conditioned place preference (CPP) in homozygous MOR-KO, although the magnitude of place preference was reduced as the number of copies of wild-type μ-opioid receptor genes was reduced. The remaining CPP of buprenorphine was abolished by pretreatment with the nonselective opioid antagonist naloxone, but only partially blocked by pretreatment with either the δ-selective opioid antagonist naltrindole or the κ-selective opioid antagonist norbinaltorphimine. These data, and biochemical confirmation of buprenorphine actions as a partial δ-, μ-, and κ-agonist, support the ideas that μ-opioid receptors mediate most of analgesic properties of buprenorphine, but that μ- and δ- and/or κ-opioid receptors are each involved in the rewarding effects of this drug.
Neuropsychopharmacology advance online publication, 21 April 2004; doi:10.1038/sj.npp.1300463

Keywords: opioid receptor; knockout mice; buprenorphine; antinociception; reward; detoxification

INTRODUCTION

Buprenorphine is a relatively long-acting nonselective partial agonist of opioid receptors that has been widely used as an analgesic and an antiaddiction therapeutic. Previous reports suggest that systemically administered buprenorphine can produce μ-opioid receptor-mediated antinociceptive actions and also antagonize morphine antinociception (Cowan *et al*, 1977; Kamei *et al*, 1995; 1997). Intrathecal (i.t.) buprenorphine administration produces antinociception that can be antagonized by κ-opioid antagonists, and it also blocks the antinociceptive effects of κ-opioid agonists in the acetic acid writhing test (Kamei *et al*, 1995; Leander, 1988; Tejwani and Rattan, 2002). Although Neilan *et al* (1999) reported buprenorphine to be a partial δ-opioid receptor agonist, Pick *et al* (1997) did not find such an effect. Each opioid receptor subtype has thus been implicated in buprenorphine antinociception, but with several inconsistencies.

Buprenorphine is also used as a therapeutic agent for patients with opioid dependence (Cheskin *et al*, 1994; Lintzeris *et al*, 2002), even though its own abuse liability is manifest by findings including its self-administration by laboratory animals (Mello *et al*, 1988; Winger and Woods, 2001). The precise molecular mechanisms underlying the therapeutic and rewarding effects of buprenorphine have not been clearly delineated, although investigators have estimated its antinociceptive and rewarding effects by using selective agonists and antagonists. Recent success in developing knockout mice with μ-opioid receptor gene deletions have allowed definition of the loss of the analgesic and rewarding effects of morphine that occurs in mice in the absence of μ-opioid receptors (Kieffer, 1999; Loh *et al*, 1998; Sora *et al*, 1997b, 2001). DPDPE, an agonist active at δ-opioid receptors with some affinity for μ-opioid receptors, has a much weaker analgesic effect in homozygous μ-opioid receptor knockout (MOR-KO) mice (Matthes *et al*, 1998; Sora *et al*, 1997a). These observations are especially interesting since the distribution of δ- and κ-opioid receptors is nearly normal in MOR-KO mice (Loh *et al*, 1998; Matthes *et al*, 1996; Sora *et al*, 1997b).

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Received 02 January 2004; revised 11 March 2004; accepted 15 March 2004
Online publication: 18 March 2004 at http://www.acnp.org/citations/Npp03180404000 1/default.pdf

We now report herein the results of further investigations into the molecular mechanisms that underlie antinociceptive and rewarding effects of buprenorphine, which we conducted by using various pharmacological agents, MOR-KO mice, and cDNAs for μ-, δ-, or κ-opioid receptors. We found abolition of buprenorphine-elicited thermal analgesia in homozygous MOR-KO mice, but retention of some naloxone-sensitive buprenorphine rewarding effects in these animals. These observations are supplemented by *in vitro* data that document partial buprenorphine agonism at δ- as well as μ- and κ-opioid receptors. Our results indicate that μ-opioid receptors play mandatory roles in buprenorphine antinociception and that δ-, κ-, and μ-opioid receptors are involved in buprenorphine reward.

METHODS

Animals

Wild-type, heterozygous, and homozygous MOR-KO mouse littermates from crosses of heterozygous/heterozygous MOR-KO mice with a C57BL/6J genetic background, as described previously (Sora *et al*, 2001), served as subjects. The experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committee, and all animals were cared for and treated humanely in accordance with our institutional animal experimentation guidelines. Naive adult (>10 weeks old) mice were housed in an animal facility maintained at 24±1°C and 50% relative humidity under a 12/12 h light/dark cycle with lights on at 0800 and off at 2000. Food and water were available *ad libitum*.

Drugs

For *in vivo* assays, all drugs were dissolved in saline and injected into animals in volumes of 10 ml/kg. Buprenorphine hydrochloride, naloxone hydrochloride, naltrindole hydrochloride, and norbinaltorphimine dihydrochloride (norBNI dihydrochloride) were purchased from SIGMA Chemical Co. (St Louis, MO). Morphine hydrochloride was purchased from Sankyo Co. (Tokyo, Japan).

For *in vitro* assays, [D-Ala^2, *N*-MePhe^4, Gly-ol^5]enkephalin (DAMGO), a μ-opioid receptor-selective agonist, and [D-Pen^2, D-Pen^5]enkephalin (DPDPE), a δ-opioid receptor 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 κ-opioid receptor-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

Hot-plate testing was performed according to the method of Woolfe and MacDonald (1944) with slight modifications. A commercially available apparatus consisting of acrylic resin cage (20 × 25 × 25 cm: width × length × height) and a thermo-controlled aluminum plate (Model MK-350A, Muromachi Kikai Co., Tokyo, Japan) were used for this test. Mice were placed on a 52±0.2°C hot plate, and latencies to paw licking were recorded with a cutoff time of 60 s. Tail-flick testing was carried out according to the method of D'Amour and Smith (1941) with slight modifications, by using a commercially available apparatus consisting of an irradiator for heat stimulation and a photosensor for the detection of the tail-flick behavior (Model MK-330A, Muromachi Kikai Co., Tokyo, Japan). Mice were loosely wrapped in a felt towel, their tails were heated, and tail-flick latencies were automatically recorded with a cutoff time of 15 s. Tail-flick and then hot-plate testing were conducted 20 min after each subcutaneous (s.c.) drug injection. Buprenorphine was administered in doses of 0.1, 0.2, 0.7, and 2.0 mg/kg, for cumulative doses of 0.1, 0.3, 1.0, and 3.0 mg/kg, respectively. Morphine was injected s.c. at a dose of 10 mg/kg. The hot-plate and tail-flick responses of each mouse in the drug-induced antinociception were converted to the percent of maximal possible effect (%MPE) according to the following formula:

$$\%\text{MPE} = \frac{(\text{post drug latency} - \text{pre drug latency})}{(\text{cut-off time} - \text{pre drug latency})} \times 100\%$$

Conditioned Place Preference (CPP) Test

CPP test was carried out according to the method of Hoffman and Beninger (1989) with some modifications. For this test, we used a two-compartment plexiglass chamber, one compartment (17.5 × 15 × 17.5 cm: width × length × height) was black with a smooth floor and the other was of the same dimensions, but white with a textured floor. For pre- and postconditioning test phases, a T-style division with double 6 × 6 cm openings allowed access to both compartments. During the conditioning phases, the openings were eliminated to restrict mice to a single compartment. Locomotion and time spent in each compartment was recorded by using an animal activity monitoring apparatus equipped with an infrared detector (Neuroscience Inc., Osaka, Japan). The compartment chamber was placed in a sound- and light-attenuated box under conditions of dim illumination (about 40 lx). Conditioned place preferences were assessed by a protocol consisting of three phases (preconditioning, conditioning, and test phases). On days 1 and 2, the mice were allowed to freely explore the two compartments through the openings for 900 s and acclimatized to the apparatus. On day 3 (preconditioning phase), the same trial was performed and the time spent in each compartment was measured for 900 s. There was no significant difference between time spent in the black compartment with a smooth floor (464±12 s, n=92) and time spent in the white compartment with a textured floor (436±12 s, n=92), indicating that there was no preference before conditioning in the apparatus itself. We selected a counterbalanced protocol in order to nullify each mouse's initial preference, as discussed previously (Tzschentke, 1998). Biased mice that spent more than 80% of the time (ie 720 s) on one side on day 3 or more than 600 s on one side on day 2 and more than 600 s on the other side on day 3 were not used for further experiments. Conditioning was conducted once daily for 4 consecutive days (days 4–7). Mice were injected with either buprenorphine (1.0 mg/kg s.c.) or saline and immediately confined to the black or

white compartment for 50 min on day 4. On day 5, the mice were injected with alternate saline or buprenorphine (1.0 mg/kg s.c.) and immediately confined to the opposite compartment for 50 min. On days 6 and 7, the same conditioning as on days 4 and 5 was repeated. Assignment of the conditioned compartment was performed randomly and counterbalanced across subjects. Naloxone (1.0 mg/kg s.c.), naltrindole (2.5 mg/kg s.c.), or norBNI (5.0 mg/kg s.c.) was injected 10 min before the injection of buprenorphine (1.0 mg/kg s.c.) or saline. During the test phase on day 8, the time spent in each compartment was measured for 900 s without drug injection. The CPP score was designated as the time spent in the drug-paired compartment on day 8 minus the time spent in the same compartment in the preconditioning phase on day 3. The scores were expressed as means ± the standard error of the mean (SEM).

Stable Expression of Human Opioid Receptors in Chinese Hamster Ovary (CHO) Cells

CHO cells were grown in F-12 medium supplemented with 10% fetal bovine serum in 5% CO_2 at 37°C. The human opioid receptor cDNAs were cloned from poly(A) + RNA obtained from human cerebrum donated by Dr R Takahashi (Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan) by using an RT-PCR-based method, subcloned into pcDNA3 (Invitrogen, Carlsbad, CA), and confirmed by sequencing using an ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Foster City, CA). CHO cells were transfected with these plasmids by using lipofectin (GibcoBRL, Gaithersburg, MD) and selected by being cultured in the presence of 500 μg/ml G418. Stable expression was confirmed by conducting binding experiments using the appropriate selective tritiated ligands.

Radioligand Binding Assay

Binding assays were performed as described (Katsumata *et al*, 1995) with slight modifications. Expressing cells were harvested after 65 h in culture, homogenized in 50 mM Tris buffer (pH 7.4) containing 10 mM $MgCl_2$ and 1 mM EDTA, pelleted by centrifugation for 20 min at 30 000 *g*, and resuspended in the same buffer. For saturation binding assays, cell membrane suspensions were incubated for 60 min at 25°C with various concentrations of [^{3}H]DAMGO for human μ-opioid receptor, [^{3}H]DPDPE for human δ-opioid receptor, or [^{3}H]U69593 for human κ-opioid receptor. Nonspecific binding was determined in the presence of 10 μM unlabeled ligands. For competitive binding assays, the cell membrane suspensions were incubated for 60 min at 25°C with 2 nM [^{3}H]DAMGO for human μ-opioid receptor, 2 nM [^{3}H]DADLE for human δ-opioid receptor, or 3 nM [^{3}H]U69593 for human κ-opioid receptor in the presence of various concentrations of ligands. After incubation for 60 min, membrane suspensions were rapidly filtrated, and the radioactivity on each filter was then measured by liquid scintillation counting. K_d values of the radiolabeled ligands were obtained by Scatchard analysis of the data from the saturation binding assay. K_i values were calculated 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 a 50% inhibition of the specific binding of radiolabeled ligand. The results of binding assays were presented as the mean ± SEM of 11–15 independent experiments.

cAMP Assay

cAMP assays were performed as described (Katsumata *et al*, 1995) 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 of HEPES-buffered saline containing 1 mM 3-isobutyl-1-methylxanthine for 10 min at 37°C. Next, they were stimulated for 10 min by the addition of 50 μl of HEPES-buffered saline containing 100 μM 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 of ice-cold 10% trichloroacetic acid to each well. Concentrations of adenosine 3′,5′-cyclic monophosphate (cAMP) were measured by radioimmunoassay as described (Amersham, Buckinghamshire, UK). cAMP accumulation was presented as a fraction of the control value obtained without addition of opiates. IC_{50} values were calculated as the concentration of ligand producing 50% of the maximal inhibition of cAMP accumulation. The values of IC_{50} and the maximal inhibitory effects (I_{max}) in cAMP assays were presented as the mean ± SEM of three to five independent experiments, each performed in triplicate.

Statistical Analyses

We combined the data of male and female mice because there were no statistically significant differences between male and female mice in the antinociceptive and rewarding effects of buprenorphine (paired *t*-test). The antinociceptive effects of buprenorphine and morphine were statistically evaluated by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls *post hoc* test. Comparisons between genotypes at each dose were analyzed by the Tukey–Kramer test. Time spent in the drug-paired compartment during pre- and postconditioning phases of CPP test were analyzed by within-group paired *t*-tests. Factors of 'genotypes' and 'treatments' were compared by the one-way ANOVA followed by the Fisher's PLSD *post hoc* test. Differences with $p < 0.05$ were considered significant.

RESULTS

Antinociceptive Effects

Buprenorphine antinociceptive dose–response relationships were analyzed in wild-type, heterozygous, and homozygous MOR-KO mice. Buprenorphine induced significant increases in the %MPE in both hot-plate (Figure 1a) and tail-flick (Figure 1b) tests in wild-type mice (ANOVA: $p < 0.0001$; $F = 8.38$; $df = 4, 75$, $p < 0.0001$; $F = 34.18$; $df = 4, 75$, respectively) and heterozygous MOR-KO mice (ANOVA: $p < 0.0001$; $F = 6.96$; $df = 4, 95$, $p < 0.0001$; $F = 16.83$; $df = 4, 95$, respectively). In contrast, buprenorphine failed to significantly change the %MPE in either hot-plate or tail-flick

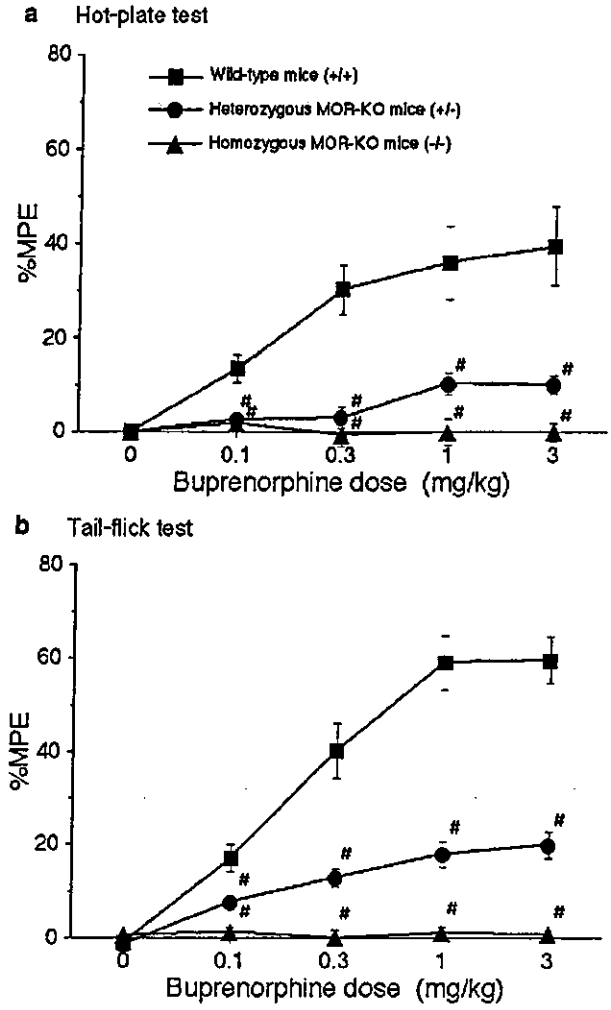

Figure 1 Antinociceptive effects of buprenorphine in wild-type, heterozygous, and homozygous MOR-KO mice. Buprenorphine-induced alterations of %MPE in the hot-plate (a) and tail-flick (b) tests in wild-type (+/+, square, $n=16$), heterozygous (+/−, circle, $n=20$), and homozygous (−/−, triangle, $n=15$) MOR-KO mice, under the cumulative dose–response paradigm. #A significant difference ($p<0.05$) from the corresponding values for wild-type mice. Data are presented as the mean ± SEM.

tests in homozygous MOR-KO mice at cumulative doses up to 3 mg/kg (Figure 1a and b). Antinociceptive effects of buprenorphine in wild-type mice were significantly ($p<0.05$) different from those of either heterozygous or homozygous MOR-KO mice in all doses in both hot-plate and tail-flick tests.

Morphine (10 mg/kg s.c.) caused a significant increase in the %MPE in both hot-plate (Figure 2a) and tail-flick (Figure 2b) tests in wild-type (ANOVA: $p<0.0001$; $F=74.79$; $df=1, 24$, $p<0.0001$; $F=7236.30$; $df=1, 24$, respectively) and heterozygous MOR-KO mice (ANOVA: $p<0.0005$; $F=19.25$; $df=1, 26$, $p<0.0001$; $F=31.18$; $df=1, 26$, respectively), whereas it had no significant effect on it in homozygous MOR-KO mice. In both hot-plate and tail-flick tests, the antinociceptive effects of morphine in wild-type mice were also significantly ($p<0.05$) different from those of heterozygous and homozygous mice at all doses.

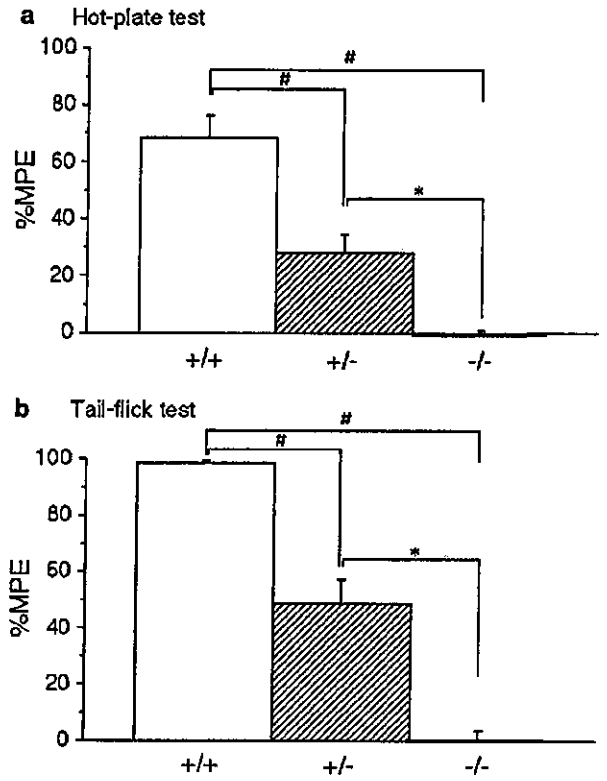

Figure 2 Antinociceptive effects of morphine in wild-type, heterozygous, and homozygous MOR-KO mice. Morphine (10 mg/kg s.c.)-induced alterations of %MPE in the hot-plate (a) and tail-flick (b) tests in wild-type (+/+, white column, $n=13$), heterozygous (+/−, hatched column, $n=14$), and homozygous (−/−, black column, $n=11$) MOR-KO mice. #A significant difference ($p<0.05$) from the values for wild-type mice. *A significant difference ($p<0.05$) from the values for homozygous MOR-KO mice. Data are presented as the mean ± SEM.

Rewarding Effects

Preferences for the places paired with 1 mg/kg buprenorphine s.c. were analyzed in wild-type, heterozygous, and homozygous MOR-KO mice. Buprenorphine induced significant increases in time spent on the previously drug-paired side in wild-type mice, as anticipated (CPP score = 154 ± 18, paired t-test, $p<0.0001$). This was also true for both heterozygous (CPP score = 96 ± 24, paired t-test, $p<0.005$) and homozygous (CPP score = 73 ± 18, paired t-test, $p<0.001$) MOR-KO mice (Figure 3). One-way ANOVA revealed significant differences between these genotype groups ($p<0.05$; $F=4.33$; $df=2, 53$). *Post hoc* comparison revealed that the buprenorphine-induced increase in CPP score for the wild-type mice was significantly higher than that for either heterozygous or homozygous MOR-KO mice ($p<0.05$). However, there was no significant difference in the place preference induced by buprenorphine between heterozygous and homozygous MOR-KO mice.

Next, we tested the influences of opioid antagonists. Mice were injected s.c. with 1.0 mg/kg of nonselective opioid antagonist naloxone, 2.5 mg/kg of δ-opioid receptor-selective antagonist naltrindole or 5.0 mg/kg of κ-opioid receptor-selective antagonist norBNI, and some of them

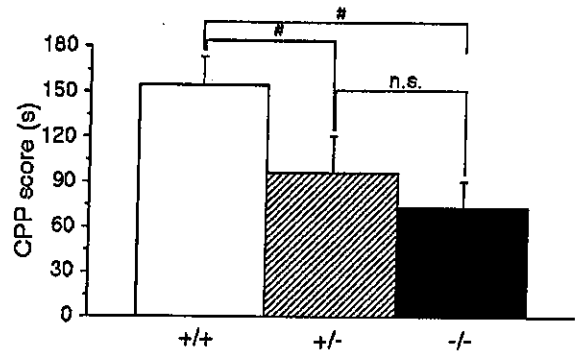

Figure 3 Rewarding effects of buprenorphine in wild-type, heterozygous, and homozygous MOR-KO mice. The CPP scores of wild-type (+/+, white column, $n=18$), heterozygous (+/−, hatched column, $n=18$) and homozygous (−/−, black column, $n=20$) MOR-KO mice. #A significant difference ($p<0.05$) from the values for wild-type mice. NS, not significant. Data are presented as the mean ± SEM.

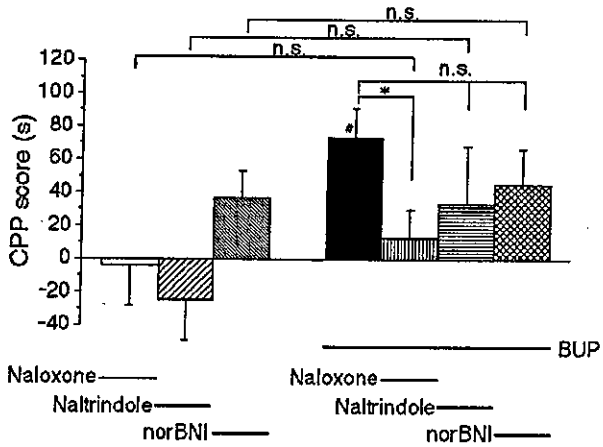

Figure 4 Inhibitory effects of naloxone, naltrindole, and norBNI on buprenorphine-induced rewarding effects in homozygous MOR-KO mice. Shown are the CPP scores of mice conditioned with naloxone ($n=18$), naltrindole ($n=8$), norBNI ($n=8$), or buprenorphine ($n=20$) alone and those of mice pretreated with naloxone ($n=18$), naltrindole ($n=10$), or norBNI ($n=10$) and conditioned with buprenorphine (BUP). #A significant difference ($p<0.05$) in the time spent in the drug-paired compartment between preconditioning and test phases in MOR-KO mice. *A significant difference ($p<0.05$) between the bracketed values. NS, not significant. Data are presented as the mean ± SEM.

were then administered buprenorphine in the CPP conditioning phase (Figure 4). When given alone, naloxone (1.0 mg/kg s.c.) did not alter place preference in homozygous MOR-KO mice (CPP score = −4 ± 29), as reported previously (Skoubis *et al*, 2001). Neither naltrindole nor norBNI significantly altered place preference when administered alone, although they produced trends toward conditioned place aversion (CPA; naltrindole CPP score = −25 ± 25) and place preference (norBNI CPP score = 36 ± 18).

Pretreatment with naloxone (1.0 mg/kg s.c.) 10 min before buprenorphine injections in the place preference conditioning phases did not change the increase in time spent on the buprenorphine-paired side in homozygous MOR-KO mice (CPP score = 13 ± 17). One-way ANOVAs demonstrated significant differences among homozygous MOR-KO mouse groups that were treated with naloxone alone, buprenorphine alone, and both buprenorphine and naloxone ($p<0.05$; $F=3.72$; $df=2, 53$). *Post hoc* comparison also revealed that naloxone pretreatment diminished buprenorphine-induced CPP in homozygous MOR-KO mice ($p<0.05$). In contrast, pretreatment with naltrindole (2.5 mg/kg s.c.) or norBNI (5.0 mg/kg s.c.) prior to buprenorphine injection did not significantly change the time spent in the buprenorphine-paired compartment after conditioning (the CPP score = 33 ± 35 and 45 ± 22, respectively). Thus, although pretreatment with naltrindole or norBNI each conferred tendencies toward lower buprenorphine place preference, one-way ANOVAs for the variously treated homozygous MOR-KO groups demonstrated no significant difference between treatment with naltrindole alone and that with it plus buprenorphine or between norBNI alone and that with it plus buprenorphine.

Binding Characteristics

In order to confirm the receptor specificity of buprenorphine, we established cell lines that stably expressed human μ-, δ-, and κ-opioid receptors (MOR/CHO, DOR/CHO, and KOR/CHO, respectively). Radiolabeled subtype-selective ligands, [^{3}H]DAMGO, [^{3}H]DPDPE, and [^{3}H]U69593, respectively, displayed saturable, high-affinity binding to membranes from these cells. K_d values of [^{3}H]DAMGO to the μ-opioid receptor, [^{3}H]DPDPE to the δ-opioid receptor, and [^{3}H]U69593 to the κ-opioid receptor were 1.7 ± 0.3 nM ($n=4$), 2.2 ± 0.2 nM ($n=4$), and 2.5 ± 0.2 nM ($n=3$), respectively. B_{max} estimates of receptor densities in these cell lines were 2300 ± 160, 3000 ± 270, and 5000 ± 450 fmol/mg protein, respectively.

Buprenorphine competition experiments using membranes prepared from MOR/CHO, DOR/CHO, and KOR/CHO cells revealed apparent binding affinities for each opioid receptor subtype (Figure 5a, Table 1). Buprenorphine bound to membranes prepared from μ-opioid receptor-expressing cells with affinity almost as high as those of morphine. In contrast, the affinities of buprenorphine for δ- and κ-opioid receptors were moderate and higher than those of morphine.

cAMP Assay

Buprenorphine effects on forskolin-stimulated cAMP accumulation in MOR/CHO, DOR/CHO, and KOR/CHO cells were also tested. Buprenorphine suppressed forskolin-stimulated cAMP accumulation in a concentration-dependent manner in all three types of cells (Figure 5b). I_{max} values for buprenorphine were lower than those of morphine for MOR/CHO and KOR/CHO cells and were slightly lower for DOR/CHO cells (Table 1). IC_{50} values of buprenorphine were apparently lower than those of morphine for all cell lines, especially for DOR/CHO cells.

DISCUSSION

Antinociceptive effects of buprenorphine were significantly reduced in heterozygous MOR-KO mice and virtually absent from homozygous MOR-KO mice in both hot-plate

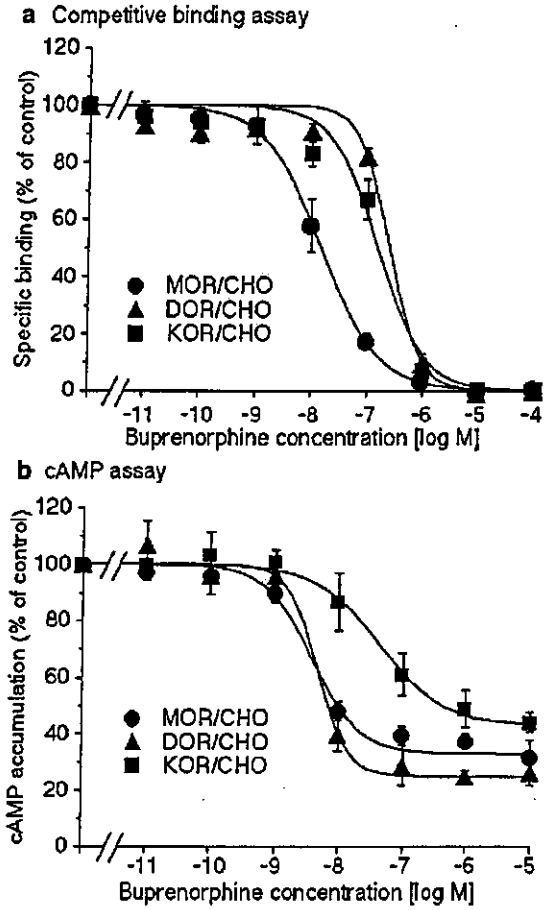

Figure 5 (a) Binding properties of buprenorphine for displacement of the specific binding of 2 nM [^{3}H]DAMGO, 2 nM [^{3}H]DADLE, and 3 nM [^{3}H]U69593 to the membranes of MOR/CHO (circle), DOR/CHO (triangle), and KOR/CHO (square) cells, respectively. The specific binding for MOR/CHO, DOR/CHO and KOR/CHO were 1800±310, 2800±340, and 4300±440 fmol/mg protein, respectively. Data are presented as the mean±SEM. $n=11$–15. (b) Agonistic effects of buprenorphine on forskolin-stimulated cAMP production in MOR/CHO (circle), DOR/CHO (triangle) cells, and KOR/CHO (square). Intracellular cAMP levels in the cells incubated with 10 μM forskolin alone served as the controls (100%). The control levels of cAMP in MOR/CHO, DOR/CHO, and KOR/CHO were 77±13, 78±6, and 75±7 pmol/well, respectively. Data are presented as the mean±SEM, $n=3$–5.

Table 1 Binding Properties and Agonistic Effects of Buprenorphine and Morphine on Human Opioid Receptor Subtypes

	MOR/CHO	DOR/CHO	KOR/CHO
Competitive binging assay			
K_i value (nM)			
Buprenorphine	12.4±3.5	154±27	108±27
Morphine	21.0±3.7	524±83	247±13
cAMP assay			
IC_{50} (nM)			
Buprenorphine	3.7±0.5	5.5±1.3	20.6±6.4
Morphine	25.0±9.0	610±220	340±160
I_{max} (%)			
Buprenorphine	66.0±4.7	75.7±2.8	57.3±4.1
Morphine	88.0±3.1	83.7±2.7	84.3±3.3

and tail-flick tests. These antinociceptive effects decreased in a μ-opioid receptor gene dose-dependent manner, even though buprenorphine activity at δ- and κ- as well as μ-opioid receptors was reconfirmed in the present study and was previously found in other *in vitro* experiments (Blake *et al*, 1997; Bot *et al*, 1998). Our data agree with those of Lutfy *et al* (2003), who also noted the absence of a thermal antinociceptive effect of buprenorphine in the tail-flick test conducted on a different strain of homozygous MOR-KO mice. Taken together, these results thus support a large role for μ-opioid receptors in both spinal and supraspinal thermal antinociceptive properties of buprenorphine. It thus seems likely that many of the nonselective opioids with moderate affinities for all subtypes of opioid receptors, such as bremazocine, pentazocine, and butorphanol, may also produce most of their analgesia through actions at the μ-opioid receptor.

Previous experiments and our present observations all suggest that the antinociceptive effects of morphine, a μ-opioid receptor agonist with low affinities for δ- and κ-opioid receptors, are reduced in each of several strains of heterozygous MOR-KO mice and completely diminished in homozygous MOR-KO mice (Loh *et al*, 1998; Sora *et al*, 1997b; 2001). We and others have identified reduced antinociceptive effects of DPDPE, a δ-opioid receptor-preferring ligand with modest affinity for the μ-opioid receptor, in MOR-KO mice (Matthes *et al*, 1998; Sora *et al*, 1997a). CXBK mice, which express μ-opioid receptors at approximately half of the level of C57BL/6 and BALB/c mouse strains, also showed reduced analgesic effects of morphine and the κ-selective agonist U50488H (Ikeda *et al*, 1999; 2001). In contrast, the antinociceptive effects of morphine were not altered in either mice lacking δ-opioid receptors (Zhu *et al*, 1999) or in those lacking κ-opioid receptors (Simonin *et al*, 1998). The present results thus add to the previous suggestions that the μ-opioid receptor is an especially key site for the analgesic effects of many opioid ligands. μ-Opioid receptor tolerance and inactivation and/or individual differences in μ-opioid receptor numbers are thus likely of importance in most of the analgesia induced by opiates.

In contrast to the abolition of buprenorphine antinociception in homozygous MOR-KO mice, significant rewarding effects were still existent. These results provide a sharp contrast to the virtually complete loss of rewarding effects of morphine in place preference assays using either these or other strains of homozygous MOR-KO mice (Matthes *et al*, 1996; Sora *et al*, 2001). Our current observations that the rewarding effects of buprenorphine in homozygous MOR-KO mice were abolished by pretreatment with naloxone, a nonselective opioid antagonist, suggest δ- and/or κ-opioid receptor involvement. Both δ- and κ-involvement in buprenorphine reward are supported by trends toward efficacies of pretreatment with naltrindole, a δ-opioid receptor

selective antagonist, and norBNI, a κ-opioid receptor selective antagonist, to reduce buprenorphine CPP in homozygous MOR-KO mice.

Previous reports documented that treatment with κ-opioid receptor-selective agonists induced CPA (Funada *et al*, 1993; Sante *et al*, 2000) and that δ-opioid receptor-selective agonists caused CPP (Longoni *et al*, 1998) in wild-type animals. A κ-opioid receptor antagonist was also reported to induce CPP in wild-type rats (Iwamoto, 1985), suggesting that dynorphin, an endogenous κ-opioid ligand, might constitutively produce aversive feelings and/or reduce rewarding feelings. Thus, μ- and δ-opioid receptors appear well poised to play positive roles, and the κ-opioid receptor, a negative role, in reward systems. Conceivably, buprenorphine could produce reward through the activation of μ- and δ-opioid receptors and inhibition of κ-opioid receptors. This κ antagonistic property of buprenorphine was also documented by the weak inhibition by buprenorphine in the CHO cells expressing κ-opioid receptors and by the complete displacement of the κ-selective ligand by buprenorphine.

The results of our *in vitro* experiments using cDNAs for human μ-, δ-, and κ-opioid receptors also suggest that buprenorphine induces rewarding effects via δ- and κ-opioid receptors in humans. Buprenorphine binds to human δ-opioid receptors with a moderate affinity, approximately 3.4-fold greater than that displayed by morphine. The ratio of buprenorphine binding affinities for μ- and δ-opioid receptors (K_i value for δ/K_i value for μ) was 12.4 in human clones and 15.8 (calculated from our unpublished results) in rodent clones. In the cAMP assays, buprenorphine showed lower IC_{50} value for δ-opioid receptors than morphine. Furthermore, buprenorphine showed the highest I_{max} value for δ-opioid receptors among the subtypes. These results suggest that not only μ- but also κ- and especially δ-opioid receptors may be involved in the rewarding effect of buprenorphine in humans as well as in rodents.

It was earlier reported that buprenorphine can serve as a reinforcer not only in laboratory animals (Mello *et al*, 1988; Winger and Woods, 2001) but also in humans (Comer *et al*, 2002), although buprenorphine has been widely used in clinical management for the detoxification in opioid abusers (Cheskin *et al*, 1994; Gibson *et al*, 2003; Lintzeris *et al*, 2002). Since the rewarding effects of buprenorphine are likely to be mediated by δ- and κ-opioid receptors in addition to μ-opioid receptors, buprenorphine might conceivably provide a prototype for clinical effectiveness through decreased μ-opioid receptor availability (Greenwald *et al*, 2003; Zubieta *et al*, 2000). Such μ-opioid receptor-selective partial agonists might even provide good adjuncts during detoxification.

In conclusion, we demonstrated abolition of antinociceptive effects of buprenorphine but retention of at least much of the rewarding effect in MOR-KO mice. Abolition of buprenorphine reward by pretreatment with naloxone and the *in vitro* data showing that buprenorphine acted significantly on δ- as well as μ- and κ-opioid receptors each support the idea that the antinociceptive effects of buprenorphine are completely dependent on μ-opioid receptor, but that its rewarding effects are mediated by its properties of being a δ- as well as μ-opioid receptor agonist and a κ-opioid receptor antagonist.

ACKNOWLEDGEMENTS

This study was supported by the Japanese Ministry of Health, Labour and Welfare; the Japanese Ministry of Education, Culture, Sports, Science, and Technology and the NIDA-IRP, NIH, DHSS. We thank Wenhua Han, Yukio Takamatsu, and Keiko Matsuoka for discussion, technical support, and animal care.

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Psychiatry Research 133 (2005) 123–128

PSYCHIATRY RESEARCH

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Cholecystokinin (CCK) and the CCKA receptor gene polymorphism, and smoking behavior

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Received 3 July 2002; received in revised form 15 May 2003; accepted 17 June 2003

Abstract

We analyzed genetic variants of the promoter region of the cholecystokinin (CCK; which modulates the release of dopamine) gene, and intron 1 and exon 5 of the CCKA receptor gene, and performed association analyses of nicotine dependence using an allele-specific amplification (ASA) method and PCR-RFLP methods. There was a significant difference between the current smoking and nonsmoking groups in the allele frequency of the CCK-45C/T polymorphism. However, there was no significant difference in the CCKA *Pst*I polymorphism, and the *Hin*cII polymorphism was not detected in our study. Our data suggest that polymorphisms of the CCK gene may be one of the risk factors for smoking behavior.

Keywords: CCK gene; CCKA receptor gene; Polymorphism; Smoking behavior

1. Introduction

The major tobacco alkaloid nicotine has important physiological and pharmacological effects on the human body (Russell and Feyerabend, 1978). Nicotine is the compound in tobacco primarily responsible for the development and maintenance of tobacco dependence (Henningfield et al., 1985). The pharmacological and behavioural bases for nicotine dependence are similar to those for cocaine and heroin dependence (Palca, 1988). Tobacco has a special status as a 'gateway' substance in the development of other drug dependencies, not only because tobacco use reliably predicts the use of other psychoactive substances such as alcohol and opioids, but also

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doi:10.1016/j.psychres.2003.06.004

because humans who use tobacco are more likely to develop dependent patterns of use than those who use most other addictive drugs (Niu et al., 2000).

Cholecystokinin (CCK) is a neuropeptide that is widely distributed in the body, including in the central nervous system, where it enhances dopaminergic function (Crawley and Corwin, 1994). CCK peptides exist in various forms (sulfated CCK8 and unsulfated CCK8 and CCK4) after posttranscriptional processing of the precursor peptide. An octapeptide to be sulfated at the tyrosine in the seventh position from the COOH terminus shows full biological activity (Crawley and Corwin, 1994). It is present in dopaminergic neurons of the mesolimbic pathway and is thought to modulate the release of dopamine and dopamine-related behavior via CCK receptors (Crawley, 1991; Moran and Schwartz, 1994). The dopaminergic system has been implicated in the maladaptive behavior associated with drug (such as alcohol and cocaine) abuse—behavior that is driven in part by dopamine-mediated reinforcement in the mesocorticolimbic reward pathway (Koob et al., 1988). A CCK antagonist significantly decreased the symptoms of nicotine withdrawal in animals (Rasmusssen et al., 1996). Individual differences in propensity to nicotine dependence are mediated in part by genetic factors (Carmelli et al., 1992). Previous studies have linked smoking behavior with genes involved in the regulation of the neurotransmitter dopamine, dopamine receptors, and serotonin transporter (Lerman et al., 2000). Sullivan and Kendler (1999) suggested that genetic factors may be important in nicotine dependence.

As for the CCK gene, a −45C/T polymorphism of the promoter region of the CCK gene has been investigated with regard to panic disorders (Wang et al., 1998), schizophrenia (Bowen et al., 1998), and alcoholism (Okubo and Harada, 2001). It has also been reported that the CCKAR gene may be associated with schizophrenia (Zhang et al., 2000; Tachikawa et al., 2000).

Two types of CCK receptors (type A and type B) have been identified on the basis of their affinities for a structurally and functionally related family of peptides with identical COOH-terminal pentapeptide sequences (Wank, 1995). These receptors were cloned, and the CCKB receptor (CCKBR) gene was found to be identical to the gastrin receptor gene (Wank et al., 1992a, 1992b).

On the other hand, the CCKA receptor (CCKAR) has been demonstrated to be present in the vagus nerve and localized areas of the central nervous system, including the nucleus tractus solitarius area postrema, interperduncular nucleus, posterior hypothalamus, and nucleus accumbens (Moran et al., 1986; Hill et al., 1990). Pharmacological studies have shown that CCK promotes dopamine release in the posterior nucleus accumbens via CCKAR, but inhibits dopamine release in the anterior nucleus accumbens via CCKBR (Marshall et al., 1991). The CCKAR gene has been mapped to 4p15.2–15.1 with the dopamine D5 receptor (DRD5) gene (de Weerth et al., 1993). Chromosome 4p was recently implicated by linkage analysis in bipolar disorders (Blackwood et al., 1996) and schizophrenia (Asherson et al., 1998).

Since a reduced CCK level in the limbic lobe and reduced high-affinity CCK binding in the hippocampus and frontal cortex have been observed in schizophrenia, the genes coding for CCK receptors can be considered as candidate genes for schizophrenia (Ferrier et al., 1985; Farmery et al., 1985). Wei and Hemmings (1999) reported that a *Pst*I polymorphism at the boundary between intron 1 and exon 2 of the CCKA receptor gene was significantly associated with auditory hallucination in schizophrenia. Although Hamann et al. (1999) reported a missense variant (*Hin*cII polymorphism) of exon 5 of the CCKA receptor gene, the frequency and relation to psychosis of this variant in Japanese have not been clarified (Hamann et al., 1999).

Based on this research, we focused on the genetic variations in CCK and the CCKA receptor gene in smoking behavior.

2. Methods

Current smokers (defined by a score above six on the Fagerstrom Test for Nicotine Dependence, FTND) in this study consisted of 36 males and 43 females who reported smoking daily for more than 5 years (Heatherton et al., 1991). The control group (nonsmokers; nicotine free for more than 5 years) consisted of 162 unrelated healthy volunteers (65 males and 97 females). The study subjects were all medical staff members with no family histories of

Table 1
Oligonucleotide primers for amplification of the CCK and CCKAR genes, and restriction fragment size

Gene	Polymorphism	Primer sequence	Restriction enzyme	Restriction fragment size
CCK	−45C/T	Common primer: 5′-GAT TAA CTC CAC CCC ACT ACA C C allele detection primer: 5′-CTG TCT CTT AAA TAC CCC CCG T allele detection primer: 5′-CTG TCT CTT AAA TAC CCC CCA	–	–
CCKAR	779T/C	Forward primer: 5′-CTG TTC ACT TGA GGA GCT TTG Reverse primer: 5′-CTG TTC ACT TGA GGA GCT TTG	*Pst*I	Allele T: 480 bp and 264 bp Allele C: 744 bp
CCKAR	365Val/Ile	Forward primer: 5′-TGC GTC AAC CCC ATC ATC Reverse primer: 5′-GAT GAT GGG GTT GAC GCA	*Hin*cII	Allele G: 236 bp and 90 bp Allele A: 326 bp

substance abuse except nicotine. Written informed consent was obtained from the tobacco smokers and healthy volunteers. Genomic DNA was obtained from peripheral blood using phenol–chloroform methods, and the DNA samples were dissolved in distilled TE buffer and stored at 4 °C.

The sequence containing the *Pst*I polymorphic site (779T/C) at the boundary between intron 1 and exon 2, and the *Hin*cII polymorphic site (365Val/Ile) in exon 5 of the CCKA receptor gene was amplified by means of polymerase chain reaction (PCR). Primers for the *Pst*I site were prepared according to methods described in a previous report (Zhang et al., 2000), and the *Hin*cII site was prepared according to accession no. U23430. The PCR was performed with 100 ng of genomic DNA, 0.2 mM dNTPs, 0.2 μM of each primer, 2.0 mM $MgCl_2$, and 1 U of Taq polymerase (Promega), in a final volume of 50 μl. PCR for the *Pst*I polymorphic site was performed for 30 cycles under the following conditions: first denaturation at 95 °C for 5 min, denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min. PCR for the *Hin*cII polymorphic site was performed for 30 cycles under the following conditions: first denaturation at 94 °C for 2 min, denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. The PCR product was digested with *Pst*I or *Hin*cII, respectively, and the digested products were subjected to electrophoresis on a 1.5% agarose gel and then stained with ethidium bromide. Each genotype was identified, followed by restriction fragment length polymorphism (RFLP) analysis (see Table 1).

The −45C/T polymorphism in the promoter region of the CCK gene was examined by the allele-specific amplification method. The PCR was performed with 100 ng of genomic DNA, 0.2 mM dNTPs, 0.2 μM of each primer, 2.0 mM $MgCl_2$, and 1 U of Taq DNA polymerase (Roche Diagnostics), in a final volume of 25 μl. PCR was performed for 30 cycles under the following conditions: first denaturation at 94 °C for 3 min, denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, and extension at 72 °C for 30 s. The PCR product was electrophoresed on a 2.0% agarose gel and then stained with ethidium bromide.

Hardy–Weinberg disequilibrium was assessed by chi-square test. Statistical differences in allelic frequency and genotype distribution between patients and controls were also assessed using chi-square test or Yate's correction.

3. Results

Tables 2 and 3 present the frequencies of the CCK and CCKA receptor genotypes. The allele frequency of the −45C/T polymorphism in the promoter region of the CCK gene showed a statistically significant difference (χ^2=7.67, *df*=1, *P*<0.005) between the

Table 2
Genotype and allele frequencies of the CCK gene −45C/T polymorphism in controls and tobacco smokers

	Genotype (%)			Alleles (%)	
	CC	CT	TT	C	T
Controls	85	74	3	244	80
(*n*=162)	(52.4)	(45.7)	(1.9)	(75)	(25)
Tobacco smokers	37	25	17	99	59
(*n*=79)	(46.8)	(31.7)	(21.5)	(62.6)	(37.4)

There was a statistically significant difference in allele frequency between the two groups (χ^2=7.76, *df*=1, *P*=0.005).

Table 3
Genotype and allele frequencies of the CCKA receptor gene 779T/C polymorphism in controls and tobacco smokers

	Genotype (%)			Alleles (%)	
	TT	TC	CC	T	C
Controls	50	75	37	175	149
(n=162)	(30.9)	(46.3)	(22.8)	(54)	(46)
Tobacco smokers	22	41	16	85	73
(n=79)	(27.8)	(51.9)	(20.3)	(53)	(47)

There was no statistically significant difference in allele frequency between the two groups.

current tobacco smokers and the nonsmokers. For the CCKA receptor, the frequency of the *Pst*I polymorphism did not significantly differ between the tobacco smokers and nonsmokers. In addition, the *Hin*cII polymorphism was not detected in this population; all subjects with this polymorphism were 365Val/Val homozygotes.

4. Discussion

In this study, we investigated the relationship between the CCK and CCKA receptor gene polymorphisms and the risk of smoking behavior. In experimental animals, nicotine causes the release of dopamine and stimulates energy metabolism in the basal ganglia, especially in the ventral tegmental area and nucleus accumbens, as do other addictive drugs such as cocaine, amphetamines, and morphine (Pontieri et al., 1996). Smoking is a familial trait (Conrad et al., 1992), and one adoption and numerous twin studies (Sullivan and Kendler, 1999) have demonstrated that genetic factors account for most of the observed familiality. Genetic factors may be particularly important for nicotine dependence, accounting for approximately 67% of the variance in liability (Sullivan and Kendler, 1999).

We have investigated three polymorphisms in the CCK gene within the promoter region, and in the CCKA receptor gene within intron 1 and exon 5. The frequency of the −45T allele in the CCK gene was significantly higher in the smoking group. However, there was no difference in the frequency of the CCKA receptor gene *Pst*I polymorphism. Our results indicated that the −45C/T polymorphism in the promoter region of the CCK gene contributes to the risk of smoking behavior. There was no association between the 799T/C (*Pst*I site) polymorphism in the CCKA receptor gene and smoking behavior. In addition, since a missense mutation 365Val/Ile, in exon 5, was not detected in this population, this variant either does not exist or is very rare in the Japanese population.

Cerebral CCK not only shows widespread expression in virtually all CNS regions, but also high peptide concentrations, rendering CCK the most abundant neuropeptide system in the human brain and in the brains of several mammals (Rehfeld, 1978). Two CCK receptor subtypes have been cloned, of which the CCKB receptor is the most abundant in the brain, including the cerebral cortex and basal ganglia (Kinze et al., 1996). The CNS distribution of the CCKA receptor is more limited but includes the mesostriatal dopaminergic pathways and the posterior part of the nucleus accumbens, both of which play important roles in reinforcement (Hill et al., 1990). One of the functions of CCK in the CNS is as a neuromodulator regulating dopamine turnover. Activation of CCKA receptors in the caudal nucleus accumbens stimulates dopamine release, whereas stimulation of CCKB receptors in the rostral part of the nucleus accumbens has the opposite effect, inhibiting dopamine release (Vaccarcino, 1994). The dopamine transporter (DAT) gene has a 3′ variable number tandem repeat (VNTR) polymorphism (Vandenbergh et al., 1992). The nine-repeat allele has been associated with cocaine-induced paranoia, a state attributed to diminished dopamine reuptake and greater availability of synaptic dopamine (Gelernter et al., 1994).

Lerman et al. (1999) and Sabol et al. (1999) reported an association of smoking with a polymorphism of the DAT gene. They found that individuals with the nine-repeat allele are less likely to be smokers than are individuals without the nine-repeat allele, and that the smokers with this allele started to smoke later and were able to quit for longer periods than were other smokers. In the opinion of Lerman et al. (1999), the nine-repeat allele might be associated with an increased synaptic dopamine level, and individuals with the nine-repeat genotype and higher levels of endogenous synaptic dopamine should therefore have less need to use exogenous substances such as nicotine to stimulate dopamine transmission.

In conclusion, this study suggested that there is a difference in the allele frequency of the −45C/T polymorphism of the CCK gene between Japanese current smokers and nonsmokers. This finding is a replication of the findings in a Caucasian population reported by Comings et al. (2001). We suggest that the −45C/T polymorphism in the promoter region of the CCK gene contributes to the risk of smoking behavior. CCK modulates the release of dopamine and dopamine-related behavior in the mesolimbic pathway of CCK dopamine coexistence (Marshall et al., 1991). Our results also support Lerman et al.'s (1999) report. Further studies in larger populations that pay attention to population stratification and the ancestry of the subjects are necessary to confirm this finding and to investigate the relationship between other novel SNPs in other genes and smoking behavior.

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Changes in Expression of the Mouse Homologues of KIAA Genes after Subchronic Methamphetamine Treatment

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ABSTRACT: **Amphetamine abuse may be associated with adaptive changes in gene expression in the brain. In the present study, a newly developed cDNA array system comprising mouse KIAA (mKIAA) cDNA clones was used to examine the gene expression affected by chronic methamphetamine treatment. Approximately 800 mKIAA clones were blotted onto a nylon membrane and hybridized with ^{33}P-labeled cDNA derived from mRNAs isolated from the whole brains of mice that had been treated daily with saline or methamphetamine (2 mg/kg, i.p.) for 2 weeks. The arrays displayed robust hybridization for almost all transcripts. The results obtained from five experiments were averaged, each performed with triplicate samples. Several clones were chosen as positive candidates for methamphetamine-induced changes; however, only Per2 and mKIAA0099 genes showed a significantly increased expression ($P < .05$). Subsequently, with the focus on the period-related proteins, the expression of these proteins in various parts of the rat brain were assessed by immunoblot analysis. Chronic administration of methamphetamine (8 mg/kg, i.p., for 10 days) caused increased Per2 protein expression in the hippocampus. Interestingly, chronic methamphetamine treatment at a lower dose (4 mg/kg, i.p., for 10 days) induced an increase in SCN circadian oscillatory protein (SCOP) expression, also in the hippocampus. These data suggest that long-lasting alterations of the period-related gene expressions in the hippocampus might play an important role in methamphetamine addiction.**

KEYWORDS: **cDNA microarray; Per2; methamphetamine; hippocampus; SCOP**

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Ann. N.Y. Acad. Sci. 1025: 92–101 (2004).
doi: 10.1196/annals.1316.012

INTRODUCTION

Amphetamines are known to alter the reuptake and release of monoamines by binding to plasmalemmal and vesicular monoamine transporters,[1] and their use results in substantial increases in the synaptic levels of dopamine, noradrenaline, and serotonin.[2] This action is the generally accepted mechanism underlying the euphorigenic effects of amphetamines. These pleasurable effects decline over a period of several hours, and then negative affective states emerge, which would be a driving force for further administration. In general, addiction to psychostimulants is a long-lasting state; however, in that state, changes in the levels of these neurotransmitters, receptors, transporters, and other proteins seem to be complex. Therefore, the identification of genes with long-lasting changes in expression would be a key for investigating the persistence of addiction. Recently, various studies that use cDNA microarrays to assess the gene-expression profile of animals treated with amphetamines have been reported. Some of these studies examined the neurotoxicity of amphetamines,[3–6] and a few of them focused on neuroadaptation elicited by chronic administration of amphetamines.[7–10] However, the results obtained were insufficient to determine the responsive gene clusters. Thus, identification of genes vulnerable to chronic low-dose administration of methamphetamines is an important task that still needs to be completed. Therefore, in the present study we examined the gene expression-altering effects of repeated administration of methamphetamine at a low dose capable of producing conditioned place preference in mice.

For the screening of novel genes whose expression levels have changed in response to drug abuse, we used a newly developed cDNA array system comprising mKIAA cDNA clones. KIAA clones were originally obtained from a human cDNA sequencing project (in progress) to accumulate protein-coding sequence (CDS) information about unidentifed human genes.[11] More than 2000 human long cDNAs already have been newly identifed and characterized by this project to date, and they are systematically designated as "KIAA" plus a 4-digit number. Mainly, KIAA clones are characterized by their long cDNA (>4 kb) sequences encoding large proteins (>50 kDa) and by the source of isolation, that is, the brain. Therefore, this large population of KIAA clones reflects an abundance of large proteins expressed in the brain. A project to isolate and determine all the sequences of mouse cDNA clones-encoding polypeptides corresponding to human KIAA proteins has been in progress since the end of 2001. The use of mKIAA genes as research materials is advantageous for exploring the physiological functions of these genes. The structural features of mKIAA cDNA clones are being released through an Internet-based database (http://www.kazusa.or.jp/rouge/) for sharing information about these genes with the worldwide research community.

Although the DNA microarray will probably become the future standard screening method because of its convenience, it is necessary to overcome the high false-positive rate associated with cDNA array experiments. For example, Delongchamp *et al.*[3] reported that 32% are expected to be false positives. The study presented here used a customized cDNA microarray to find candidate genes whose expression was affected by chronic methamphetamine treatment. Two beneficial aspects of this customized microarray are as follow: first, mouse homologues of the KIAA cDNA are plotted on membranes. To obtain reliable data, we conducted multiple biological repeats and made multiple membrane replicates, according to the "Standardization of

Protocols in cDNA Microarray Analysis" by Benes and Muckenthaler.[12] Second, to verify the results, selected gene products were analyzed by semiquantitative Western blot analysis using specific antibodies.

MATERIALS AND METHODS

Treatment Schedules: Subchronic Methamphetamine Treatment

For all experiments, ethical approval was obtained prior to the experiments, according to the requirements of the Institutional Animal Care and Use Committee of the Tokyo Institute of Psychiatry (Japan). All animals were housed five to a cage (mouse) or three to a cage (rat) with free access to food and water in an animal room maintained on a 12-h light/dark cycle (lights on at 0800). Nine-week-old male C57BL/6J mice receiveda saline or methamphetamine injection (2 mg/kg, i.p.) between 1300 and 1500 once a day for 14 days, and were killed 24 h after the last injection. In separate experiments, 8-week-old male Wistar rats received a saline or methamphetamine injection (4 mg/kg, i.p., or 8 mg/kg, i.p.) once a day for 10 days, and were killed 24 h after the last injection, between 1300 and 1500.

Expression of Fusion Proteins and Antibody Production

A specific antibody against Per2 was commercially purchased from BD Biosciences (Franklin Lakes, Nevada). Anti-mouse SCOP antiserum was used against glutathione S-transferase (GST)-SCOP fusion proteins containing the amino acids 1523–1640 of the corresponding rat SCOP protein. The GST fusion protein was produced in *Escherichia coli* Rosetta (pLysS) cells (Novagen, Madison, Wisconsin) transfected with pGEX-4TDES.[13] The GST fusion protein was mixed with Freund's complete adjuvant and injected into rabbits. The IgG fraction of the resulting antiserum against GST-SCOP protein was purified by using a HiTrap Protein A Sepharose column (Amersham, Piscataway, New Jersey).

Tissue Preparation, RNA Isolation, Probe Labeling, and Microarray Hybridization

After decapitation, the whole brains from five mice per treatment group were removed and immediately frozen at −80°C until RNA isolation could be performed. Rat brains were also removed, and various parts of the brain were dissected on ice, and then immediately frozen at −80°C prior to SDS-PAGE.

All the RNA was isolated from each whole brain of the mice ($n = 5$ per group) by using an RNeasy kit (Qiagen Inc., Valencia, California), following the manufacturer's protocol. Further, poly A^+ mRNA-enriched RNA was isolated from total RNA by using a Dinabeads mRNA Purification Kit (Dynal Biotech, Lake Success, New York). The quantity and quality of the RNA were measured by spectrophotometry and electrophoresis on a denaturing agarose gel. Radiolabeled cDNA probes were prepared from 1.0 μg of heat-denatured (70°C, 10 min) poly A^+ RNA or 0.1 μg Tet1-PolyA$^+$ RNA in the presence of random nonamer, dATP, dTTP, dGTP, [^{33}P]dCTP, Superscript II reverse transcriptase, and RNase inhibitor by incubation of the mixture at 43°C for 1 h. RNA in the reaction mixture was then decomposed by incuba-