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Dopamine D_1 Receptors Participate in Cocaine-Induced Place Preference via Regulation of Ryanodine Receptor Expression

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Abstract. Ryanodine receptors (RyRs) with three different isoforms in the brain play a role to facilitate Ca^{2+} release from the intracellular Ca^{2+} pool. Although cocaine is a strongly addictive psychostimulant that dramatically affects the central nervous system function, the role of RyRs and regulation of their expression by cocaine-induced place preference have not yet been defined well. The present study investigated the regulation of RyR expression in mice under intermittent cocaine treatment using the place preference procedure. The cocaine-induced place preference was inhibited by intracerebroventricular pretreatment with dantrolene, a RyRs antagonist, in a dose-dependent manner. The levels of RyR-1 and -2 in the limbic forebrain and frontal cortex significantly increased in the cocaine-conditioned mice, whereas that of RyR-3 in these two brain regions showed no changes. Although the up-regulation of RyRs was not affected by blockade of L-type voltage-gated calcium channels, the increase of RyR-1 and -2 in the limbic forebrain and frontal cortex was completely abolished by SCH23390, a selective antagonist of dopamine D_1 receptors, but not by sulpiride, a selective antagonist of dopamine D_2 receptors. These findings indicate that RyRs play a critical role in the development of cocaine-induced place preference and that the up-regulation of RyRs in the brain of a mouse showing cocaine-induced place preference is regulated by dopamine D_1 receptors.

Keywords: cocaine, ryanodine receptor, dantrolene, dopamine D_1 receptor, conditioned place preference

Introduction

Cocaine is a strong addictive psychostimulant that dramatically alters the central nervous system (CNS) function. A growing body of evidence suggests that the mesolimbic dopamine system, which originates in the ventral tegmental area and mainly projects to the nucleus accumbens and medial prefrontal cortex, plays an essential role in the development of dependence on psychostimulants (1, 2).

In addition to the pivotal role of Ca^{2+} signaling via NMDA receptors, Ca^{2+} influx via L-type voltage-gated Ca^{2+} channels (VGCCs) is important for psychostimulant-induced behavioral and neurochemical changes (3 – 5). VGCCs are classified into distinct subtypes (L, N, P/Q, R, and T) based on their pharmacological and biophysical properties (6). L-type VGCCs (α_{1c} and α_{1d} subunits) are heterometric complexes and show electrophysiological and pharmacological diversities (7). α_{1c} and α_{1d} subunits are dominant calcium channel–forming subunits of L-type VGCCs and expressed in many types of neurons (8). We previously demonstrated that blockers of L-type VGCCs, which bind to α_1 subunits of VGCCs to suppress Ca^{2+} entry into cells, inhibit the development of psychological dependence on drugs of abuse, suggesting that up-regulation of L-type VGCCs (especially α_{1c} and α_{1d} subunits) occurs during the development of psychological dependence (9).

In the CNS, changes in intracellular Ca^{2+} concentration play an regulatory role in various CNS functions such as learning and memory (10). Channels for intracellular Ca^{2+} release can be divided into two major categories: inositol-1,4,5-trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs), both of which are present on the

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endoplasmic reticulum, and release Ca^{2+} into intracellular spaces from intracellular Ca^{2+} pools in response to a variety of stimuli. IP_3Rs and RyRs are activated by Ca^{2+} in the process of Ca^{2+}-induced Ca^{2+} release (CICR), as well as by a change in membrane voltage (11, 12). Three different RyR isoforms have been identified in mammalian tissues (13). RyR-1 is expressed preferentially in skeletal muscles, while RyR-2 is expressed mainly in the heart (14), although all three isoforms are present in the brain (15 – 17). In addition, functional interaction between L-type VGCCs and RyRs in the CNS has also been suggested (18, 19). However, little is known about the role of RyRs in psychological dependence on cocaine.

In the present study, we examined whether RyRs were involved in the development of the cocaine-induced place preference and how expression of RyRs was regulated under such conditions.

Materials and Methods

Animals

Male ddY mice (8-week-old; Japan SLC, Inc., Hamamatsu), which were housed in a room maintained at 22 ± 1°C and 55 ± 0.5% humidity, with a 12-h light/dark cycle (light on 8:00 A.M. to 8:00 P.M.) for 1 week prior to the experiments, were used. Food and water were available ad libitum.

All experiments presented in this study were approved by the Animal Research Committee of Kawasaki Medical School and conducted according to the "Guide for Care and Use of Laboratory Animals" of Kawasaki Medical School that is based on the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996.

Intracerebroventricular injection

Intracerebroventricular (i.c.v.) administration was carried out as described previously (20 – 22). Nifedipine, dantrolene, SCH23390, and sulpiride were injected into the lateral cerebral ventricle of mice. One day before the beginning of drug or vehicle injection, mice were anesthetized with diethyl ether and a 2-mm double-needle (tip: 27G × 2 mm and base: 22G × 10 mm; Natsume Seisakusyo Co., Ltd., Tokyo) attached to a 25-μl Hamilton microsyringe was inserted into the unilateral injection site; as a result, a hole was made in the skull for the injection. A hole on the mouse head was made using the 2-mm double-needle and no implantation of cannulas or needles was done in this study. The unilateral injection site was approximately 2 mm from either side of the midline between the anterior roots of the ears. During the experiment, no leakage of body fluid and blood from the hole was observed, which may be due to the small size of the hole.

On i.c.v. injection of drugs, the mouse head was fixed with a V-shaped holder and the drugs were injected through the hole. Therefore, the measurement of conditioned place preference (CPP) was not affected by the drug injection method used here. The volume of drug solution injected was 4 μl/mouse (9, 23).

The confirmation of the injection site through the hole in the brain was carried out by ink injection after all experiments.

Place conditioning

The place-conditioning procedure was carried out according to the method reported previously (24 – 26). Place-conditioning studies were conducted using an apparatus consisting of a shuttle box (15.5 cm × 35 cm × 17 cm, w × l × h), which was made of acrylic resin board and divided into two equal-sized compartments. One compartment was white with a textured floor and the other was black with a smooth floor to create equally inviting compartments. The schedule for place-conditioning preference consisted of three phases (preconditioning test, conditioning test, and post-conditioning test).

Preconditioning test (Day 1): Before the preconditioning test, the mice were placed on the border of both compartments and the time mice spent was measured. The time mice spent in each compartment during a 900-s session was then recorded automatically using an infrared beam sensor (BS-CPP-MS; BrainScienceIdea, Co., Ltd., Osaka). The compartment in which mice spent a longer time was defined as the preferred compartment and the other compartment was defined as the non-preferred one. Mice that spent more than 600 s in one compartment were excluded from the experiment. The remaining mice were divided into two groups according to place preference, that is, one group consisted of the mice preferring the black compartment and the other consisted of those preferring the white compartment. The baseline preference in each test group for the black and white compartments was approximately 50%. With such assignment, some mice would be conditioned in their preferred compartment and others would be conditioned in their non-preferred compartment.

Conditioning test (Days 2 – 7): After the preconditioning test, conditioning sessions were carried out. During this test, mice administered cocaine were conditioned in the non-preferred compartment (drug-paired compartment) and those administered saline were placed in the preferred compartment (saline-paired compartment). Mice were subjected to three cocaine-paired days (Days 2, 4, and 6 or 3, 5, and 7) and three saline-paired days (Days 3, 5, and 7 or 2, 4, and 6), respectively. That is, on

Day 2, the mice were placed in the cocaine-paired compartment for 1 h immediately after s.c. injection of cocaine (10 mg/kg) or placed in the saline-paired compartment immediately after s.c. injection of saline, and then on an alternative day (Day 3), the mice were conditioned in the saline-paired compartment with saline injection or in the cocaine-paired compartment with cocaine treatment, respectively. Such a cycle of conditioning was carried out for six days. Vehicle, dantrolene (a non-selective inhibitor for RyRs; 1, 3, 10 nmol/mouse), nifedipine (L-type VGCCs antagonist, 30 nmol/mouse), SCH23390 (a dopamine D_1 receptor antagonist; 3, 10, 30 nmol/mouse), or sulpiride (a dopamine D_2 receptor antagonist; 3, 10, 30 nmol/mouse) was daily i.c.v. administered 30 min before s.c. treatment with cocaine (10 mg/kg) or saline.

Vehicle administration did not produce any changes in the cocaine-induced place preference.

Post-conditioning test (Day 8): On the 8th day after the final conditioning session (Day 7), the post-conditioning test identical to the preconditioning test was performed for 900 s. The preference for the drug-paired place was expressed as the mean difference between the duration spent in it during the pre- and post-conditioning tests.

In the experiments carried out, the number of mice in each group; for dantrolene pretreatment of the experiments, vehicle + saline group (n = 10), dantrolene (10 nmol/mouse) + saline group (n = 10), vehicle + cocaine group (n = 10), dantrolene (1 nmol/mouse) + cocaine group (n = 10), dantrolene (3 nmol/mouse) + cocaine group (n = 10), and dantrolene (10 nmol/mouse) + cocaine group (n = 10) were used for this study. For nifedipine pretreatment of the experiments, vehicle + saline group (n = 10), nifedipin (30 nmol/mouse) + saline group (n = 10), vehicle + cocaine group (n = 10), nifedipin (30 nmol/mouse) + cocaine group (n = 10) were used for this study. For SCH23390 pretreatment of the experiments, vehicle + saline group (n = 10), SCH23390 (30 nmol/mouse) + saline group (n = 10), vehicle + cocaine group (n = 10), SCH23390 (3 nmol/mouse) + cocaine group (n = 10), SCH23390 (10 nmol/mouse) + cocaine group (n = 10), and SCH23390 (30 nmol/mouse) + cocaine group (n = 10) were used for this study. For sulpiride pretreatment of the experiments, vehicle + saline group (n = 10), sulpiride (30 nmol/mouse) + saline group (n = 10), vehicle + cocaine group (n = 10), sulpiride (3 nmol/mouse) + cocaine group (n = 10), sulpiride (10 nmol/mouse) + cocaine group (n = 10), and sulpiride (30 nmol/mouse) + cocaine group (n = 10) were used for this study.

Tissue dissection and preparation for western blot

Immediately after the post-conditioning test, the animal was decapitated, and the limbic forebrain (including the nucleus accumbens and olfactory tubercle), frontal cortex, and cerebellum were dissected on an ice-cold glass plate. Briefly, the brain was placed to expose the dorsal surface and a vertical cut was made through the anterior commissure. The resulting frontal part was turned to expose the ventral surface. A vertical cut was passed through the rhinal fissure and the small part including the accessory olfactory bulb and olfactory nucleus was removed. The remaining block of the brain tissue was turned to expose the section and the area bordered by the caudate putaman and nucleus accumbens was vertically cut. The block of the brain tissue including the nucleus accumbens and olfactory tubercle was taken as the main part of the limbic forebrain. The dissected brain tissues were homogenized with ice-cold lysis buffer (pH 7.5) containing 10 mM Tris-HCl, 150 mM NaCl, 0.5 mM EDTA, 10 mM NaF, and 0.5% Triton X-100 with a protease-inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). The homogenate was centrifuged at 1,000 × *g* for 10 min, and the resultant supernatant was further centrifuged at 100,000 × *g* for 60 min at 4°C. The supernatant and pellet thus obtained were stored at −80°C until the experimental use. The membrane fractions were highly enriched in synaptic plasma membranes, endoplasmic reticulum, and Golgi complexes.

Western blotting

After electrophoresis (applied proteins, 10 μg/lane) using a 3% – 8% Tris-acetate gel (Invitrogen, Carlsbad, CA, USA) with a size of 8 × 8 cm and thickness of 1.0 mm at 150 V for 60 min, proteins separated on the gel were transferred to a nitrocellulose filter with a wet-type transblotter (90 V, 60 min). For immunoblot detection of separated proteins, the membrane was incubated overnight at 4°C with primary antibodies against α_{1c} (rabbit polyclonal anti-α_{1c}, 1:1000) and α_{1d} (rabbit polyclonal anti-α_{1d}, 1:1000), RyR-1 (mouse monoclonal anti-ryanodine receptor type-1, 1:1000), RyR-2 (mouse monoclonal anti-ryanodine receptor type-2, 1:1000), and RyR-3 (rabbit polyclonal anti-ryanodine receptor type-3, 1:1000) diluted in phosphate-buffered saline (PBS) containing 5% nonfat dried milk and then further incubated for 2 h at room temperature with horseradish peroxidase–conjugated goat anti-rabbit IgG or horseradish peroxidase–conjugated goat anti-mouse IgG diluted 1:5000 in PBS containing 5% nonfat dried milk. Finally, proteins were detected by chemiluminescence (Pierce, Rockford, IL, USA).

Statistical analyses

All data are presented as the mean ± S.E.M. Statistical analyses were by performed by Prism 5 (GraphPad, Inc., San Diego, CA, USA). The statistical significance of differences was assessed by the methods described in each figure legend after the application of one-way ANOVA followed by the Bonferroni multiple comparisons test or Dunnett *post hoc* test.

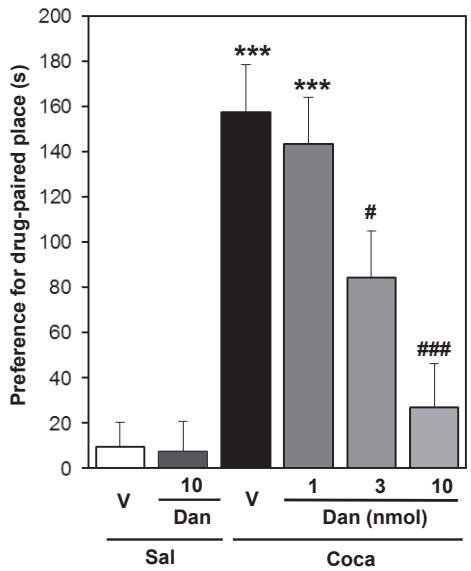

Fig. 1. Effect of dantrolene on cocaine-induced place preference in mice. Dantrolene (Dan; 1, 3, or 10 nmol/mouse) or vehicle (V) was i.c.v. administered 30 min before s.c. treatment with saline (Sal) and cocaine (Coca, 10 mg/kg). Each column represents the mean ± S.E.M. obtained from ten mice. ***$P < 0.001$ vs. vehicle-saline group (*post hoc* Bonferroni multiple comparison test). $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. vehicle-cocaine group (*post hoc* Dunnett's test).

Drugs

Cocaine hydrochloride and nifedipine were the products of Shionogi Co. (Osaka) and Wako Pure Chemicals (Osaka), respectively. Dantrolene, SCH23390, and sulpiride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against α_{1c} (rabbit polyclonal anti-α_{1c}) and α_{1d} (rabbit polyclonal anti-α_{1d}) were purchased from Alomone Labs, Ltd. (Jerusalem, Israel). Antibodies for RyR-1 (mouse monoclonal anti-ryanodine receptor type-1) and RyR-2 (mouse monoclonal anti-ryanodine receptor type-2) were the products of Sigma-Aldrich. Antibody against RyR-3 (rabbit polyclonal anti-ryanodine receptor type-3) was obtained from Millipore Bioscience Research Reagents (Temecula, CA, USA). Antibody against horseradish peroxidase–conjugated goat anti-rabbit IgG and horseradish peroxidase–conjugated goat anti-mouse IgG were the products of Southern Biotechnology Associates, Inc. (Birmingham, AL, USA). Cocaine and SCH23390 were dissolved in saline. Nifedipine, dantrolene, and sulpiride were dissolved in 5% dimethyl sulfoxide in saline.

Results

Blockade of development of cocaine-induced place preference by the RyR antagonist dantrolene

We investigated the effect of dantrolene, a RyR antagonist, on the place preference by cocaine. Mice conditioned with cocaine (10 mg/kg, s.c.) exhibited a significant preference for the drug-associated place ($F_{(5,54)} = 13.82$, $P < 0.0001$, Fig. 1). Dantrolene (10 nmol/mouse, i.c.v.)

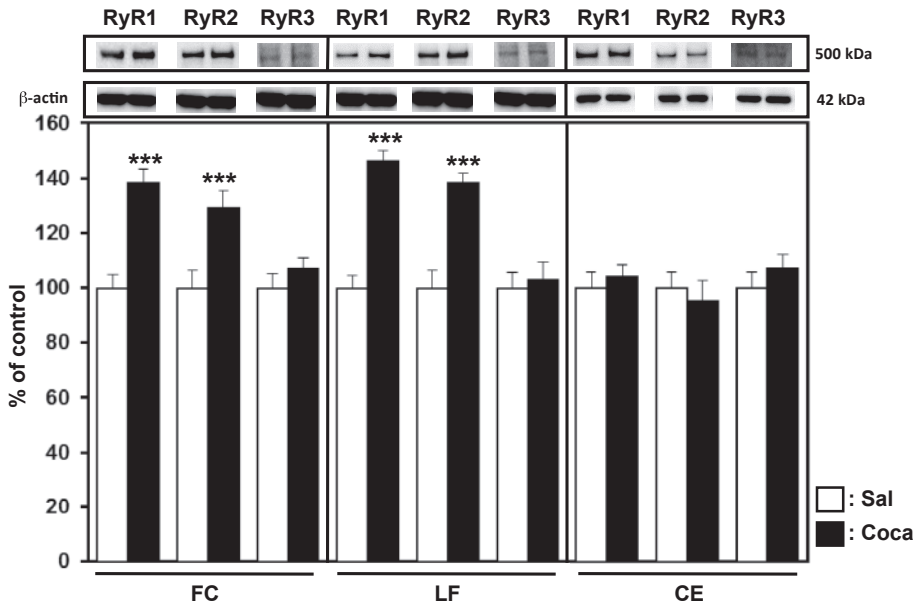

Fig. 2. Changes in protein levels of ryanodine receptors (RyRs) in the membrane fractions of the frontal cortex (FC), limbic forebrain (LF), and cerebellum (CE) of mice following cocaine-conditioning. Mice were s.c. injected with saline (Sal) or cocaine (Coca, 10 mg/kg) every other day for 6 days according to the conditioning schedule described in the text in detail. After the post-conditioning test, membrane fractions were prepared from the brains of mice treated with cocaine or saline. Each column represents the mean ± S.E.M. of four mice. ***$P <$ 0.001 vs. saline (Student's *t*-test).

alone induced neither significant place preference nor place aversion in mice. Under these conditions, the significant place preference produced by cocaine was suppressed by i.c.v. pretreatment with dantrolene in a dose-dependent manner (3 nmol: q = 2.85, $P < 0.05$; 10 nmol: q = 5.10, $P < 0.001$ versus vehicle-cocaine group by the *post hoc* test, respectively; Fig. 1).

Changes in protein levels of RyRs in the membrane fractions of the mouse frontal cortex, limbic forebrain, and cerebellum following cocaine-conditioning

The levels of proteins of three RyR isoforms in the frontal cortex and limbic forebrain obtained from cocaine-conditioned mice were also examined using western blot analysis. RyR-1 and -2 in the frontal cortex and limbic forebrain significantly increased by cocaine conditioning ($P < 0.001$ versus saline group by Student's *t*-test), although there were no changes in the expressions of these two types of RyRs in the cerebellum (Fig. 2). In contrast, the levels of RyR-3 in the frontal cortex and limbic forebrain as well as the cerebellum obtained from the cocaine-conditioned mice showed no changes (Fig. 2).

Effect of nifedipine on increase in RyR-1 and -2 expression in the frontal cortex and limbic forebrain obtained from cocaine-conditioned mice

We examined how the blockade of L-type VGCCs by nifedipine affected the increased expression of these RyRs because our previous study demonstrated that L-type VGCC blockers inhibited the development of the rewarding effect of METH (9) and there is functional interaction between L-type VGCCs and RyRs known as CICR (27). Nifedipine pre-administered i.c.v. at the dose that significantly blocked the cocaine-induced place preference did not produce any changes in the increase of RyR-1 and -2 in the frontal cortex and limbic forebrain (Fig. 3).

Effects of dantrolene on increase in α_{1c} and α_{1d} subunits of L-type VGCCs in the frontal cortex and limbic forebrain of cocaine-conditioned mice

We investigated effects of dantrolene on the increase in α_{1c} and α_{1d} subunits of L-type VGCCs protein expression in the frontal cortex and limbic forebrain of cocaine-conditioned mice because our previous report demonstrated that the protein levels of α_{1c} and α_{1d} subunits of L-type VGCCs in these brain regions were significantly increased by cocaine-induced place preference (9). Under these conditions, the i.c.v. pretreatment with dantrolene at the dose that blocked the cocaine-induced place preference had no effects on the increase in α_{1c} and α_{1d} subunits of L-type VGCCs proteins (Fig. 4).

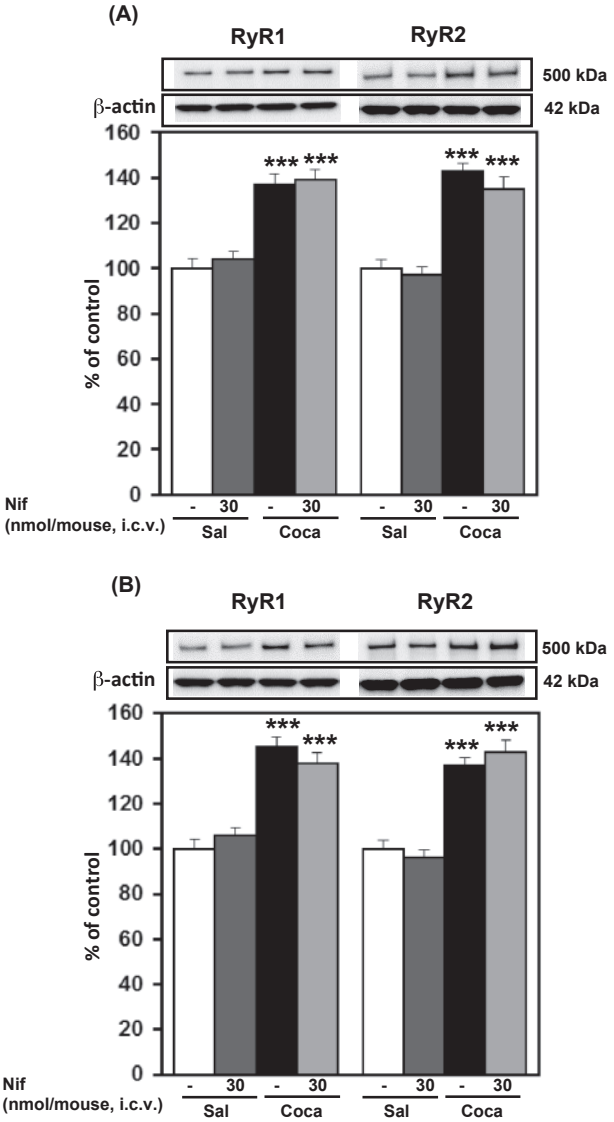

Fig. 3. Effects of nifedipine on increased expression of ryanodine receptors (RyRs) (types 1 and 2) in the frontal cortex (A) and limbic forebrain (B) obtained from cocaine-conditioned mice. Mice were s.c. treated with saline (Sal) or cocaine (Coca, 10 mg/kg) every other day over the period for 6 days. Mice were i.c.v. infused with vehicle or nifedipine (Nif, 30 nmol/mouse) 30 min before s.c. injection of cocaine or saline. After the post-conditioning test, membrane fractions were prepared from brains of mice treated with cocaine or saline. Each column represents the mean ± S.E.M. of four mice. ***$P < 0.001$ vs. saline (Bonferroni multiple comparison test).

Blockade of development of cocaine-induced place preference by dopamine receptor antagonists

The cocaine-induced place preference was suppressed by i.c.v. pretreatment with a dopamine D_1 receptor antagonist SCH23390 ($F_{(5,54)} = 11.27$, $P < 0.0001$, q = 4.363, $P < 0.05$; 30 nmol, $P < 0.001$ versus vehicle-cocaine group by the *post hoc* test; Fig. 5A), and a dopamine D_2 receptor antagonist sulpiride ($F_{(5,54)} = 19.48$, $P <$

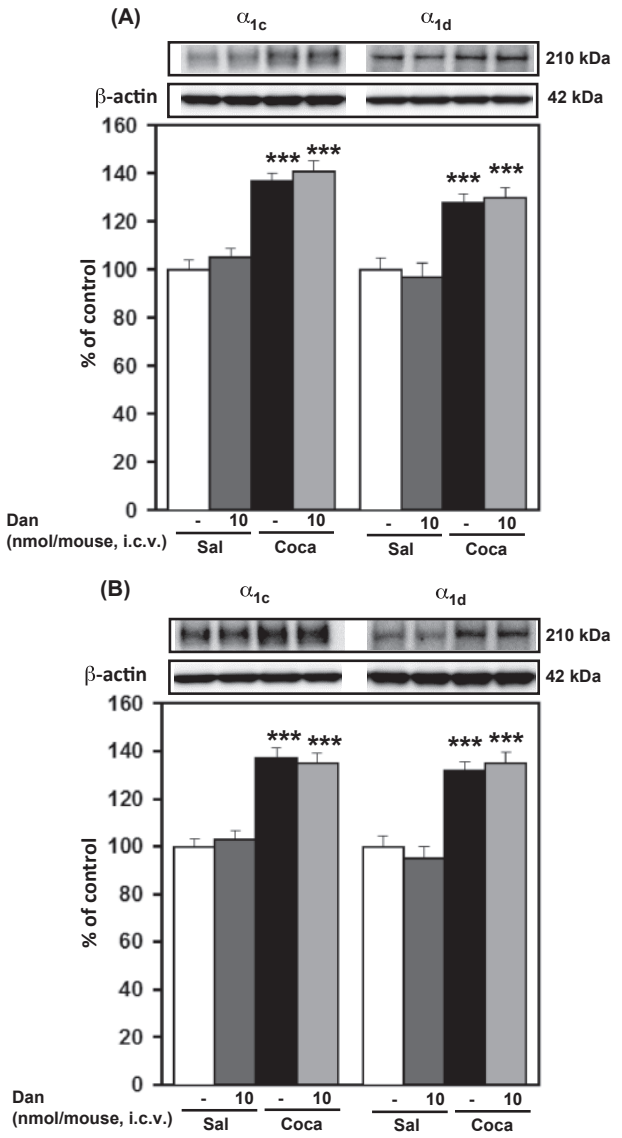

Fig. 4. Effect of dantrolene on increased expression of α_{1c} and α_{1d} subunits of L-type VGCCs in the frontal cortex (A) and limbic forebrain (B) obtained from cocaine-conditioned mice. Mice were treated s.c. with saline (Sal) or cocaine (Coca, 10 mg/kg) every other day over the period for 6 days. Mice were i.c.v. treated with vehicle or dantrolene (Dan, 10 nmol/mouse) 30 min before s.c. injection of cocaine or saline. After the post-conditioning test, membrane fractions were prepared from brains of mice treated with cocaine or saline. Each column represents the mean ± S.E.M. obtained from four mice. ***$P < 0.001$ vs. saline (Bonferroni multiple comparison test).

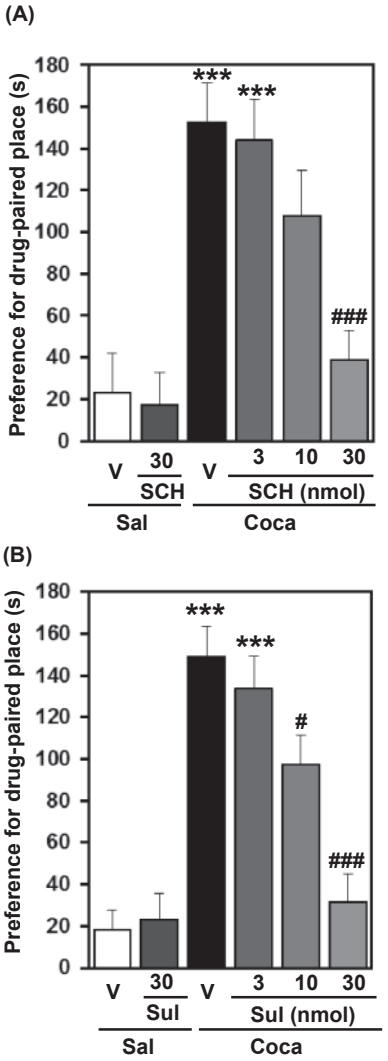

Fig. 5. Effects of SCH23390 (A) and sulpiride (B) on cocaine-induced place preference in mice. SCH23390 (SCH; 3, 10, and 30 nmol/mouse), sulpiride (Sul; 3, 10, and 30 nmol/mouse), or vehicle (V) was i.c.v. administered 30 min before s.c. injection of saline (Sal) and cocaine (Coca, 10 mg/kg). Each column represents the mean ± S.E.M. obtained from ten mice. ***$P < 0.001$ vs. vehicle-saline group (*post hoc* Bonferroni multiple comparison test). $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. vehicle-cocaine group (*post hoc* Dunnett's test).

0.0001; 10 nmol, q = 2.73, $P < 0.05$; 30 nmol, q = 6.24, $P < 0.001$ versus vehicle-cocaine group by the *post hoc* test; Fig. 5B), in a dose-dependent manner. Administration of SCH23390 or sulpiride alone did not change place preference or aversion (Fig. 5: A and B). In addition, mice showed almost normal locomotor activity and no sedation after i.c.v. injection of SCH23390 and sulpiride alone (data not shown).

Effect of SCH23390 and sulpiride on increased expression of RyR-1 and -2 in frontal cortex and limbic forebrain of cocaine-conditioned mice

Effects of SCH23390 on the increase in RyR-1 and -2 proteins in the frontal cortex and limbic forebrain in cocaine-conditioned mice were examined. Increased expression of RyRs was completely abolished by i.c.v. pretreatment with SCH23390 at the dose that blocked the cocaine-induced place preference (frontal cortex: RyR1,

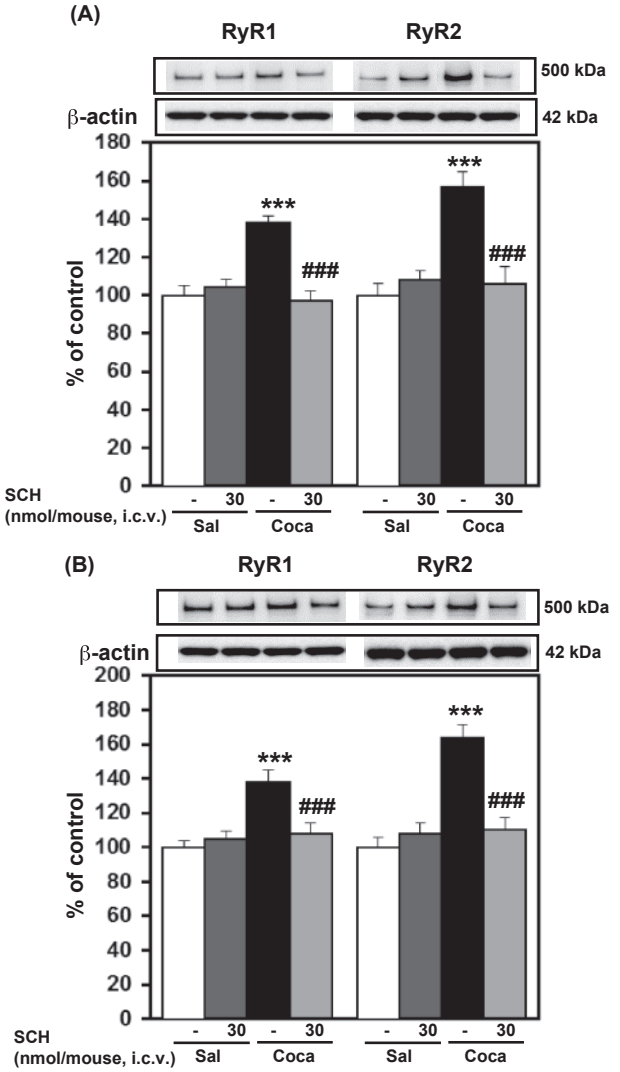

Fig. 6. Effect of SCH23390 on increased expression of ryanodine receptors (RyRs) (types 1 and 2) in the frontal cortex (A) and limbic forebrain (B) obtained from cocaine-conditioned mice. Mice were s.c. injected with saline (Sal) or cocaine (Coca, 10 mg/kg) every other day over the period for 6 days according to the conditioning schedule described in the text. Mice were i.c.v. administered vehicle or SCH23390 (SCH, 30 nmol/mouse) 30 min before s.c. injection of cocaine or saline. The membrane fractions used for measuring RyRs were prepared 24 h after the last conditioning with cocaine or saline. Each column represents the mean ± S.E.M. of four mice. ***$P < 0.001$ vs. vehicle-saline. $^{###}P < 0.001$ vs. vehicle-cocaine group (Bonferroni multiple comparison test).

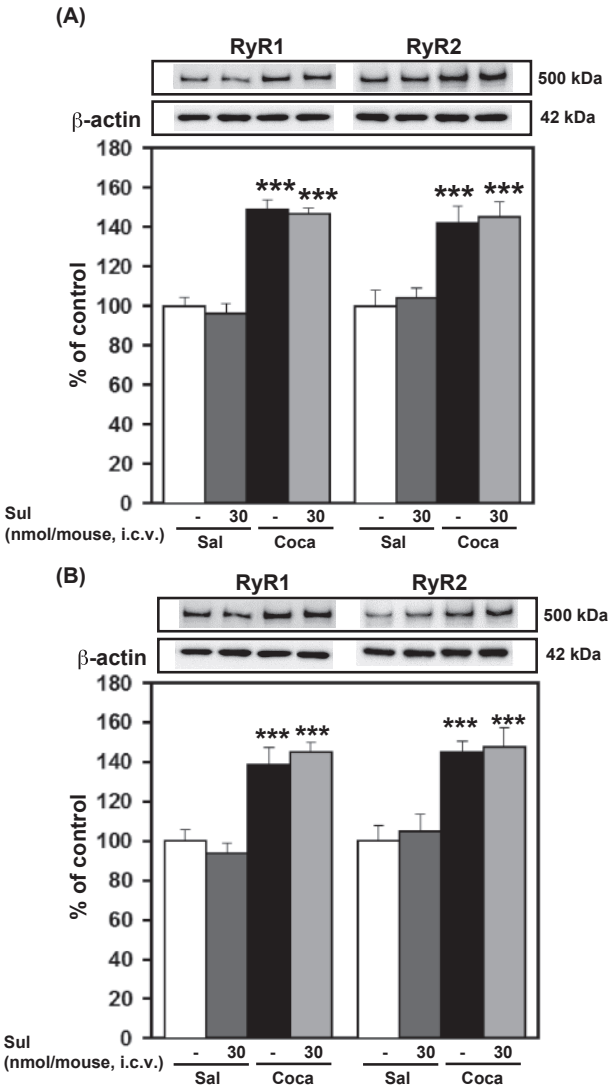

Fig. 7. Effect of sulpiride on increase in ryanodine receptors (RyRs) (types 1 and 2) proteins in frontal cortex (A) and limbic forebrain (B) obtained from cocaine-conditioned mice. Mice were s.c. treated with saline (Sal) or cocaine (Coca, 10 mg/kg) every other day for 6 days according to the conditioning schedule described in the text. Intracerebroventricular administration of vehicle or sulpiride (Sul, 30 nmol/mouse) was carried out 30 min before s.c. injection of cocaine or saline. The membrane fractions used for measuring RyRs were prepared 24 h after the last conditioning with cocaine or saline. Each column represents the mean ± S.E.M. of four mice. ***$P < 0.001$ vs. vehicle-saline (Bonferroni multiple comparison test).

$F_{(3,12)} = 23.06$ and RyR2, $F_{(3,12)} = 67.13$; limbic forebrain: RyR1, $F_{(3,12)} = 23.40$ and RyR2, $F_{(3,12)} = 71.22$; $P < 0.001$ versus vehicle-cocaine group by the *post hoc* test; Fig. 6). Similarly, the effects of sulpiride on the increase in RyR-1 and -2 proteins in the frontal cortex and limbic forebrain in cocaine-conditioned mice were examined. The increased expression of RyRs was not affected by i.c.v. pretreatment with sulpiride at the dose that blocked the cocaine-induced place preference (Fig. 7).

Discussion

In the present study, we found that a new pathway participating in the cocaine-induced up-regulation of RyRs, which can lead to the expression of place preference by cocaine, is regulated by dopamine D_1 receptors.

Intracerebroventricular administration of dantrolene, a specific antagonist for RyRs, significantly suppressed the place preference produced by cocaine, which is considered to be due to increased expression of RyRs. Indeed, we found significant increases of RyR-1 and -2 in the frontal cortex and limbic forebrain of the cocaine-conditioned mice, although cocaine-conditioning failed to affect the level of RyR-3 in the frontal cortex and limbic forebrain. In contrast, there were no changes in RyR levels in the cerebellum, the brain region that does not have an established role in drug abuse. These data clearly indicate that chronic administration of cocaine changes the expression of RyRs. These results also suggest that RyR-1 and -2 may be key players in the development of cocaine-induced place preference.

Three RyR isoforms have been identified in mammalian tissues, and all of them are expressed to various degrees in the CNS (13, 15, 17, 28, 29). Until now, it was known that only RyR-1, known as the skeletal muscle isoform, does not require an influx of extracellular Ca^{2+} for its activation and that RyR-1 is co-immunoprecipitated with the α_{1c} subunit. The latter provides direct evidence for functional coupling between these two proteins (27). That is, surface membrane depolarization sensed by L-type VGCCs is transduced to activate RyR-1. Several studies have reported that dantrolene and nifedipine block the KCl-evoked increase in Ca^{2+} concentration (30). The stimulating effect of KCl on the increase in intracellular Ca^{2+} concentration presumably results from membrane depolarization with subsequent activation of L-type VGCCs and associated facilitation of CICR from ryanodine-sensitizing Ca^{2+} stores (31, 32). Because such functional interaction between L-type VGCCs and RyRs raises a possibility of bidirectional regulatory mechanisms for their increased expression observed in the cocaine-induced place preference, we investigated such possible mutual regulatory mechanisms. Under the cocaine-induced place conditioning that significantly increased RyR-1 and -2 proteins in the frontal cortex and limbic forebrain, we found that i.c.v. pretreatment with nifedipine at the dose that significantly blocked the cocaine-induced place preference had no effects on the increase in RyR expression. Furthermore, the present study demonstrated that dantrolene had no potential to modify increased α_{1c} and α_{1d} subunit proteins in the frontal cortex and limbic forebrain of the cocaine-conditioned mice. These results therefore suggest that the increased expression of RyRs and α_1 subunits of L-type VGCCs may be regulated by independent mechanisms.

As shown in the present study, the place preference produced by repeated cocaine injection was significantly suppressed by dantrolene, indicating that the up-regulation of RyRs participates in the development of the cocaine-induced place preference. In addition, the blockade of dopamine D_1 and D_2 receptors by their respective antagonists, SCH23390 and sulpiride, significantly attenuated place preference induced by cocaine. Similar inhibitory roles of both dopamine D_1 and D_2 receptors in the development of the place preference of cocaine have been also reported (33, 34). These data therefore suggest a possibility that there may be a functional and/or regulatory pathway between the expression of RyRs involved in place preference of cocaine and dopamine receptors. As demonstrated in this study, administration of SCH23390, but not sulpiride, resulted in significant inhibition of the increased expression of RyR-1 and -2 in the frontal cortex and limbic forebrain evoked by cocaine, although both dopamine receptor antagonists have the potential to suppress the cocaine-induced place preference. According to the results shown here, it is reasonable to conclude that the up-regulation of RyRs is mediated through the activation of dopamine D_1 receptors during cocaine treatment.

Although the exact mechanisms of the regulatory effects of dopamine D_1 receptors on RyRs expression are not clear at present, a neurochemical pathway involving cAMP is supposed. Among the dopamine receptors generally grouped into two subfamilies, D_1-like and D_2-like receptors (35), dopamine D_1 receptors facilitate cAMP production to activate protein A kinase (PKA) signaling, which may finally activate the neurochemical events to up-regulate RyRs. However, the exact mechanisms remain to be elucidated at present.

One of the interesting findings in this study is that the increased expression of RyRs in the brains of mice with cocaine-induced place preference is regulated by dopamine D_1 receptors, but not dopamine D_2 receptors. Although both types of dopamine receptors have the potential to produce the cocaine-induced rewarding effect (33, 34) as well as to mediate reinforcing signals of abuse (36), opposing effects of dopamine D_1 and D_2 receptor activation on cAMP-dependent signaling have been reported in many studies (37). That is, dopamine D_1 receptors activate adenylate cyclase through stimulatory Gαs protein, and dopamine D_2 receptors inhibit adenylate cyclase via inhibitory Gαi/o protein. In addition, PKA signaling in the nucleus accumbens is involved in expression of animal behaviors (38 – 40). Therefore, dopamine D_1 receptors are concluded to be involved in increased expression of RyRs via a cAMP-linked metabolic pathway. However, as demonstrated here, dopamine D_2 receptors linked to inhibitory pathway of cAMP production have no activity to regulate RyR expression in the cocaine-induced place preference, which may provide a possible explanation for why dopamine D_2 receptors induce the cocaine-induced place preference through a

metabolic pathway different from that activated by dopamine D_1 receptors with increased production of cAMP, although the exact neurochemical pathway through which dopamine D_2 receptors facilitate cocaine-induced place preference remains to be elucidated.

The activity of RyRs is modulated by phosphorylation and by associated proteins, including calmodulin, calsequestrin, and FK506-binding proteins (41, 42). The increased proteins of RyR-1 and -2 by cocaine-conditioning may be also mediated through the activation of these associated proteins. However, the regulatory and pathophysiological roles of these associated proteins including calmodulin, calsequestrin, and FK506-binding proteins in the changes in RyR expression in brain remain to be elucidated.

Recent investigations also reported nicotine-mediated RyR-2 up-regulation by CREB in the VTA (43) and *N*-methyl-D-aspartate receptors–mediated and L-type calcium channel activator–mediated increase of RyR-2 (44). Furthermore, RyRs are linked to nicotine-induced neuronal plasticity (43, 45). These data therefore suggest that the changes in RyR function may be an important event linking to neurochemical and behavioral adaptation in association with the alteration of neuronal plasticity occurring in drug abuse.

The present study shows the increased levels of RyR-1 and -2, but not RyR-3, in the frontal cortex and limbic forebrain of mice with cocaine-induced place preference. It is considered to be difficult to define that cocaine-conditioning fail to affect RyR-3 expression in the brains of mice with cocaine-induced place preference are neurochemical events specific to cocaine-conditioning at present. On the other hand, we have reported that there are significant increases of RyR-1 and -2 in the frontal cortex, significant increases of RyR-1 in the limbic forebrain, and no changes in RyR-3 in both regions of mice treated with methamphetamine, which are very similar to those in mice with cocaine-induced place preference (21). Recent investigations also reported nicotine-mediated RyR-2 up-regulation in the ventral tegmental area (43) and *N*-methyl-D-aspartate receptors–mediated and L-type calcium channel activator–mediated increase of RyR-2 in the rat hippocampus (44). Modification of RyR-2 and -3 expressions has been demonstrated under conditions of cerebral ischemia (46). The mouse model of Alzheimer's disease showed increased RyR-3 expression in cortical neurons (47). Based on these data, it is likely that difference in drugs administered and differences in pathophysiological conditions may produce different pattern of changes in RyR isoform expression. However, available data on the exact mechanisms by which isoforms of RyRs are up-regulated under different pathophysiological conditions are few at present.

The present study demonstrates that the increase of RyRs in the frontal cortex and limbic forebrain of mice under intermittent administration of cocaine is significantly suppressed by a dopamine D_1 receptor antagonist SCH23390, which indicates that the expression of RyRs is positively regulated by dopamine D_1 receptors. However, as these effects of SCH23390 were induced by its i.c.v. administration, but not direct injection into the frontal cortex and limbic forebrain, global dopamine D_1 receptor blockade could be produced to modify the functions of reward circuitry activated during intermittent cocaine administration in any parts of the brain with the exception of the two brain regions described above. Therefore, it is noted that the effect of i.c.v. injected SCH23390 on RyR expression in the frontal cortex and limbic forebrain demonstrated here may be due to possible indirect effects mediated through modified function of other brain regions projecting to the frontal cortex and limbic forebrain, although such a possibility remains to be elucidated.

In conclusion, the present study demonstrated that repeated treatment of mice with cocaine increased RyR-1 and -2 levels in the frontal cortex and limbic forebrain, but not in the cerebellum, which was significantly suppressed by dantrolene. The increased expression of RyR-1 and -2 in these two brain regions in the cocaine-conditioned mice were completely suppressed by the blockade of dopamine D_1 receptors administered prior to cocaine, but not by dopamine D_2 receptors. These findings indicate that the critical roles of RyR-1 and -2 in the development of cocaine-induced place preference are regulated by dopamine D_1 receptors and that antagonists of RyRs including dantrolene may be potential therapeutics for the treatment and prevention of cocaine dependence.

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