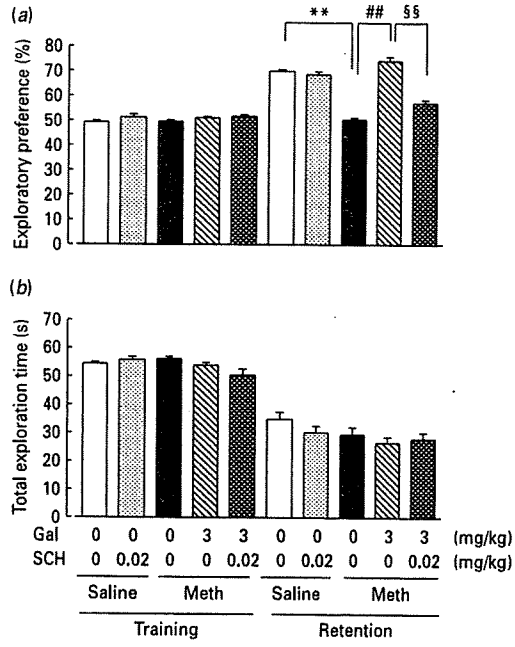

Fig. 6. Involvement of dopaminergic systems in the cognitive-improving effect of galantamine on methamphetamine (Meth)-treated mice: (*a*) exploratory preference and (*b*) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the novel object recognition test. Galantamine (Gal; 3 mg/kg p.o.) and SCH 23390 (SCH; 0.02 mg/kg s.c.) were administered 1 h and 30 min, respectively, before the training session. Values indicate the mean ± S.E. ($n = 10$–15). One-way ANOVA, (*a*) training: $F(4, 50) = 1.422$, $p = 0.240$; retention: $F(4, 55) = 40.622$, $p < 0.01$; (*b*) training: $F(4, 50) = 1.520$, $p = 0.211$; retention: $F(4, 55) = 1.943$, $p = 0.116$. ** $p < 0.01$ compared to saline + saline/saline-treated group (Bonferroni's test). ## $p < 0.01$ compared to Meth + saline/saline-treated group (Bonferroni's test). §§ $p < 0.01$ compared to Meth + galantamine/saline-treated group (Bonferroni's test).

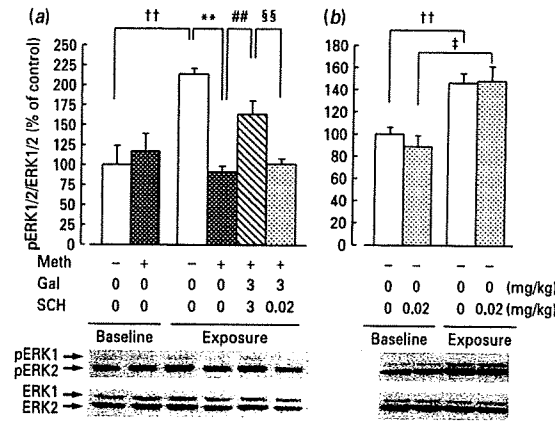

Fig. 7. Effect of galantamine on the defect of novelty-induced ERK1/2 phosphorylation in the PFC of methamphetamine (Meth)-treated mice. One hour before exposure to novel objects, galantamine (Gal; 3 mg/kg p.o.) or saline was administered to mice that had been previously treated with either saline or Meth (1 mg/kg s.c.) for 7 d. SCH 23390 (SCH; 0.02 mg/kg s.c.) was administered 30 min before exposure to novel objects. Values indicate the mean ± S.E. ($n = 4$–5). †† $p < 0.01$ compared to saline + saline/saline-treated group that was not exposed to novel objects (baseline) (Student's t test). ‡ $p < 0.05$ compared to saline + saline/SCH23390-treated group that was not exposed to novel objects (baseline) (Student's t test). One-way ANOVA: $F(3, 16) = 28.286$, $p < 0.01$. ** $p < 0.01$ compared to saline + saline/saline-treated group (exposure) (Bonferroni's test). ## $p < 0.01$ compared to Meth + saline/saline-treated group (exposure) (Bonferroni's test). §§ $p < 0.01$ compared to Meth + galantamine/saline-treated group (exposure) (Bonferroni's test).

mice. Therefore, it is unlikely that the observed improvement in performance in the task brought about by galantamine is due to changes in motivation in Meth-treated mice, and it is apparently true that galantamine ameliorates learning and memory deficits caused by repeated Meth treatment in mice. The improving effects of galantamine on the performance of Meth-treated mice were prevented by treatment with mecamylamine, a nAChR antagonist, at a dose that did not significantly affect the performance of saline-treated mice. These findings support the notion that galantamine improves Meth-induced cognitive impairment via activation of nAChRs. Alternatively, the roles of mAChRs in the effects of galantamine were also investigated in the present study. The effects of galantamine on the performance of Meth-treated mice in the NOR task were not blocked by scopolamine at the dose that impaired the performance of saline-treated mice. Although mAChR agonists improve cognitive dysfunctions in patients with Alzheimer's disease and schizophrenia (Friedman, 2004), the present result indicated that mAChRs have little influence on the effects of galantamine for this particular cognitive task. On the other hand, the activation of nAChRs may be due to an increase in the levels of ACh caused by AChE inhibition of galantamine. We investigated the effect of donepezil, which is 3–15 times more potent in AChE inhibition than that of galantamine *in vivo* (Geerts *et al.* 2005), on Meth-induced cognitive impairment. Although donepezil at 1 mg/kg caused about a 2-fold increase from basal extracellular ACh levels in the PFC of Meth-treated mice, it had no effect on behavioural performance in Meth-treated mice. From the

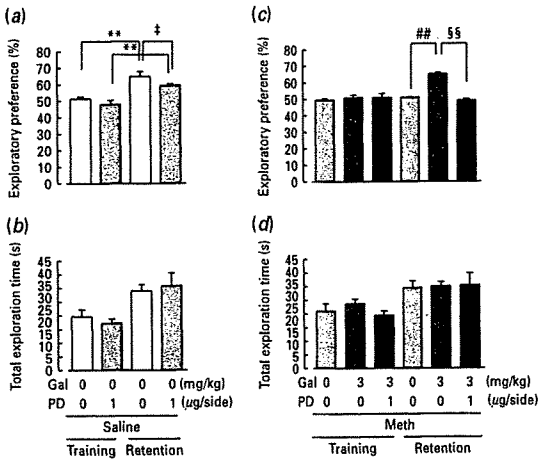

Fig. 8. Influence of an ERK inhibitor on the cognitive-improving effect of galantamine on methamphetamine (Meth)-treated mice: (*a*, *c*) exploratory preference and (*b*, *d*) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the novel object recognition test. Galantamine (Gal; 3 mg/kg p.o.) and PD98059 (PD; 1 μg/0.5 μl per side) were administered 1 h and 30 min, respectively, before the training session. Values indicate the mean ± S.E. (*a*, *b*; $n=8$) (*c*, *d*; $n=9$–10). One-way ANOVA, (*c*) training: $F(2,25)=0.309$, $p=0.737$; retention: $F(2,25)=27.986$, $p<0.01$; (*d*) training: $F(2,25)=0.399$, $p=0.676$; retention: $F(2,25)=0.015$, $p=0.985$. ** $p<0.01$ compared to corresponding saline-treated training group (Student's *t* test). ‡ $p<0.05$ compared to saline + saline/vehicle-treated retention group (Student's *t* test). ## $p<0.01$ compared to Meth + saline/vehicle-treated group (Bonferroni's test). §§ $p<0.01$ compared to Meth + galantamine/vehicle-treated group (Bonferroni's test).

present results and a report that there is only 1–12% brain AChE inhibition 1 h after s.c. injection of 3 mg/kg galantamine (Geerts *et al.* 2005), our conclusion is that galantamine induces the ameliorating effect on impairment of memory mainly by allosterically modulating the function of nAChRs, but not by AChE inhibition. However, further experiments are needed to exclude the involvement of AChE inhibition by galantamine in the ameliorating effect of it on cognitive impairment in Meth-treated mice, since the allosteric potentiating effect of nAChRs can be detected at lower doses (Geerts *et al.* 2005).

Accumulating evidence suggests that the dopaminergic system in the PFC is involved in cognitive function. For instance, disruption of dopamine transmission in the PFC by infusions of dopamine D_1R antagonists or by excitotoxic lesions impairs the performance of object retrieval-detour tasks, as well as delayed response tasks in non-human primates (Dias *et al.* 1996*a*,*b*; Sawaguchi & Goldman-Rakic, 1991). A previous study with functional magnetic resonance imaging has shown that dysfunction in the PFC of Meth abusers is related to cognitive impairment (Paulus *et al.* 2002). Accordingly, cognitive impairment in Meth abusers may be associated with deficits in dopamine transmission in the PFC. Our previous findings in *in-vivo* microdialysis experiments demonstrated that galantamine increases the extracellular dopamine release in the hippocampus and PFC and that the increasing effects of galantamine on dopamine release in the hippocampus are potentiated by nicotine and antagonized by mecamylamine (Wang *et al.* 2007*a*). The present *in-vivo* microdialysis experiment show that galantamine significantly increased extracellular dopamine release in the PFC of Meth-treated mice. The effects of galantamine on increasing dopamine release were antagonized by mecamylamine. These results strongly suggest that galantamine ameliorates Meth-induced learning and memory deficits by activating nAChRs, and thereby stimulates release of dopamine in the PFC. Further, we found that the improving effects of galantamine were prevented by SCH 23390, a dopamine D_1R antagonist. Galantamine enhances dopaminergic neurotransmission *in vivo* via allosteric potentiation of nAChRs. These findings provide the *in-vivo* evidence that galantamine augments dopaminergic neurotransmission in the PFC through the allosteric activation of nAChRs. The present results are supported by the results published by Schilström *et al.* (2007) that effects of galantamine on dopamine cell firing are mediated by allosteric potentiation of nAChRs. Taken together, our results suggest that the PFC-dependent behaviour task was impaired due to dysfunction of dopaminergic systems induced by Meth, since the PFC is involved in object recognition behaviour (Kamei *et al.* 2006). In fact, Kamei *et al.* (2006) have already demonstrated that repeated administration of Meth in mice induces object recognition impairment, which is associated with the dopamine D_1Rs, but not dopamine D_2Rs in the PFC. However, the object recognition memory is ascribed to the perirhinal cortex and its interactions with the hippocampus (Winters *et al.* 2008). We will investigate the functional role of the perirhinal cortex in Meth-induced cognitive deficits, in the ameliorating effects of galantamine and D_1R/ERK signalling in the NOR test.

Previous studies have demonstrated that the ERK1/2 signalling pathway linked to dopamine D_1Rs (Valjent *et al.* 2000; Zanassi *et al.* 2001) is involved in the rewarding effects induced by Meth (Mizoguchi *et al.* 2004) and the behavioural sensitization and

rewarding effects induced by cocaine (Valjent *et al.* 2000). Regarding the mechanism underlying the repeated Meth-induced memory impairment, Kamei *et al.* (2006) have already demonstrated dysfunction of the ERK1/2 pathway in the PFC. Hyperphosphorylation of ERK1/2 was found in the PFC when control mice were exposed to novel objects, whereas this activation was abolished in repeated Meth-treated mice. Inhibition of ERK1/2 by the microinjection of PD98059 (4 μg/mouse/bilateral), a selective MEK inhibitor, into the PFC resulted in cognitive impairment (Kamei *et al.* 2006). Ito *et al.* (2007) have also found that another MEK1/2 inhibitor, SL327 (30 and 50 mg/kg i.p.), significantly impairs long-term recognition memory 24 h after a training session in naive mice. In this study, galantamine ameliorated the Meth-induced defect of ERK1/2 hyperphosphorylation in the PFC of mice exposed to novel objects. In addition, the ameliorating effect of galantamine on Meth-induced object recognition impairment was completely blocked by pretreatment with the ERK inhibitor PD98059 at the dose used, slightly affecting the performance of saline-treated mice. Accordingly, these results suggest that the ameliorating effect of galantamine on Meth-induced cognitive impairment is related to the activation of ERK1/2 in the PFC.

As discussed above, our findings suggest that dopamine D_1R-ERK1/2 systems are required for the effects of galantamine. Since dopamine the D_1R antagonist and ERK inhibitor impaired recognition memory based on phosphorylation of ERK in the PFC of normal mice (Kamei *et al.* 2006), dopamine D_1R-ERK1/2 systems are critical in recognition memory. If the action site of galantamine is downstream of dopamine D_1R-ERK1/2 systems, dopamine D_1R antagonists or the ERK inhibitor would fail to reverse the effect of galantamine. Accordingly, our data suggest that galantamine acts upstream of dopamine D_1R-ERK1/2 systems.

In conclusion, the ameliorating effect of galantamine on Meth-induced memory impairment is associated with indirect activation of dopamine D_1R-ERK1/2 following augmentation with dopaminergic neurotransmission in the PFC through the allosteric activation of nAChRs. Galantamine could prove to be a useful therapeutic drug for treating cognitive deficits in schizophrenia/Meth psychosis, as well as Alzheimer's disease.

Acknowledgements

This study was supported in part by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO05-27), by the Academic Frontier Project for Private Universities (2007–2011) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by the International Research Project supported by the Meijo Asian Research Center (MARC), by a Grant-in-Aid for Exploratory Research from the Japan Society for the Promotion of Science, by a Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science, by a Grant-in-Aid on Priority Areas from the Japan Society for the Promotion of Science, by a Health and Labour Sciences Research Grant for Research on Regulatory Science of Pharmaceuticals and Medical Devices and Research on Risk of Chemical Substances from the Ministry of Health, Labour and Welfare of Japan, and by a grant from the Smoking Research Foundation.

Statement of Interest

None.

References

Cretzmeyer M, Sarrazin MV, Huber DL, Block RI, *et al.* (2003). Treatment of methamphetamine abuse: research findings and clinical directions. *Journal of Substance Abuse Treatment* **24**, 267–277.

Dias R, Robbins TW, Roberts AC (1996*a*). Dissociation in prefrontal cortex of affective and attentional shifts. *Nature* **380**, 69–72.

Dias R, Robbins TW, Roberts AC (1996*b*). Primate analogue of the Wisconsin card sorting test: effects of excitotoxic lesions of the prefrontal cortex in the marmoset. *Behavioral Neuroscience* **110**, 872–886.

Eisele JL, Bertrand S, Galzi JL, Devillers-Thiery A, *et al.* (1993). Chimaeric nicotinic-serotoninergic receptor combines distinct ligand binding and channel specificities. *Nature* **366**, 479–483.

Franklin KBJ, Paxinos G (1996). *The Mouse Brain in Stereotaxic Coordinates*. San Diego: Academic Press.

Friedman JI (2004). Cholinergic targets for cognitive enhancement in schizophrenia: focus on cholinesterase inhibitors and muscarinic agonists. *Psychopharmacology (Berlin)* **174**, 45–53.

Geerts H, Guillaumat PO, Grantham C, Bode W, *et al.* (2005). Brain levels and acetylcholinesterase inhibition with galantamine and donepezil in rats, mice and rabbits. *Brain Research* **1033**, 186–193.

Ito Y, Takuma K, Mizoguchi H, Nagai T, *et al.* (2007). A novel azaindolizinone derivative ZSET1446 [spiro[imidazo[1,2-a]pyridine-3,2-indan]-2(3H)-one] improves methamphetamine-induced impairment of recognition memory in mice by activating extracellular signal-regulated kinase 1/2. *Journal of Pharmacology and Experimental Therapeutics* **320**, 819–827.

Kalechstein AD, Newton TF, Green M (2003). Methamphetamine dependence is associated with neurocognitive impairment in the initial phases of abstinence. *Journal of Neuropsychiatry and Clinical Neurosciences* **15**, 215–220.

Kamei H, Nagai T, Nakano H, Togan Y, *et al.* (2006). Repeated methamphetamine treatment impairs recognition memory through a failure of novelty-induced ERK1/2 activation in the prefrontal cortex of mice. *Biological Psychiatry* **59**, 75–84.

Mizoguchi H, Yamada K, Mizuno M, Mizuno T, *et al.* (2004). Regulations of methamphetamine reward by extracellular signal-regulated kinase 1/2/ets-like gene-1 signaling pathway via the activation of dopamine receptors. *Molecular Pharmacology* **65**, 1293–1301.

Mouri A, Noda Y, Noda A, Nakamura T, *et al.* (2007). Involvement of a dysfunctional dopamine-D1/*N*-methyl-*d*-aspartate-NR1 and Ca^{2+}/calmodulin-dependent protein kinase II pathway in the impairment of latent learning in a model of schizophrenia induced by phencyclidine. *Molecular Pharmacology* **71**, 1598–1609.

Mouri A, Zou LB, Iwata N, Saido TC, *et al.* (2006). Inhibition of neprilysin by thiorphan (i.c.v.) causes an accumulation of amyloid beta and impairment of learning and memory. *Behavioral Brain Research* **168**, 83–91.

Nordahl TE, Salo R, Leamon M (2003). Neuropsychological effects of chronic methamphetamine use on neurotransmitters and cognition: a review. *Journal of Neuropsychiatry and Clinical Neurosciences* **15**, 317–325.

Paulus MP, Hozack NE, Zauscher BE, Frank L, *et al.* (2002). Behavioral and functional neuroimaging evidence for prefrontal dysfunction in methamphetamine-dependent subjects. *Neuropsychopharmacology* **26**, 53–63.

Rawson RA, Gonzales R, Brethen P (2002). Treatment of methamphetamine use disorders: an update. *Journal of Substance Abuse Treatment* **23**, 145–150.

Santos MD, Alkondon M, Aracava Y, Eisenberg HM, *et al.* (2002). The nicotinic allosteric potentiating ligand galantamine facilitates synaptic transmission in the mammalian central nervous system. *Molecular Pharmacology* **61**, 1222–1234.

Sato M, Chen CC, Akiyama K, Otsuki S (1983). Acute exacerbation of paranoid psychotic state after long-term abstinence in patients with previous methamphetamine psychosis. *Biological Psychiatry* **18**, 429–440.

Sawaguchi T, Goldman-Rakic PS (1991). D1 dopamine receptors in prefrontal cortex: involvement in working memory. *Science* **251**, 947–950.

Schilström B, Ivanov VB, Wiker C, Svensson TH (2007). Galantamine enhances dopaminergic neurotransmission in vivo via allosteric potentiation of nicotinic acetylcholine receptors. *Neuropsychopharmacology* **32**, 43–53.

Shintani F, Kanba S, Nakaki T, Nibuya M, *et al.* (1993). Interleukin-1b augments release of norepinephrine, dopamine, and serotonin in the rat anterior hypothalamus. *Journal of Neuroscience* **13**, 3574–3581.

Simon SL, Domier C, Carnell J, Brethen P, *et al.* (2000). Cognitive impairment in individuals currently using methamphetamine. *American Journal on Addictions* **9**, 222–231.

Srisurapanont M, Ali R, Marsden J, Sunga A, *et al.* (2003). Psychotic symptoms in methamphetamine psychotic in-patients. *International Journal of Neuropsychopharmacology* **6**, 347–352.

Valjent E, Corvol JC, Pages C, Besson MJ, *et al.* (2000). Involvement of the extracellular signal-regulated kinase cascade for cocaine-rewarding properties. *Journal of Neuroscience* **20**, 8701–8709.

Wang D, Noda Y, Zhou Y, Mouri A, *et al.* (2007*a*). The allosteric potentiation of nicotinic acetylcholine receptors by galantamine ameliorates the cognitive dysfunction in beta $amyloid_{25\text{-}35}$ i.c.v.-injected mice: involvement of dopaminergic systems. *Neuropsychopharmacology* **32**, 1261–1271.

Wang D, Noda Y, Zhou Y, Nitta A, *et al.* (2007*b*). Synergistic effect of combined treatment with risperidone and galantamine on phencyclidine-induced impairment of latent visuospatial learning and memory: role of nAChR activation-dependent increase of dopamine D1 receptor-mediated neurotransmission. *Neuropharmacology* **53**, 379–389.

Winters BD, Saksida LM, Bussey TJ (2008). Object recognition memory: neurobiological mechanisms of encoding, consolidation and retrieval. *Neuroscience and Biobehavioral Reviews* **32**, 1055–1070.

Yui K, Ikemoto S, Goto K, Nishijima K, *et al.* (2002). Spontaneous recurrence of methamphetamine-induced paranoid-hallucinatory states in female subjects: susceptibility to psychotic states and implications for relapse of schizophrenia. *Pharmacopsychiatry* **35**, 62–71.

Zanassi P, Paolillo M, Feliciello A, Avvedimento EV, *et al.* (2001). cAMP-dependent protein kinase induces cAMP-response element-binding protein phosphorylation via an intracellular calcium release/ERK-dependent pathway in striatal neurons. *Journal of Biological Chemistry* **276**, 11487–11495.

Neuropharmacology 55 (2008) 1300–1306

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Methamphetamine-seeking behavior is due to inhibition of nicotinic cholinergic transmission by activation of cannabinoid CB1 receptors

Takato Hiranita [a,b,c], Yoko Nawata [a], Katsuya Sakimura [b], Tsuneyuki Yamamoto [a,*]

[a] Department of Pharmacology, Faculty of Pharmaceutical Sciences, Nagasaki International University, 2825-7 Huis Ten Bosch, Sasebo, Nagasaki 859-3298, Japan
[b] Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[c] Psychobiology Section, Medications Discovery Research Branch, Intramural Research Program, Department of Health and Human Services, National Institute on Drug Abuse, National Institutes of Health, Baltimore, MD 21224, United States

ARTICLE INFO

Article history:
Received 15 September 2007
Received in revised form 12 August 2008
Accepted 14 August 2008

Keywords:
Cannabinoid CB1 receptors
Craving
Drug-seeking behavior
Methamphetamine
Nicotinic ACh receptors
Relapse

ABSTRACT

We previously reported the involvement of cannabinoid CB1 receptors (CB1Rs) and nicotinic acetylcholine receptors (nAChRs) in the reinstatement of methamphetamine (MAP)-seeking behavior (lever-pressing response for MAP reinforcement under saline infusion). The present study examined whether the reinstatement involves interactions between these receptors. Rats were trained to self-administer MAP with a light and tone (MAP-associated cues). Then, extinction sessions under saline infusion without cues were conducted. After that, a reinstatement tests were conducted by either presenting the cues or a MAP-priming injection. Systemic and intracranial administration of HU210, a cannabinoid CB1R agonist, into the nucleus accumbens core (NAC) and prelimbic cortex (PrC) reinstated MAP-seeking behavior. The reinstatement caused by the systemic HU210 treatment was attenuated by intracranial administration of AM251, a cannabinoid CB1R antagonist, into each region mentioned above. Meanwhile, reinstatement induced by the MAP-associated cues and MAP-priming injection was also attenuated by intracranial administration of AM251 in each region. In these regions, the attenuating effects of AM251 on the reinstatement induced by each stimulus were blocked by the intracranial administration of mecamylamine, a non-selective nAChR antagonist, but not by scopolamine, a muscarinic ACh receptor (mAChR) antagonist. Furthermore, the intracranial administration of DHβE, an α4β2 nAChR antagonist, but not MLA, an α7 nAChR antagonist, into each region blocked the AM251-induced attenuation of the reinstatement. These findings suggest that relapses to MAP-seeking behavior may be due to two steps, first inhibition of ACh transmission by the activation of cannabinoid CB1Rs and then the inactivation of α4β2 nAChRs.

1. Introduction

Relapse to drug-seeking behavior is a hallmark of drug dependence, but an effective treatment has yet to be developed. In human addicts and animal models of relapse, three different kinds of stimuli are capable of eliciting drug-seeking behavior: stress, cues predicting drug availability, and re-exposure to a previously self-administered drug (Shalev et al., 2002). Understanding the neural mechanisms by which these stimuli elicit relapse is a prerequisite to creating adequate pharmacotherapies for drug dependence.

We have previously demonstrated that the systemic administration of a cannabinoid CB1R antagonist SR141716A (rimonabant) (Anggadiredja et al., 2004a) and nicotine (Hiranita et al., 2004, 2006) attenuates the reinstatement of MAP-seeking behavior induced by re-exposure to MAP-associated cues, previously paired with MAP-taking, as well as a MAP-priming injection (1.0 mg/kg, i.p.); however, the site of action of the antagonist responsible for attenuating the reinstatement is unknown. Therefore, the first purpose of this study is to identify this region. We reported that the nucleus accumbens core (NAC) and prelimbic cortex (PrC) were involved in the reinstatement of MAP-seeking behavior (Hiranita et al., 2006). Recently it was reported that endocannabinoid was important for the neural plasticity of glutamatergic neurons between these regions (Robbe et al., 2003). Therefore, we focused on these two regions. Meanwhile, it has been shown that SR141716A, a cannabinoid CB1R antagonist, stimulates the release of ACh in the medial prefrontal cortex (mPFC) (Gessa et al., 1998; Tzavara et al., 2003a). Electrophysiological studies reported that endocannabinoids functioned as antagonists at nAChRs. Thus, anandamide and 2-arachidonoylglycerol (2-AG), endogenous cannabinoid CB1R agonists, inhibited the function of nAChRs (Oz, 2006; Spivak et al., 2007). Furthermore, a cannabinoid CB1R antagonist AM251 attenuated

* Corresponding author. Tel.: +81 956 20 5629; fax: +81 956 39 3111.
E-mail address: tyamamot@niu.ac.jp (T. Yamamoto).

0028-3908/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuropharm.2008.08.012

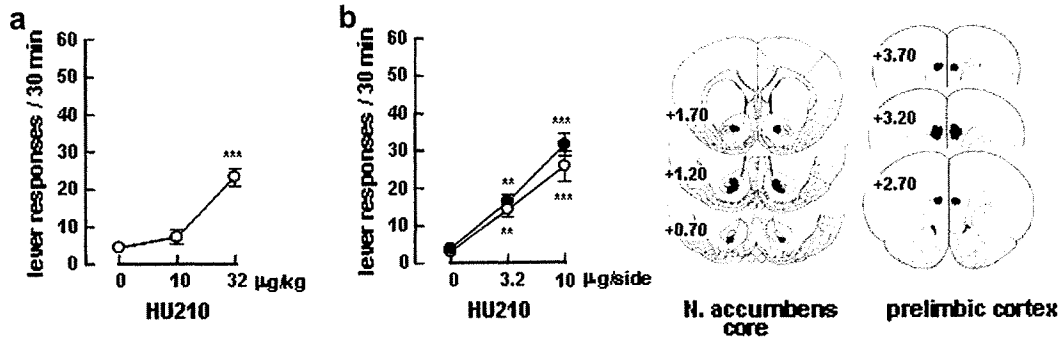

Fig. 1. Effect of HU210, a cannabinoid CB1 receptor agonist, in MAP self-administered rats. (a) Effect of systemic treatment with HU210 in MAP self-administered rats ($n = 6$). ***, $P < 0.001$ compared with vehicle. (b) Effect of the intracranial administration of HU210 into the nucleus accumbens core (open circles) and prelimbic cortex (closed circles) in MAP self-administered rats ($n = 7$, each). **, $P < 0.01$ and ***, $P < 0.001$ compared with vehicle. Coronal brain maps show a schematic representation of where the annuluses were placed. The numbers indicate the distance from the bregma in the anteroposterior plane. Mapping also includes the location of the tips of the cannulas used in all experiments.

nicotine self-administration and nicotine-seeking behavior in rats (Shoaib, 2008). Such findings indicate that the cannabinoid system modulates AChrgic transmission. Consequently, it is possible that a cannabinoid CB1R antagonist SR141716A (Anggadiredja et al., 2004a) attenuates reinstatement of MAP-seeking behavior by mediating AChrgic transmission. However, little is known about the interaction between the cannabinoid system and nAChRs during relapse to drug-seeking behavior. The second purpose of this study is to clarify the interrelation between cannabinoid CB1Rs and nAChRs.

Neuronal nAChRs are comprised of combinations of α (2–9) and β (2–4) subunits arranged to form a pentameric receptor (Grottick et al., 2000). The principal subtypes in the central nervous system are believed to be α4β2 and homomeric α7 nAChRs (Grottick et al., 2000). Their distribution in the PFC and striatum, including the PrC and NAC, respectively, is known to be similar (Gotti et al., 2006). Although it is well established that nicotine has a rewarding effect, the nAChR subtypes involved in the reinstatement of drug-seeking behavior are unknown. Therefore, the third purpose of this study was to identify the nAChR subtypes responsible for the CB1R–nAChR interaction.

2. Materials and methods

2.1. Subjects

One hundred thirty-three subjects were used. Male Wistar/ST (Nippon SLC Co., Hamamatsu, Japan) rats (250–350 g, 10 weeks old) were individually housed in a temperature- and humidity-controlled environment under a 12-h light/dark cycle (lights on at 7:00 a.m.). Food and water were available ad libitum in the home cage except when daily food intake was limited to 15–20 g after the implantation of catheters to fix the distance between the proximal position of a catheter in the vein and the surface of the atrial auricle. Rats were trained and tested between 9:00 a.m. and 5:00 p.m. Procedures for animal treatments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the Declaration of Helsinki and Faculty of Pharmaceutical Sciences, Kyushu University Publication, enacted 1988. In all studies, within-subject designs are used so that each animal served as its own control, and the overall number of subjects was minimal.

2.2. Surgery

Silascon catheters (inner and outer diameter: 0.5 and 1.0 mm, respectively; Kaneka Medix Co., Japan) were surgically implanted into the jugular vein under sodium pentobarbital (40 mg/kg, intraperitoneal (i.p.), Kyoritsu Seiyaku Co., Japan) anesthesia as described previously (Hiranita et al., 2006). After the surgery, catheter patency was maintained by daily infusion of 0.15 ml of a saline solution containing heparin (30 U/ml) after each session. After catheterization in the jugular vein, rats were fixed in a stereotaxic apparatus. Two guide cannulas (inner and outer diameter: 0.4 and 0.7 mm, respectively; stainless steel pipe) were bilaterally implanted 1 mm above the NAC (coordinates: anteroposterior, mediolateral, and dorsoventral, +1.2, ±1.6, and −7.8 mm relative to the bregma, midline, and skull surface, respectively), and PrC (+3.2, ±0.75, and −4.7). Two stainless steel screws were implanted in the skull for support. The cannulas and screws were held in place with dental cement. An obturator (stainless steel) was inserted into each guide cannula to prevent blockage.

2.3. Drugs

MAP HCl (Dainippon Pharmaceutical, Osaka), (Nattick, MA), mecamylamine (a non-specific nAChR antagonist, Sigma–Aldrich), (−)-scopolamine (a muscarinic AChR (mAChR) antagonist, Sigma–Aldrich), dihydro-β-erythroidine HBr (DHβE, an α4β2 nAChR antagonist, Sigma–Aldrich) and methyllycaconitine citrate (MLA, an α7 nAChR antagonist, Sigma–Aldrich) were dissolved in saline, while AM251 and HU210, a cannabinoid CB1R antagonist and agonist, respectively, were dissolved in dimethyl sulfoxide. MAP was delivered intravenous (i.v.) for self-administration (0.02 mg/0.1 ml/infusion) and i.p. for priming injections (1.0 mg/kg) 30 min before tests. Systemic administration of HU210 (10–32 µg/kg) was done subcutaneously (s.c.) 15 min before the sessions. Drugs administered intracranially (0.5 µl/side) were microinjected into the brain 5 min before sessions through an injection cannula (inner and outer diameters were 0.1 and 0.35 mm, respectively; a stainless steel tube was used) that extended 1 mm below the guide cannula (stainless steel) using a microsyringe (Hamilton).

2.4. Apparatus

The injector system contained a fluid swivel (Instech Lab., Inc. PA) mounted on the top of each operant chamber (Neuroscience, Inc. Japan). One end of the swivel was connected via polyethylene tubing (Kaneka Medix Co., Japan) encased in a protective stainless steel spring tether (Instech Laboratories, Inc. PA) to the animal's catheter while the other end of the swivel was connected via polyethylene tubing to the infusion pump. The operant chambers were enclosed in ventilated, sound-attenuating cubicles and controlled by computer software (Med Associates Inc., VT). The chamber's light was switched on throughout the session. Lever-pressing responses resulted in the infusion of MAP (0.02 mg/infusion over 6 s) accompanied by light (mounted 4 cm above the lever, 200 lux) and tone (85 dB/2.9 kHz) for 26 s (MAP-associated cues). The subsequent 20 s was a 'time out' period during which lever presses were still recorded but not accompanied with infusions.

2.5. MAP self-administration, extinction, and reinstatement

Two days after surgery, rats were trained to self-administer MAP under a fixed ratio (FR-1) schedule of reinforcement (each lever-press is reinforced) in a 2-h daily session for 10 days (MAP-taking). Each injection was accompanied by light and tone (MAP-associated cues). During this time, inactive lever responses had no programmed consequences, but were recorded. After MAP 10 days of self-administration, 5 daily extinction sessions (1-h), were conducted during which active lever responding resulted in an infusion of saline instead of MAP without presentation of the MAP-associated cues (or until the rats achieved the extinction criterion of less than 10 responses per session on the previously active lever). Reinstatement (drug-seeking behavior) tests under saline infusions were carried out for 30 min from day

Table 1
Effect of intracranial injection of HU210 on food-taking responses (responses/min)

Nucleus accumbens core			Prelimbic cortex		
HU210 (µg/side)			HU210 (µg/side)		
0	3.2	10	0	3.2	10
25.0 ± 0.9	26.3 ± 1.1	25.2 ± 1.1	25.2 ± 1.1	24.7 ± 1.1	25.4 ± 0.7

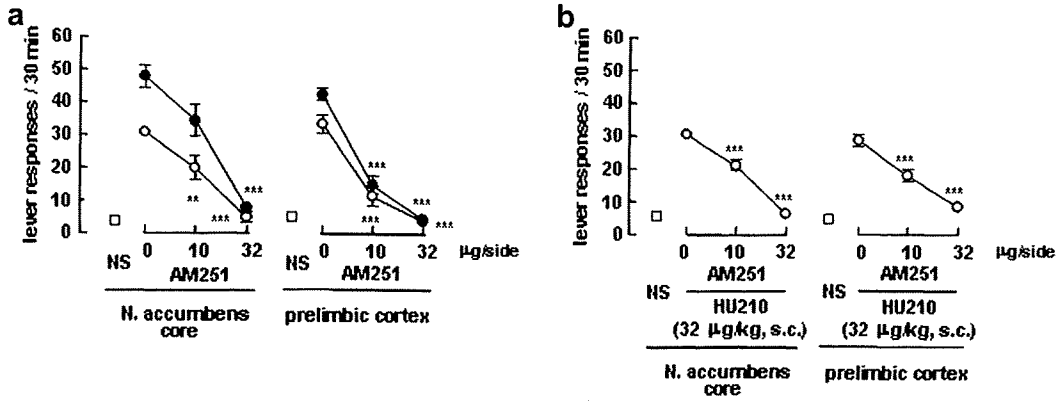

Fig. 2. Effects of the intracranial administration of AM251, a cannabinoid CB1 receptor antagonist, into the nucleus accumbens core and prelimbic cortex on the reinstatement of MAP-seeking behavior induced by MAP-associated cues, MAP-priming injections, and systemic administration of HU210. (a) Effects of the intracranial administration of AM251 into the nucleus accumbens core and prelimbic cortex on the reinstatement induced by MAP-associated cues and MAP-priming injections ($n = 7$, each). Open squares, and open and closed circles represent groups given non-stimuli (NS), MAP-associated cues, and MAP-priming injections. **, $P < 0.01$, and ***, $P < 0.001$ compared with the cue presentation and MAP-priming injection alone. (b) Effects of the intracranial administration of AM251 into the nucleus accumbens core and prelimbic cortex on the reinstatement induced by systemic treatment with HU210 (32 μg/kg, s.c.) ($n = 7$, each). Open squares and circles represent groups given non-stimuli (NS) and HU210, respectively. ***, $P < 0.001$ compared with HU210 alone. NS means lever responses under the extinction condition.

6 of extinction (or the day after rats achieved the extinction criterion) every 6 days under an FR-1 schedule. In the cue-induced test, immediately after the onset of the session, rats were re-exposed to the MAP-associated cues, and each press on the active lever resulted in presentation of the cues. In the MAP primed reinstatement test, MAP (1.0 mg/kg i.p.) was injected 30 min before the test. Each response during the test session resulted in an infusion of saline but not the MAP-associated cues. Drugs were preadministered in a counterbalanced order. Each rat was evaluated by both cue- and MAP-induced reinstatement. In our pilot study, levels of active lever responses induced by MAP-associated cues and MAP-priming injections did not change during at least the third time. Therefore, each rat was given either the cue-stimulus or MAP-priming injection alternately and had 6 reinstatement tests in total, that is, 3 tests per stimulus.

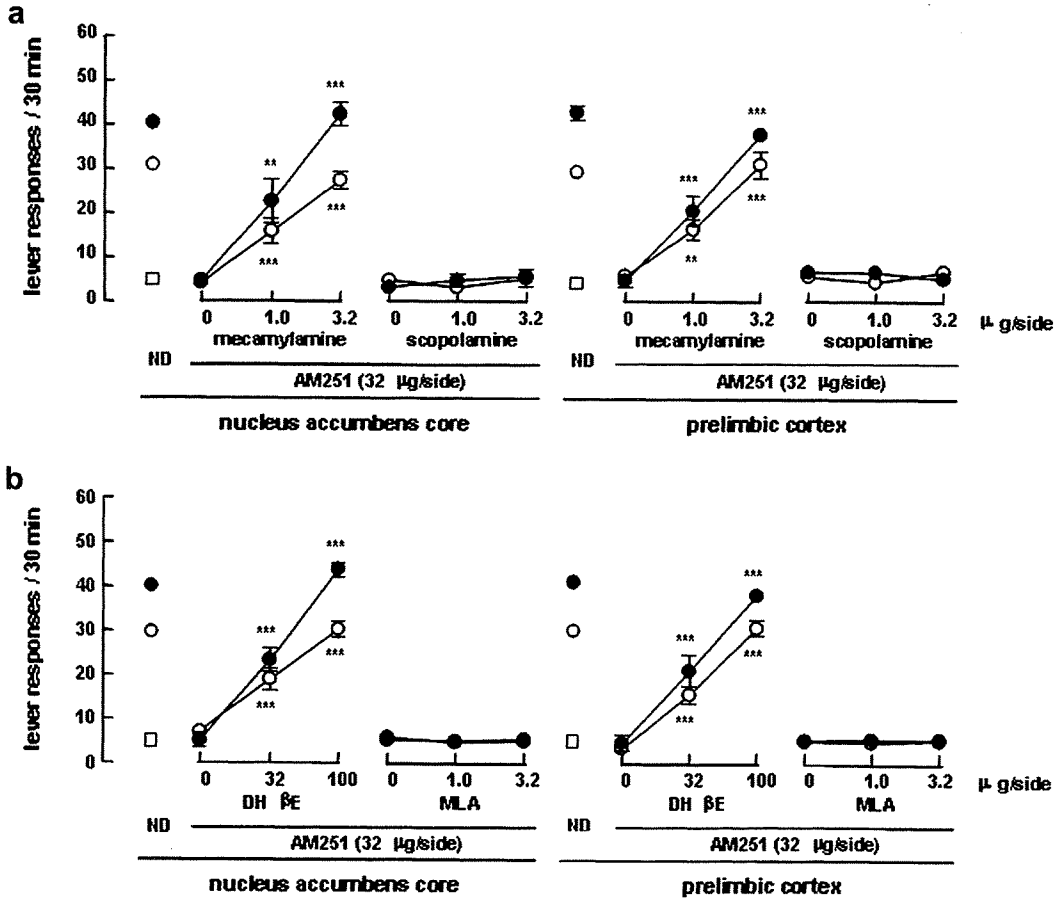

Fig. 3. Effects of the intracranial administration of cholinergic antagonists into the nucleus accumbens core and the prelimbic cortex on the AM251-induced attenuation of the reinstatement induced by the cues and MAP-priming injections. Open squares, and open and closed circles represent groups given non-stimuli (NS), MAP-associated cues, and MAP-priming injections, respectively. ND: non-drugs. **, $P < 0.01$, and ***, $P < 0.001$ compared with AM251-pretreated groups given the cues and MAP-priming injections ($n = 7$, each). (a) Effects of a non-selective nicotinic and muscarinic acetylcholine receptor antagonist (mecamylamine and scopolamine, respectively) on the reinstatement. (b) Effects of a selective α4β2 and α7 nicotinic acetylcholine receptor antagonist (DHβE and MLA, respectively) on the reinstatement. NS means lever responses under the extinction condition, whereas ND means no pretreatment with test drugs.

Table 2
Effect of intracranial injection of AM251 on food-taking responses in rats given the inductive stimuli (responses/min)

Inductive stimuli of the reinstatement	Nucleus accumbens core			Prelimbic cortex		
	AM251 (μg/side)			AM251 (μg/side)		
	0	10	32	0	10	32
MAP-associated cues	22.4 ± 1.9	21.1 ± 1.8	20.1 ± 2.1	24.1 ± 1.4	24.9 ± 0.7	25.9 ± 1.2
MAP-priming injection	22.3 ± 1.1	20.5 ± 2.0	22.5 ± 1.5	24.9 ± 1.1	24.6 ± 1.4	24.2 ± 1.2
HU210 (32 μg/kg, s.c.)	22.4 ± 1.9	21.1 ± 1.8	20.1 ± 2.1	24.1 ± 1.4	24.9 ± 0.7	25.9 ± 1.2

Table 3
Effect of coadministration of AM251 with cholinergic antagonists on food-taking responses in rats given the inductive stimuli (responses/min)

Inductive stimuli of the reinstatement	Nucleus accumbens core			Prelimbic cortex		
	AM251 (32 μg/side)			AM251 (32 μg/side)		
	Mecamylamine (μg/side)			Mecamylamine (μg/side)		
	0	1	3.2	0	1	3.2
MAP-associated cues	25.4 ± 1.2	24.0 ± 1.5	21.2 ± 1.8	25.6 ± 1.1	24.3 ± 0.8	25.1 ± 1.1
MAP-priming injection	21.2 ± 1.6	23.5 ± 2.0	23.3 ± 1.2	25.5 ± 0.9	26.1 ± 1.4	25.6 ± 1.0
	DHβE (μg/side)			DHβE (μg/side)		
	0	32	100	0	32	100
MAP-associated cues	25.6 ± 0.8	21.1 ± 0.8	22.1 ± 1.8	22.8 ± 1.6	24.3 ± 1.3	24.7 ± 1.1
MAP-priming injection	24.3 ± 1.3	24.7 ± 1.1	25.6 ± 0.8	23.9 ± 1.5	22.6 ± 1.1	22.0 ± 1.4
	MLA (μg/side)			MLA (μg/side)		
	0	1	3.2	0	1	3.2
MAP-associated cues	25.1 ± 0.8	25.0 ± 1.0	22.2 ± 1.0	25.6 ± 0.8	22.6 ± 1.3	21.7 ± 1.5
MAP-priming injection	22.9 ± 1.0	22.1 ± 1.6	25.1 ± 0.8	22.9 ± 1.1	22.5 ± 1.0	22.9 ± 1.0
	Scopolamine (μg/side)			Scopolamine (μg/side)		
	0	1	3.2	0	1	3.2
MAP-associated cues	22.7 ± 1.9	24.4 ± 1.9	24.3 ± 1.5	24.9 ± 0.8	25.3 ± 1.1	25.0 ± 1.1
MAP-priming injection	21.2 ± 1.6	23.5 ± 2.0	22.5 ± 1.6	24.6 ± 1.0	25.9 ± 1.1	25.0 ± 1.2

2.6. Operant task performance for food pellets

All subjects pressed a lever for food-pellet reinforcement under the FR-1 schedule 5 min after the self-administration session. Each test ended when rats had received 30 pellets. The time limit was 1200 s.

Table 4
Effect of AM251 or cholinergic antagonists alone on food-taking responses (responses/min)

Brain regions	AM251 (μg/side)		
	0	10	32
Nucleus accumbens core	25.3 ± 1.0	24.4 ± 1.1	26.0 ± 1.1
Prelimbic cortex	24.8 ± 0.9	23.8 ± 1.2	24.0 ± 0.9
	Mecamylamine (μg/side)		
	0	1	3.2
Nucleus accumbens core	23.8 ± 0.8	24.3 ± 1.0	25.2 ± 1.2
Prelimbic cortex	24.6 ± 1.3	24.1 ± 0.9	23.4 ± 0.8
	DHβE (μg/side)		
	0	32	100
Nucleus accumbens core	24.2 ± 1.0	25.1 ± 0.9	24.4 ± 1.3
Prelimbic cortex	24.3 ± 1.3	24.0 ± 1.3	23.5 ± 1.0
	MLA (μg/side)		
	0	1	3.2
Nucleus accumbens core	24.6 ± 101	24.6 ± 0.7	24.4 ± 0.9
Prelimbic cortex	24.4 ± 1.1	25.1 ± 1.1	25.1 ± 0.8
	Scopolamine (μg/side)		
	0	1	3.2
Nucleus accumbens core	22.5 ± 0.9	24.3 ± 1.9	24.7 ± 0.9
Prelimbic cortex	24.0 ± 1.2	25.1 ± 1.2	24.8 ± 1.0

2.7. Data analysis

Data represent the mean ± SEM number of lever responses. Response totals were analyzed by ANOVA (a within-subjects design). A one-way ANOVA was used to compare means, and Bonferroni–Dunn tests were used for post hoc analyses. Differences were considered significant at $P < 0.05$. All statistical analyses were performed by using the Stat View software program (v. 5.0; SAS Institute Inc., Cary, NC).

2.8. Histology

After the experiments, all rats were deeply anesthetized with pentobarbital (52 mg/kg, i.p.) and transcardially perfused with phosphate-buffered saline followed by 4% PLP (periodate lysine paraformaldehyde). The brains were removed, soaked in PLP for at least 24 h, sliced at a thickness of 80 μm, mounted on MAS-coated slides, and stained with cresyl violet. The positions of the injection cannulas were inspected under a light microscope.

3. Results

The total amount of MAP-intake was 3.3 ± 0.7 mg, i.v. for 10 days. Systemic administration of HU210 dose-dependently increased number of the lever responses in the MAP self-administration paradigm [MAP-seeking behavior, from 4.3 ± 0.7 to 23.2 ± 2.4, $F(2, 15) = 34.247$, $P < 0.001$] (Fig. 1a). Intracranial administration of HU210 into the NAC and PrC also increased lever responses [$F(2, 18) = 18.620$, $P < 0.001$, and $F(2, 18) = 40.443$, $P < 0.001$, respectively, Fig. 1b]. On the other hand, intracranial administration of HU210 into the NAC and PrC did not affect responses for food-reinforcement [food-taking responses] (Table 1, $P > 0.1$).

Re-exposure to the MAP-associated cues increased lever responses from 3.4 ± 0.7 to 30.7 ± 0.8 [$F(1, 16) = 515.165$, $P < 0.001$ compared with the NS group, Fig. 2a]. This increase was dose-dependently attenuated by the intracranial administration of

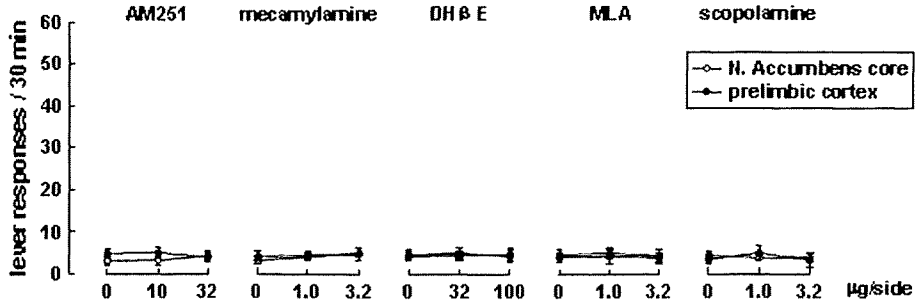

Fig. 4. Effects of intracranial priming-administration of AM251 and cholinergic antagonists alone into the nucleus accumbens core and the prelimbic cortex in MAP self-administered rats. Open and closed circles represent drug-priming injections into the nucleus accumbens core and prelimbic cortex, respectively ($n = 6$, each).

AM251 into the NAC [$F(2, 15) = 30.973$, $P < 0.001$, Fig. 2a]. MAP-priming injections also increased lever responses from 3.4 ± 0.7 to 47.8 ± 3.4 [$F(1, 16) = 308.145$, $P < 0.001$, Fig. 2a]. Similar to the cue-induced increase in lever responses, the MAP-priming injection-induced increase was also attenuated by the intracranial administration of AM251 into the NAC [$F(2, 15) = 34.475$, $P < 0.001$, Fig. 2a]. Correlation coefficients between the total amount of MAP-intake and number of active lever responses induced by MAP-associated cues ($r = 0.343$, $P < 0.01$) and MAP-priming injections ($r = -0.481$, $P < 0.001$) were revealed. As to the PrC, increased lever responses induced by MAP-associated cues and MAP-priming injections were also attenuated by the intracranial administration of AM251 [$F(2, 15) = 41.524$, $P < 0.001$ and $F(2, 15) = 95.203$, $P < 0.001$, respectively, Fig. 2a]. The intracranial administration of AM251 into the NAC and PrC also attenuated the increase in lever responses induced by systemic HU210 treatment [$F(2, 15) = 69.847$, $P < 0.001$, and $F(2, 15) = 40.745$, $P < 0.001$, respectively, Fig. 2b]. In contrast, intracranial administration of AM251 into the NAC and PrC did not affect food-taking responses in rats given the MAP-associated cues, and MAP- and HU210 (32 μg/kg, s.c.)-priming injections (Table 2, $P > 0.1$).

Mecamylamine (1.0–3.2 μg/side, intra-NAC) dose-dependently antagonized the attenuation of lever responding induced by intra-NAC AM251 [$F(2, 15) = 35.002$ (cues) and 31.138 (MAP-priming injection), respectively, $P < 0.01$, Fig. 3a]. With regard to the PrC, intracranial administration of mecamylamine also blocked the attenuating effect of the intracranial administration of AM251 on lever pressing [$F(2, 15) = 28.078$ (cues) and 51.028 (MAP-priming injection), respectively, $P < 0.001$ Fig. 3a]. However, scopolamine (1.0–3.2 μg/side, intra-NAC and intra-PrC, respectively) did not block the intra-NAC and intra-PrC AM251-induced attenuation of the lever responses induced by each stimulus ($P > 0.3$, Fig. 3a). On the other hand, intra-NAC DHβE blocked the attenuation of lever pressing induced by intra-NAC AM251 [$F(2, 15) = 38.504$ (cues) and 94.037 (MAP-priming injection), $P < 0.001$, Fig. 3b]. In the PrC, intracranial administration of DHβE also antagonized the attenuation induced by the intracranial administration of AM215 [$F(2, 15) = 67.111$ (cues) and 48.347 (MAP-priming injection), $P < 0.001$, Fig. 3b]. However, MLA (1.0–3.2 μg/side, intra-NAC and intra-PrC, respectively) did not block the intra-NAC and intra-PrC AM251-induced attenuation ($P > 0.7$, Fig. 3b). Meanwhile, the coadministration of AM251 with cholinergic antagonists did not affect food-taking responses (Table 3, $P > 0.1$).

Microinjection of neither AM251 nor cholinergic antagonists alone into the NAC and PrC reinstated MAP-seeking behavior (Fig. 4). In addition, these treatments did not alter food-taking responses (Table 4, $P > 0.1$).

4. Discussion

MAP-associated cues and MAP-priming injections reinstated MAP-seeking behavior. Systemic administration of HU210, a cannabinoid CB1R agonist, also reinstated this behavior. Reinstatement produced by these three stimuli was attenuated by the intracranial administration of AM251, a CB1R antagonist, into the NAC and PrC. The treatments did not affect food-taking behavior, indicating these effects of AM251 to be due to specific behavioral effect on MAP. These findings suggest that CB1Rs in these two regions have an important role in reinstatement of MAP-seeking behavior. Regarding the cannabinoid system, this is the first identification of the regions responsible for MAP cravings. In each region, the attenuating effect of AM251 was blocked by mecamylamine, a non-selective nAChR antagonist, but not by scopolamine, an mAChR antagonist. The effects of mecamylamine were mimicked by DHβE, but not by MLA, an α4β2 and α7 nAChR antagonist, respectively. These findings suggest an important role of interaction between CB1Rs and α4β2 nAChRs in reinstatement of MAP-seeking behavior. Recently, CB1R agonists, such as Δ^9-tetrahydrocannabinol (THC) and WIN55,212-2, inhibited ACh release in the mPFC and hippocampus (Gessa et al., 1998; Tzavara et al., 2003b). These inhibitory effects were suppressed by SR141716A, a CB1R antagonist. SR141716A alone promoted ACh release in both regions (Gessa et al., 1998; Tzavara et al., 2003b). These findings suggest the cannabinoid system to be an inhibitory modulator of AChrgic transmission. Considering this inhibitory regulation of CB1Rs, our findings suggest that relapses to MAP-seeking behavior may be due to two steps, first inhibition of ACh transmission by the activation of CB1Rs and then the inactivation of α4β2 nAChRs. Additionally, we previously reported that SR141716A, nicotine, and donepezil, an acetylcholinesterase inhibitor, attenuated reinstatement of MAP-seeking behavior (Anggadiredja et al., 2004a; Hiranita et al., 2004, 2006). Nicotine and ACh have greater affinity for α4β2 (K_i values: 0.79 and 44 nM) than for α7 nAChRs (5000 and 14,300 nM) (Decker et al., 1995; Gotti et al., 2006). Considering this preference of nicotine and ACh for α4β2 nAChRs, our previous and present findings support an important role for α4β2 nAChRs in the reinstatement of drug-seeking behavior.

In our previous study, systemic administration of mecamylamine alone neither reinstated MAP-seeking behavior nor potentiated the reinstatement induced by cues and MAP-priming injections (Hiranita et al., 2006). The present study demonstrates that intracranial injection of neither mecamylamine nor DHβE alone reinstated MAP-seeking behavior. Therefore, these results suggest the functional normalization of α4β2 nAChRs to be important for the blockade of reinstatement of MAP-seeking behavior. However, ACh and nicotine have less affinity for α7 than for α4β2 nAChRs (Decker et al., 1995; Gotti et al., 2006). Therefore, these findings suggest that activation of α7 nAChRs through 'endogenous' ACh might not be enough to produce a behavioral effect. Meanwhile, CB1Rs in the NAC and PrC have an inhibitory role in glutamatergic transmission (Mackie, 2005; Robbe et al., 2001), whereas endocannabinoid is involved in the neural plasticity of glutamatergic neurons between these two regions (Robbe et al., 2003). Recently, the activation of glutamatergic neurons was

reported to reinstate cocaine-seeking behavior (Kalivas and McFarland, 2003). Although the present results did not demonstrate a role for α7 nAChRs in reinstatement, α7 nAChRs are expressed in glutamatergic terminals from the cortex to the striatum and ventral tegmental area (VTA) (Mansvelder et al., 2002). Therefore, the distribution of α7 nAChRs has a very similar distribution to that of CB1Rs. This finding suggests the activation of glutamatergic transmission via α7 nAChRs to contribute to the reinstatement of drug-seeking behavior. Therefore, α4β2 and α7 nAChRs might have opposite roles in the reinstatement of drug-seeking behavior, inhibitory and facilitatory, respectively.

This report is the first indication that α4β2, but not α7, nAChRs have an inhibitory role in relapse to drug-seeking behavior. To attenuate the relapse, it may be important to target the rewarding property of α4β2 nAChRs. Indeed, regarding nicotine, DHβE reduced self-administration (Corrigall et al., 1994; Grottick et al., 2000) and conditioned place preference (Walters et al., 2006), whereas MLA affected neither self-administration (Grottick et al., 2000) nor the threshold elevation of electric brain stimulation during nicotine withdrawal (Markou and Paterson, 2001). α4β2 nAChR knockout mice showed decreases in nicotine place preference (Cincotta et al., 2008). Mutant mice lacking the β2 subunit showed decreased nicotine self-administration relative to the wild-type mice (Picciotto et al., 1998). The mice did not exhibit nicotine place preference, while α7 nAChR knockout mice did (Cincotta et al., 2008; Walters et al., 2006). Considering the rewarding property of α4β2 nAChRs, activation of this receptor may be important in preventing cravings for not only MAP but also other abusive drugs.

There is evidence demonstrating an involvement of α7 nAChRs in the effects of CB1R agonists. In rats, systemic administration of MLA, but not DHβE, antagonized the discriminative effects of Δ^9-THC and reduced self-administration of WIN55,212-2 (Solinas et al., 2007). Meanwhile, we previously showed that effect of a cannabinoid agonist altered the MAP withdrawal/extinction state. Repeated administration of the cannabinoid agonist, Δ^8-THC, during the extinction phase, suppressed reinstatement of MAP-seeking behavior induced by cues and a MAP-priming injection (Anggadiredja et al., 2004a). However, after extinction training, although Δ^8-THC had no effect by itself, coadministration of the agonist and MAP at small doses reinstated MAP-seeking behavior (Anggadiredja et al., 2004a). These findings suggest that the interaction between nAChR subtypes and the cannabinoid system may switch from α7 to α4β2 nAChRs before and after MAP withdrawal/extinction. In contrast to the cannabinoid system, α4β2, but not α7, nAChR, contributed to the effect of nicotine effects before and after withdrawal. As shown above, α4β2, but not α7, nAChRs contribute to nicotine reinforcement before nicotine withdrawal (Corrigall et al., 1994; Grottick et al., 2000). During the nicotine withdrawal phase, the administration of DHβE precipitated the signs of withdrawal (Malin et al., 1998), whereas MLA did not affect the threshold elevation of electric brain stimulation during nicotine withdrawal (Markou and Paterson, 2001).

The present study showed an interaction between CB1Rs and α4β2 nAChRs in the NAC and PrC for the reinstatement of MAP-seeking behavior. Other regions might also be involved. We previously demonstrated the involvement of the amygdala and hippocampus in reinstatement of MAP-seeking behavior (Hiranita et al., 2006). The involvement of information processing from these two regions to the PrC or NAC in reinstatement is reported (Di Ciano and Everitt, 2004; Fuchs et al., 2007; Miller and Marshall, 2005). Additionally, intra-amygdala SR141716A failed to affect cue-induced reinstatement of heroin-seeking behavior (Alvarez-Jaimes et al., 2007). Therefore, it is unlikely that there is interaction between CB1Rs and nAChRs in the amygdala as there is in the NAC and PrC. Whether such interaction occurs in the hippocampus, however, remains to be elucidated. Meanwhile, several studies reported the involvement of nAChRs in the VTA in the behavioral effects of nicotine. Microinjection of DHβE into the VTA reduced nicotine self-administration (Corrigall et al., 1994). In a place conditioning procedure, DHβE was found to block both the rewarding and the aversive properties of intra-VTA nicotine (Laviolette and van der Kooy, 2003). However, MLA blocked nicotine reward and switched the motivational valence from rewarding to aversive (Laviolette and van der Kooy, 2003). Nicotine produced a reduction in intracranial self-stimulation threshold, while intra-VTA MLA attenuated the effect of nicotine (Panagis et al., 2000). On the other hand, rats self-administered Δ^9-THC into the VTA (Zangen et al., 2006). As to the VTA, these findings may suggest the involvement of not only α4β2 but also α7 nAChRs in the cannabinoid system. Therefore, the manner in which CB1R and nAChR subtypes interact might not be the same in each region of the brain.

Despite differences in brain regions and incentive stimuli, AM251 and DHβE on reinstatement of MAP-seeking behavior were equipotent in their effect. In our previous study, nicotine equipotently attenuated reinstatement induced by cues or MAP-priming injections (Hiranita et al., 2006). The effects of nicotine were attenuated by mecamylamine at the same dose range (Hiranita et al., 2006). In other laboratories, the microinjection of SR141716A into different brain regions equipotently attenuated heroin- (Alvarez-Jaimes et al., 2007) and nicotine-seeking behavior (Kodas et al., 2007) induced by cues. Additionally, systemic administration of SR141716A equipotently attenuated the reinstatement of heroin-seeking behavior induced by cues or heroin-priming injections (De Vries et al., 2003). However, cannabinoid CB1Rs are expressed more densely in the cortex than in the nucleus accumbens (Mackie, 2005), whereas both α4β2 and α7 nAChRs are expressed densely in the PFC and striatum (Gotti et al., 2006). We previously reported that an opioid receptor antagonist, naltrexone, attenuated the reinstatement of MAP-seeking behavior induced by cues, but not MAP-priming injections (Anggadiredja et al., 2004b). A corticotrophin-releasing factor receptor antagonist, CP-154,526, attenuated reinstatement induced by cues more effectively than that induced by MAP-priming injections (Moffett and Goeders, 2007). Considering these differences, it is very surprising that singular mechanisms regulate reinstatement.

In summary, the present study demonstrated that CB1Rs and α4β2 nAChRs play a key role in the relapse to MAP-seeking behavior. These findings further provide support for considering substances that inactivate cannabinoid CB1Rs and α4β2 nAChR agonists for relieving drug cravings.

Acknowledgements

We thank Dr. Paul L. Soto, National Institute on Drug Abuse, for checking grammar and spelling of this manuscript. This study was supported by Grants-in-Aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labor, and Welfare and Japan and Smoking Research Foundation, Japan.

References

Alvarez-Jaimes, L., Polis, I., Parsons, L.H., 2007. Attenuation of cue-induced heroin-seeking behavior by cannabinoid CB(1) antagonist infusions into the nucleus accumbens core and prefrontal cortex, but not basolateral amygdala. Neuropsychopharmacology.

Anggadiredja, K., Nakamichi, M., Hiranita, T., Tanaka, H., Shoyama, Y., Watanabe, S., Yamamoto, T., 2004a. Endocannabinoid system modulates relapse to methamphetamine seeking: possible mediation by the arachidonic acid cascade. Neuropsychopharmacology 29, 1470–1478.

Anggadiredja, K., Sakimura, K., Hiranita, T., Yamamoto, T., 2004b. Naltrexone attenuates cue- but not drug-induced methamphetamine seeking: a possible

mechanism for the dissociation of primary and secondary reward. Brain Res. 1021, 272–276.
Cincotta, S.L., Yorek, M.S., Moschak, T.M., Lewis, S.R., Rodefer, J.S., 2008. Selective nicotinic acetylcholine receptor agonists: potential therapies for neuropsychiatric disorders with cognitive dysfunction. Curr. Opin. Investig. Drugs 9, 47–56.
Corrigall, W.A., Coen, K.M., Adamson, K.L., 1994. Self-administered nicotine activates the mesolimbic dopamine system through the ventral tegmental area. Brain Res. 653, 278–284.
De Vries, T.J., Homberg, J.R., Binnekade, R., Raaso, H., Schoffelmeer, A.N., 2003. Cannabinoid modulation of the reinforcing and motivational properties of heroin and heroin-associated cues in rats. Psychopharmacology (Berl.) 168, 164–169.
Decker, M.W., Brioni, J.D., Bannon, A.W., Arneric, S.P., 1995. Diversity of neuronal nicotinic acetylcholine receptors: lessons from behavior and implications for CNS therapeutics. Life Sci. 56, 545–570.
Di Ciano, P., Everitt, B.J., 2004. Direct interactions between the basolateral amygdala and nucleus accumbens core underlie cocaine-seeking behavior by rats. J. Neurosci. 24, 7167–7173.
Fuchs, R.A., Eaddy, J.L., Su, Z.I., Bell, G.H., 2007. Interactions of the basolateral amygdala with the dorsal hippocampus and dorsomedial prefrontal cortex regulate drug context-induced reinstatement of cocaine-seeking in rats. Eur. J. Neurosci. 26, 487–498.
Gessa, G.L., Casu, M.A., Carta, G., Mascia, M.S., 1998. Cannabinoids decrease acetylcholine release in the medial–prefrontal cortex and hippocampus, reversal by SR 141716A. Eur. J. Pharmacol. 355, 119–124.
Gotti, C., Zoli, M., Clementi, F., 2006. Brain nicotinic acetylcholine receptors: native subtypes and their relevance. Trends Pharmacol. Sci. 27, 482–491.
Grottick, A.J., Wyler, R., Higgins, G.A., 2000. The alpha4beta2 agonist SIB 1765F, but not the alpha7 agonist AR-R 17779, cross-sensitises to the psychostimulant effects of nicotine. Psychopharmacology (Berl.) 150, 233–236.
Hiranita, T., Anggadiredja, K., Fujisaki, C., Watanabe, S., Yamamoto, T., 2004. Nicotine attenuates relapse to methamphetamine-seeking behavior (craving) in rats. Ann. N.Y. Acad. Sci. 1025, 504–507.
Hiranita, T., Nawata, Y., Sakimura, K., Anggadiredja, K., Yamamoto, T., 2006. Suppression of methamphetamine-seeking behavior by nicotinic agonists. Proc. Natl. Acad. Sci. U.S.A. 103, 8523–8527.
Kalivas, P.W., McFarland, K., 2003. Brain circuitry and the reinstatement of cocaine-seeking behavior. Psychopharmacology (Berl.) 168, 44–56.
Kodas, E., Cohen, C., Louis, C., Griebel, G., 2007. Cortico-limbic circuitry for conditioned nicotine-seeking behavior in rats involves endocannabinoid signaling. Psychopharmacology (Berl.) 194, 161–171.
Laviolette, S.R., van der Kooy, D., 2003. The motivational valence of nicotine in the rat ventral tegmental area is switched from rewarding to aversive following blockade of the alpha7-subunit-containing nicotinic acetylcholine receptor. Psychopharmacology (Berl.) 166, 306–313.
Mackie, K., 2005. Distribution of cannabinoid receptors in the central and peripheral nervous system. Handb. Exp. Pharmacol., 299–325.
Malin, D.H., Lake, J.R., Upchurch, T.P., Shenoi, M., Rajan, N., Schweinle, W.E., 1998. Nicotine abstinence syndrome precipitated by the competitive nicotinic antagonist dihydro-beta-erythroidine. Pharmacol. Biochem. Behav. 60, 609–613.
Mansvelder, H.D., Keath, J.R., McGehee, D.S., 2002. Synaptic mechanisms underlie nicotine-induced excitability of brain reward areas. Neuron 33, 905–919.
Markou, A., Paterson, N.E., 2001. The nicotinic antagonist methyllycaconitine has differential effects on nicotine self-administration and nicotine withdrawal in the rat. Nicotine Tob. Res. 3, 361–373.
Miller, C.A., Marshall, J.F., 2005. Altered Fos expression in neural pathways underlying cue-elicited drug seeking in the rat. Eur. J. Neurosci. 21, 1385–1393.
Moffett, M.C., Goeders, N.E., 2007. CP-154,526, a CRF type-1 receptor antagonist, attenuates the cue-and methamphetamine-induced reinstatement of extinguished methamphetamine-seeking behavior in rats. Psychopharmacology (Berl.) 190, 171–180.
Oz, M., 2006. Receptor-independent actions of cannabinoids on cell membranes: focus on endocannabinoids. Pharmacol. Ther. 111, 114–144.
Panagis, G., Kastellakis, A., Spyraki, C., Nomikos, G., 2000. Effects of methyllycaconitine (MLA), an alpha 7 nicotinic receptor antagonist, on nicotine- and cocaine-induced potentiation of brain stimulation reward. Psychopharmacology (Berl.) 149, 388–396.
Picciotto, M.R., Zoli, M., Rimondini, R., Lena, C., Marubio, L.M., Pich, E.M., Fuxe, K., Changeux, J.P., 1998. Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. Nature 391, 173–177.
Robbe, D., Alonso, G., Duchamp, F., Bockaert, J., Manzoni, O.J., 2001. Localization and mechanisms of action of cannabinoid receptors at the glutamatergic synapses of the mouse nucleus accumbens. J. Neurosci. 21, 109–116.
Robbe, D., Alonso, G., Manzoni, O.J., 2003. Exogenous and endogenous cannabinoids control synaptic transmission in mice nucleus accumbens. Ann. N.Y. Acad. Sci. 1003, 212–225.
Shalev, U., Grimm, J.W., Shaham, Y., 2002. Neurobiology of relapse to heroin and cocaine seeking: a review. Pharmacol. Rev. 54, 1–42.
Shoaib, M., 2008. The cannabinoid antagonist AM251 attenuates nicotine self-administration and nicotine-seeking behaviour in rats. Neuropharmacology 54, 438–444.
Solinas, M., Scherma, M., Fattore, L., Stroik, J., Wertheim, C., Tanda, G., Fratta, W., Goldberg, S.R., 2007. Nicotinic alpha 7 receptors as a new target for treatment of cannabis abuse. J. Neurosci. 27, 5615–5620.
Spivak, C.E., Lupica, C.R., Oz, M., 2007. The endocannabinoid anandamide inhibits the function of alpha4beta2 nicotinic acetylcholine receptors. Mol. Pharmacol. 72, 1024–1032.
Tzavara, E.T., Davis, R.J., Perry, K.W., Li, X., Salhoff, C., Bymaster, F.P., Witkin, J.M., Nomikos, G.G., 2003a. The CB1 receptor antagonist SR141716A selectively increases monoaminergic neurotransmission in the medial prefrontal cortex: implications for therapeutic actions. Br. J. Pharmacol. 138, 544–553.
Tzavara, E.T., Wade, M., Nomikos, G.G., 2003b. Biphasic effects of cannabinoids on acetylcholine release in the hippocampus: site and mechanism of action. J. Neurosci. 23, 9374–9384.
Walters, C.L., Brown, S., Changeux, J.P., Martin, B., Damaj, M.I., 2006. The beta2 but not alpha7 subunit of the nicotinic acetylcholine receptor is required for nicotine-conditioned place preference in mice. Psychopharmacology (Berl.) 184, 339–344.
Zangen, A., Solinas, M., Ikemoto, S., Goldberg, S.R., Wise, R.A., 2006. Two brain sites for cannabinoid reward. J. Neurosci. 26, 4901–4907.

Endocannabinoids selectively enhance sweet taste

Ryusuke Yoshida[a,1], Tadahiro Ohkuri[a,1], Masafumi Jyotaki[a], Toshiaki Yasuo[a], Nao Horio[a], Keiko Yasumatsu[a], Keisuke Sanematsu[a], Noriatsu Shigemura[a], Tsuneyuki Yamamoto[b], Robert F. Margolskee[c], and Yuzo Ninomiya[a,2]

[a]Section of Oral Neuroscience, Graduate School of Dental Sciences, Kyushu University, Fukuoka 812-8582, Japan; [b]Department of Pharmacology, Faculty of Pharmaceutical Sciences, Nagasaki International University, Nagasaki 859-3298, Japan; and [c]Monell Chemical Senses Center, Philadelphia, PA 19104-3308

Edited by Linda M. Bartoshuk, University of Florida, Gainesville, FL, and approved November 30, 2009 (received for review October 19, 2009)

Endocannabinoids such as anandamide [N-arachidonoylethanolamine (AEA)] and 2-arachidonoyl glycerol (2-AG) are known orexigenic mediators that act via CB_1 receptors in hypothalamus and limbic forebrain to induce appetite and stimulate food intake. Circulating endocannabinoid levels inversely correlate with plasma levels of leptin, an anorexigenic mediator that reduces food intake by acting on hypothalamic receptors. Recently, taste has been found to be a peripheral target of leptin. Leptin selectively suppresses sweet taste responses in wild-type mice but not in leptin receptor-deficient *db/db* mice. Here, we show that endocannabinoids oppose the action of leptin to act as enhancers of sweet taste. We found that administration of AEA or 2-AG increases gustatory nerve responses to sweeteners in a concentration-dependent manner without affecting responses to salty, sour, bitter, and umami compounds. The cannabinoids increase behavioral responses to sweet-bitter mixtures and electrophysiological responses of taste receptor cells to sweet compounds. Mice genetically lacking CB_1 receptors show no enhancement by endocannnabinoids of sweet taste responses at cellular, nerve, or behavioral levels. In addition, the effects of endocannabinoids on sweet taste responses of taste cells are diminished by AM251, a CB_1 receptor antagonist, but not by AM630, a CB_2 receptor antagonist. Immunohistochemistry shows that CB_1 receptors are expressed in type II taste cells that also express the T1r3 sweet taste receptor component. Taken together, these observations suggest that the taste organ is a peripheral target of endocannabinoids. Reciprocal regulation of peripheral sweet taste reception by endocannabinoids and leptin may contribute to their opposing actions on food intake and play an important role in regulating energy homeostasis.

energy homeostasis | gustation | reciprocal regulation

Endocannabinoids such as anandamide [N-arachidonoylethanolamine (AEA)] and 2-arachidonoyl glycerol (2-AG) are known orexigenic mediators that act via CB_1 receptors in hypothalamus and limbic forebrain to induce appetite (1, 2) and stimulate food intake (3). Systemic administration of exogenous cannabinoids or endocannabinoids in rodents causes hyperphagia (4) and increases the preference for palatable substances such as sucrose solution or food pellets (5, 6). These effects are mediated by the CB_1 receptor: pretreatment with the CB_1 antagonist SR141716 inhibited hyperphagia and reduced consumption of both bland and palatable foods (4–6). The natural "liking" reactions of rats to sweet compounds were amplified by endogenous cannabinoid signals in nucleus accumbens (7). Thus, endocannabinoids may be related to hedonic aspects of sweet taste.

There is growing evidence that taste function can be modulated by hormones or other factors that act on receptors present in the peripheral gustatory system. Leptin, an anorexigenic mediator that reduces food intake by acting on hypothalamic receptors (8), selectively suppresses sweet taste responses and these effects may be mediated by leptin receptor, Ob-Rb (9–11). GLP-1, an incretin that influences glucose transport, metabolism, and homeostasis (12), normally acts to maintain or enhance sweet taste sensitivity by its paracrine activity (13). We sought to determine whether cannabinoids affect peripheral sweet taste reception. In the present study, we investigated neural, behavioral, and cellular responses to taste stimuli before and after administration of endocannabinoids. We demonstrated that sweet taste responses are selectively enhanced by administration of endocannabinoids AEA and 2-AG, and that the sweet enhancing effect of enndocannabinoids was mediated by CB_1 receptors, which are coexpressed in taste cells with the sweet receptor component T1r3.

Results and Discussion

Gustatory Nerve Responses. We examined potential effects of endocannabinoids on gustatory nerve responses to various taste stimuli and involvement of CB_1 receptors in the effects by using wild-type (WT) and $CB_1^{-/-}$ mice (14). Because mouse responses to sweet substances are much larger in the chorda tympani (CT) nerve innervating the anterior tongue than in the glossopharyngeal (GL) nerve innervating the posterior tongue (15, 16) we focused on CT nerve responses. We recorded CT taste responses after administration of vehicle (saline with less than 0.5% ethanol) or cannabinoids AEA and 2-AG. After i.p. injection of 2-AG, CT nerve responses of WT mice to sweeteners increased significantly [Fig. 1*A*: sucrose ($P < 0.001$, t test), saccharin ($P < 0.01$), glucose and SC45647 ($P < 0.05$)]. After injection of endocannabinoids, increased responses to sweet compounds (~150% of control for 500 mM sucrose) were observed at 10–30 min postinjection and then recovered to the control level at 60–120 min postinjection (Fig. S1). In marked contrast, 2-AG had no such effect in WT mice on responses to salty (NaCl), bitter (quinine), sour (HCl), or umami [MSG: monosodium l-glutamate (in the presence of 10 μM amiloride, a sodium response inhibitor)] substances ($P > 0.1$; Fig. 1*B* and Fig. S2), suggesting that the effect of 2-AG is highly specific for sweet taste. The sweet enhancing effect of 2-AG was absent in $CB_1^{-/-}$ mice ($P > 0.1$; Fig. 1 *A* and *C*), indicating that 2-AG is acting on sweet taste via the CB_1 receptor. The effect of endocannabinoids on sweet responses in WT mice was dose dependent (Fig. 1*D*), saturated at ≈1 mg/kg body weight (bw) of the AEA or 2-AG injected. 2-AG has a higher affinity than AEA for the CB_1 receptor (17): consistent with the published report, the EC_{50} of AEA (0.185 mg/kg bw) was approximately 3-fold greater than that of 2-AG (0.055 mg/kg bw), but the maximum effect at the saturating concentration was not significantly different between 2-AG and AEA. At a dose of 1 mg/kg bw of 2-AG, the CT nerve responses to ~10–500 mM sucrose were significantly enhanced in WT ($F_{(1,79)} = 5.68, P < 0.05$) but not in $CB_1^{-/-}$ mice ($F_{(1,76)} = 0.73$, $P > 0.1$; Fig. 1*E*). Similar effects of 2-AG were observed in GL nerve responses (Fig. S3).

Author contributions: Y.N. designed research; R.Y., T.O., M.J., T. Yasuo, N.H., K.Y., K.S., and N.S. performed research; T. Yamamoto and R.F.M. contributed new reagents/analytic tools; R.Y., T.O., and Y.N. analyzed data; and R.Y., T.O., R.F.M., and Y.N. wrote the paper.

Conflict of interest statement: R.F.M. has a personal financial interest in the form of stock ownership in the Redpoint Bio company and is an inventor on patents and patent applications, which have been licensed to Redpoint Bio. All other authors declare no competing financial interests.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]R.Y. and T.O. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: yuninom@dent.kyushu-u.ac.jp.

This article contains supporting information online at www.pnas.org/cgi/content/full/0912048107/DCSupplemental.

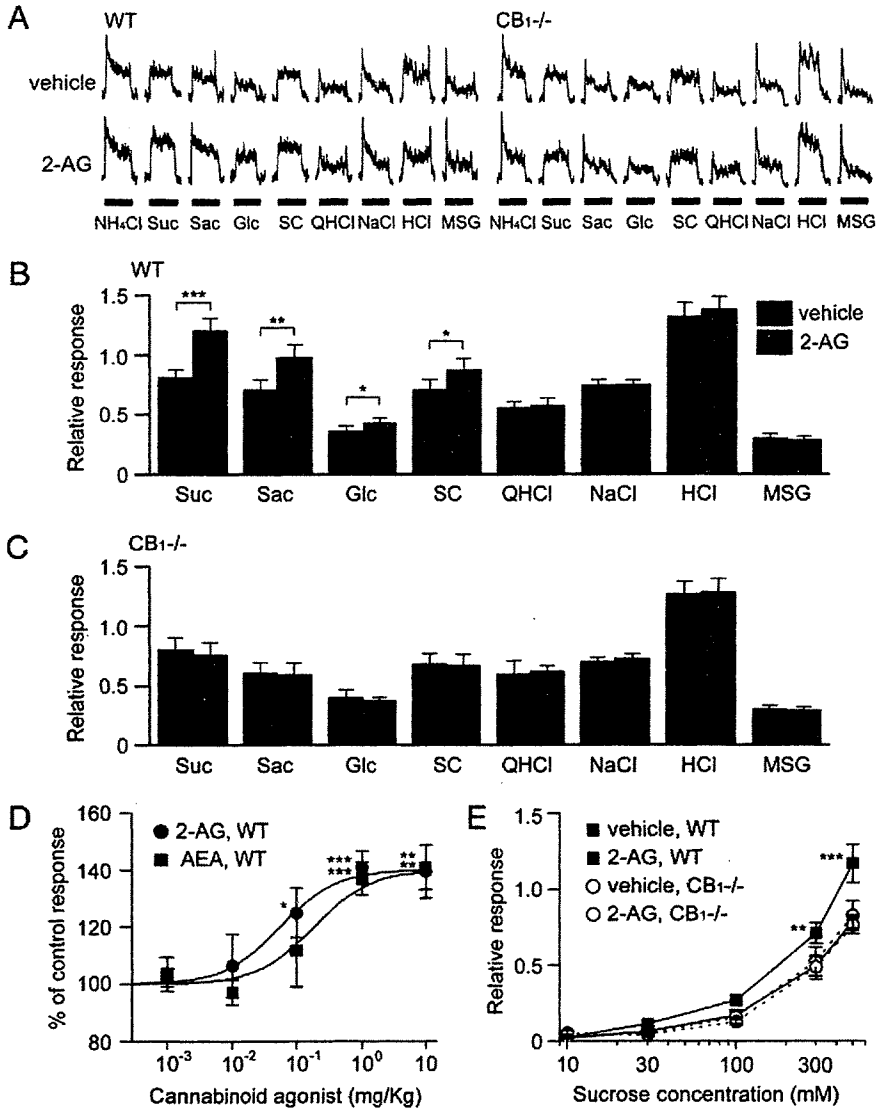

Fig. 1. Endocannabinoids enhance gustatory nerve responses to sweeteners. (*A*) Typical examples of CT nerve responses of WT and $CB_1^{-/-}$ mice showing the effect of i.p. injection of 1 mg/kg bw of 2-AG (*Lower* traces) vs. vehicle-injected control (*Upper* traces). CT nerve responses (normalized to response to 100 mM NH_4Cl) of WT (*B*) and $CB_1^{-/-}$ (*C*) mice stimulated by sweet (Suc, 500 mM sucrose; Sac, 20 mM saccharin; Glc, 500 mM glucose; SC, 1 mM SC45647), bitter (QHCl, 20 mM quinine-HCl), salty (NaCl, 100 mM NaCl), sour (HCl, 10 mM HCl), and umami (MSG, 100 mM monosodium glutamate + 10 μM amiloride) compounds 10–30 min after administration of vehicle (black bars) or 1 mg/kg bw of 2-AG (red bars) (n = 5–10). (*D*) Dose-dependent effect of AEA (blue symbols) or 2-AG (red symbols) treatment on normalized chorda tympani nerve responses to 500 mM sucrose (n = 5–14). (*E*) Concentration-dependent responses to sucrose 10–30 min after administration of vehicle (black symbols) or 1 mg/kg bw of 2-AG (red symbols) in WT (squares) (n = 7) and $CB_1^{-/-}$ (circles) mice (n = 5). Asterisks indicate significant differences from control (*P < 0.05; **P < 0.01; ***P < 0.001; Fisher's PLSD post hoc test or t test). All data are presented as the mean ± SEM.

Behavioral Responses. Next, we asked whether the enhancement of sweet responses of gustatory nerves by endocannabinoids would alter behavioral responses of mice to sweet stimuli. We used a short-term lick test and measured the number of licks (per 10 s) for test stimuli after administration of vehicle or 2-AG. Numbers of licks per 10 s for distilled water (DW) and sucrose at various concentrations in water-deprived mice are typically similar, within a range of about 60–80, whereas lick responses for 1 mM quinine are around 10. To more clearly detect concentration-dependent changes in lick rates for sucrose, we used mixtures of 1 mM quinine and ~30–500 mM sucrose (a sweet-bitter mixture paradigm) (10) as test solutions. As shown in Fig. 2, lick rates for sucrose-quinine mixtures increased with increasing sucrose concentration in both WT and $CB_1^{-/-}$ mice (Fig. 2 *A* and *B*), indicating clear concentration dependencies. Thirty minutes after i.p. injection of 1 mg/kg bw of 2-AG (Fig. 2*A*) or AEA (Fig. 2*B*) in WT mice, mean lick rates for the sucrose-quinine mixtures at different concentrations of sucrose were significantly greater than before injection with 2-AG ($F_{(1,39)}$ = 16.6, P < 0.01) or AEA ($F_{(1,39)}$ = 16.4, P < 0.01). However, administration of 2-AG and AEA in WT mice did not affect lick rates for NaCl (300 and 1,000 mM), HCl (3 and 10 mM), quinine (0.3 and 1 mM), or MSG (100 and 300 mM) + quinine (1 mM) (Fig. 2 *C* and *D*), indicating a selective increase in the responses to the sucrose component of the sweet-bitter mixtures. In marked contrast, the sweet enhancing effect of 2-AG and AEA was not observed in $CB_1^{-/-}$ mice ($F_{(1,39)}$ = 1.35 for 2-AG, $F_{(1,47)}$ = 0.10 for AEA, P > 0.1, Fig. 2 *A* and *B*), again indicating the involvement of CB_1 receptors in the 2-AG effect on sweet responses. The time course for the effect of injected 2-AG on lick rates was comparable with that of CT nerve responses (Fig. S4). Lick rates for 500 mM sucrose plus 1 mM quinine started increasing ~10 min after injection, reached a maximum level of enhancement 10–30 min after injection (about 140% of control for 500 mM sucrose), and recovered to the control level ≈2 h after injection of 2-AG (Fig. S4). Collectively, both nerve and behavioral response measurements indicate that administration of endocannabinoids selectively enhances sweet taste responses and the endocannabinoid effect is mediated by their receptor, CB_1.

Taste Cell Responses. We next sought to determine whether the endocannabinoid effect occurred at the taste cell level. In fungiform taste buds, which are located on the anterior tongue innervated by the CT nerve, we found many taste cells (TCs) that responded to a sweetener (~1–20 mM saccharin) with action potentials (18). To identify taste cells expressing T1r3, a component of both sweet and umami receptors (19–23), we used trans-

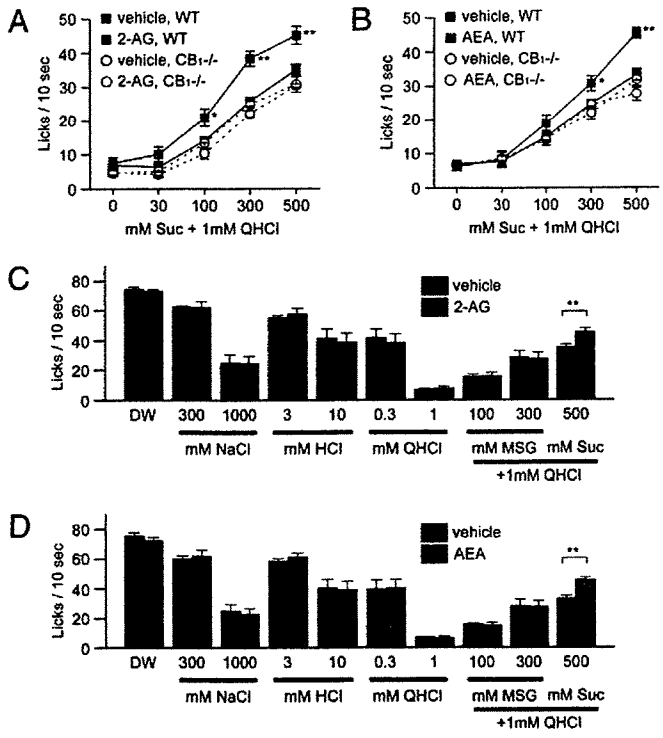

Fig. 2. Endocannabinoids enhance behavioral responses to sweeteners. Concentration response relationships to varying concentrations of sucrose in mixtures with 1 mM quinine 30 min after i.p. injection of vehicle (black symbols) or 1 mg/kg bw 2-AG (red symbols) (*A*) and AEA (blue symbols) (*B*) in WT (squares) and $CB_1^{-/-}$ (circles) mice ($n = 5$). Lick responses to distilled water (DW), NaCl (300 and 1,000 mM), HCl (3 and 10 mM), quinine (QHCl; 0.3 and 1 mM), MSG + 1 mM quinine (MSG; 100 and 300 mM), and sucrose + 1 mM quinine (Suc; 500 mM) 30 min after administration of vehicle (black bars), 1 mg/kg bw 2-AG (red bars) (*C*), or AEA (blue bars) (*D*) in WT mice ($n = 5$). Asterisks indicate significant differences (*$P < 0.05$; **$P < 0.01$; Fisher's PLSD post hoc test or *t* test). All data are presented as the mean ± SEM.

genic mice that express green fluorescent protein (GFP) from the T1r3 promoter (24) and recorded taste responses from these cells (Fig. 3*A*). As expected, T1r3-GFP taste cells responded to multiple sweeteners such as sucrose, glucose, saccharin, SC45647, and D-phenylalanine (Fig. S5); responses to saccharin were enhanced by basolateral treatment of 1 μg/mL 2-AG (Fig. 3*A*). We tested the effect of basolateral application of AEA and 2-AG on responses of T1r3-GFP taste cells and sweet sensitive taste cells in WT mice. In total, 27 of 47 (57%, 22 of 39 in WT, 5 of 8 in T1r3-GFP mice) cells showed enhancement of responses to sweeteners (>120% of control) after application of 1 μg/mL AEA or 2-AG to the basolateral side of taste cell membrane. Responses of TCs to saccharin were significantly increased after application of 1 μg/mL AEA ($P < 0.01$, $n = 28$) or 2-AG ($P < 0.01$, $n = 19$, *t* test). The enhancing effects of AEA and 2-AG on sweet responses of TCs in WT mice saturated at ~1 μg/mL (Fig. 3*B*). We found the half maximal effective concentration (EC_{50}) for enhancing sweet responses of WT TCs by AEA (0.112 μg/mL) was about 6-fold greater than that of 2-AG (0.017 μg/mL). The effective concentrations of the endocannabinoids are within physiological ranges found in various tissues (25). In $CB_1^{-/-}$ mice, sweet responses of TCs were not affected by 1 μg/mL AEA (95.3 ± 5.3%, $n = 7$, Fig. 3*B*, open circle) or 2-AG (99.8 ± 6.3%, $n = 5$, Fig. 3*B*, open rectangle). A pharmacological blocker of CB_1 receptors, AM251, suppressed the sweet enhancing effect of 1 μg/mL 2-AG ($P < 0.05$, $n = 6$, Fig. 3*C*); however, the CB_2 receptor blocker AM630 did not ($P > 0.1$, $n = 5$, Fig. 3*D*). These data indicate that endocannabinoids act on CB_1 receptors to enhance sweet taste responses of TCs.

Expression of the CB_1 Receptor. We next tested whether TCs express the CB_1 receptor. In RT-PCR experiments (Fig. 4*A*), the mRNAs for α-gustducin (a taste selective G protein α-subunit) (26), CB_1 and CB_2 receptors were all expressed in taste buds in both fungiform papillae (FP) and circumvallate (CV) papillae. The mRNA for CB_2, but not those for CB_1 and gustducin, were expressed in epithelial tissues (ET) adjacent to taste buds. Control experiments in which reverse transcriptase was omitted yielded no specific products (Fig S6). We used immunohistochemistry to determine whether CB_1 receptors were coexpressed with T1r3, a component of sweet and umami receptors. In WT mice about 70% of TCs in both FP and CV expressing CB_1 receptors coexpressed T1r3; ~60% of TCs expressing T1r3 also expressed CB_1 (Fig. 4*B*, Fig. S7, and Table S1). In $CB_1^{-/-}$ mice CB_1 immunoreactivity in TCs was absent (Fig. S7*E*). The average number of CB_1 positive taste cells in a fungiform or circumvallate taste bud in our slice preparation was 1.67 ± 0.08 ($n = 67$) and 1.73 ± 0.08 ($n = 137$), respectively: there is no significant difference between these numbers ($P > 0.1$).

In the central nervous system, CB_1 receptors are expressed in presynaptic cells and underlie modulation (inhibition) of transmitter release from presynaptic cells (27). In the peripheral taste organ, CB_1 immnoreactivity was observed in fewer than 12% of GAD67-expressing TCs, which in mice are thought to be presynaptic cells (28) (Fig. 4*B* and Table S1). GAD67-expressing presynaptic cells are reported to be primarily sensitive to sour taste stimuli (29, 30). Endocannabinoids did not affect sour taste responses, indicating that presynaptic cells are not the major target for endocannabinoids in the taste organ. Instead, the majority of TCs expressing CB_1 receptors are sweet-sensitive cells expressing T1r3: endocannabinoids act to enhance sweet taste responses through these type II taste receptor cells known to lack well-elaborated synapses.

To date, leptin (9–11), CCK (31, 32), VIP (32), NPY (33), and GLP-1 (13) are implicated in the modulation of peripheral taste sensitivity. Leptin and GLP-1 are known to be modulators for sweet taste. Leptin specifically suppresses sweet taste responses and these effects may be mediated by leptin receptors (Ob-Rb) on TCs (9–11). GLP-1 signaling increases sweet and sour taste sensitivity and these effects may be mediated by GLP-1 receptors on adjacent intragemmal afferent nerve fibers (13). Our findings indicate that endocannabinoids selectively enhance sweet taste sensitivity via CB_1 receptors on the TCs. Both endocannabinoids and GLP-1 enhance sweet taste but their specificity (sweet vs. sweet-and-sour) and targets (TCs vs. afferent fibers) differ, suggesting that these modulators have different roles in modulating sweet taste. Circulating endocannabinoid levels inversely correlate with plasma levels of leptin (34). Both endocannabinoids and leptin affect responses of TCs via their cognate receptors. Therefore, endocannabinoids and leptin may reciprocally regulate peripheral sweet taste sensitivity.

Exogenous cannabinoid agonists and antagonists are known to affect the preference for sweet compounds. Administration of cannabinoid agonists increase the intake of sucrose solutions (5). Systemic administration of the CB_1 antagonist SR141716A decreases intake of a sweet milk diet (35), sucrose solution (5, 36), and sweetened pellets without affecting the intake of normal pellets (37, 38). These results suggest an interaction of cannabinoid-induced modifications in feeding behavior with the sensation of palatability component (i.e., sweet taste) of food stimuli. Infusions of AEA into the nucleus accumbens enhance taste reactivity to sucrose, although standard chow intake is also enhanced (7). Infusions of 2-AG into the pontine parabrachial nucleus that contains third order gustatory neurons increase intake of sweet food without affecting the intake of normal chow

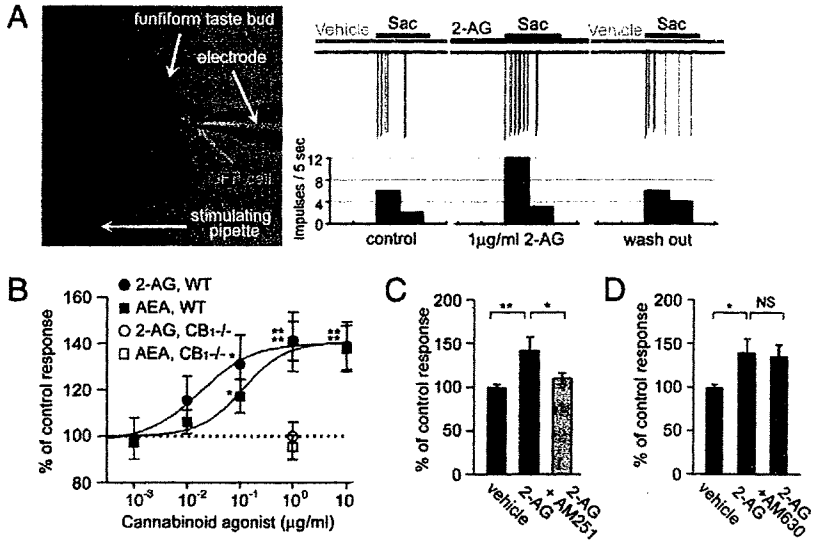

Fig. 3. Endocannabinoids enhance sweet responses of taste bud cells. (*A*) The effect of 1 μg/mL 2-AG on the response of a T1r3-GFP taste cell in the isolated fungiform taste bud with the epithelium to the sweet compound saccharin. The picture shows a T1r3-GFP taste cell from which taste responses were recorded. In this cell, the response to 5 mM saccharin was increased about 2-fold by bath application of 2-AG for 2 min and returned to the control level 2 min after wash-out of 2-AG. (*B*) Dose-dependent effect of AEA and 2-AG on responses to saccharin of taste bud cells from WT and T1r3-GFP mice (labeled WT, $n = 7$–28). Responses to saccharin of taste bud cell in $CB_1^{-/-}$ mice were not affected by 1 μg/mL AEA (blue open rectangles, $n = 7$) or 2-AG (red open circles, $n = 5$). (*C*) The CB_1 antagonist AM251 inhibited the enhancing effects of 2-AG on responses to saccharin of TCs from WT and T1r3-GFP mice ($n = 10$). (*D*) The CB_2 antagonist AM630 did not inhibit the enhancing effects of 2-AG on response to saccharin of TCs from WT and T1r3-GFP mice ($n = 9$). Asterisks indicate significant differences (NS: $P > 0.1$; $^*P < 0.05$; $^{**}P < 0.01$, *t* test). All data are presented as the mean ± SEM.

during the first 30 min after infusion (39). Our findings provide evidence that the peripheral taste organ is also a target of cannabinoids. Increases in taste cell responses, nerve responses, and lick responses to sucrose especially at its higher (more palatable) concentrations found in this study are in line with the previous findings mentioned above. This modulation of peripheral sweet taste sensitivities by endocannabinoids may play a significant role in regulating feeding behavior.

In conclusion, we have identified endocannabinoids as modulators of the peripheral components of sweet taste. The positive effect of endocannabinoids on sweet sensitivity was opposed to that of leptin, which suppresses sweet sensitivity (9–11). Endocannabinoids, therefore, not only stimulate food intake via central systems but also may increase palatability of foods by enhancing peripheral sweet taste responses. We found that the sweet enhancing effect of enndocannabinoids was mediated by CB_1 receptors, which were coexpressed with the sweet receptor component T1r3 in TCs. Orexigenic and anorexigenic factors such as endocannnabinoids and leptin may affect energy homeostasis by regulating taste sensitivity.

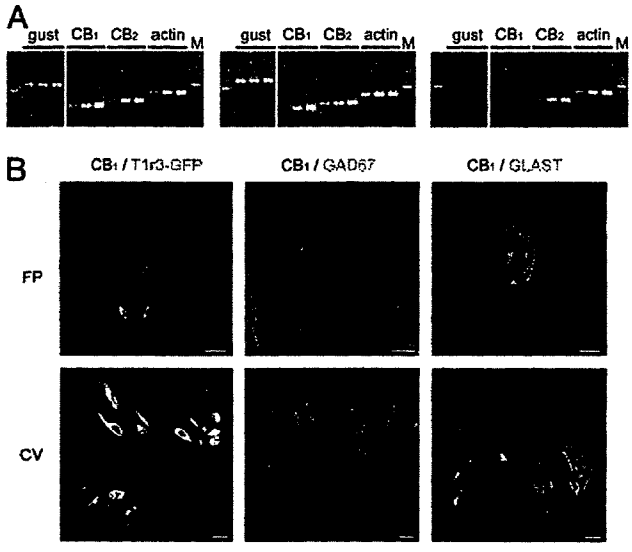

Fig. 4. CB_1 and T1r3 are coexpressed in taste bud cells. (*A*) Expression of gustducin (40, 45, and 50 cycles), CB_1 (40, 45, and 50 cycles), CB_2 (40, 45, and 50 cycles), and β-actin (25, 30, and 35 cycles) mRNAs in fungiform taste buds (FP), circumvallate taste buds (CV), and tongue epithelium devoid of TCs (ET). M, 100-bp marker. (*B*) Coexpression patterns of CB_1 with: T1r3 (*Left*), GAD67 (*Middle*), and GLAST (*Right*) in FP and CV of T1r3-GFP or WT mice. Immunostaining for CB_1 is shown in red. T1r3-GFP expression and immunostaining for GAD and GLAST are shown in green. (Scale bar, 10 μm.) Negative control and immunostaining in $CB_1^{-/-}$ mice are shown in Fig. S7.

Methods

Full methods are in *SI Methods*.

All experimental procedures were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the committee for Laboratory Animal Care and Use at Kyushu University, Japan.

Animals. T1r3-GFP mice were as described previously (24). CB_1-KO mice on a CD1 background (14) were backcrossed to C57BL/6N mice for five generations to breed heterozygous mice. These mice were interbred to generate $CB_1^{-/-}$ mice.

Nerve Recordings. Whole nerve responses to lingual application of tastants were recorded from the CT and the GL nerve as described (9, 15, 16). Responses to tastants were normalized to responses to NH_4Cl. A series of taste responses was recorded before and ~5–120 min after i.p. injection of AEA, 2-AG, or vehicle (physiological saline/ethanol, 99:1). Significant effects of AEA or 2-AG in neural and behavioral experiments were tested with repeated ANOVA, the Fisher's PLSD post hoc test and Student's *t* test. All data are presented as the mean ± SEM.

Behavioral Tests. Taste behavior was assayed by a short-term lick test with sweet-bitter mixtures as test stimuli (10). On training days (from the first to the fifth day), the mouse was placed in the test cage and trained to drink distilled water on an interval schedule (10-s DW presentation, 20-s interval) for 1-h session after 23-h water deprivation. On the test day, the number of licks for each test stimulus and DW was counted during the first 10 s after the animal's first lick before and ~5–120 min after administration of AEA, 2-AG, or vehicle.

Taste Cell Recordings. Taste responses of fungiform TCs were recorded as previously described (18, 30). Action potentials of TCs in isolated taste buds were recorded extracellularly from the basolateral side at room temperature (25°C). TCs were adapted to vehicle (Tyrode with <0.1% ethanol). Numbers of impulses/10 s subtracting spontaneous activities were used to assess the effect of 2-AG, AEA, AM251, and AM630.

RT-PCR and Immunostaining. RT-PCR and immunostaining were as described previously (9, 10). Primer sequences for each PCR are listed in Table S2. Antibodies for immunostaining were obtained from commercial sources (Table S3).

ACKNOWLEDGMENTS. This work was supported by Japan Society for the Promotion of Science, Grant-in-Aid for Scientific Research (KAKENHI) 18109013 and 18077004 (to Y.N.) and National Institutes of Health DC03155 (to R.F.M.).

1. Jamshidi N, Taylor DA (2001) Anandamide administration into the ventromedial hypothalamus stimulates appetite in rats. *Br J Pharmacol* 134:1151–1154.
2. Cota D, et al. (2003) The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. *J Clin Invest* 112:423–431.
3. Kirkham TC, Williams CM, Fezza F, Di Marzo V (2002) Endocannabinoid levels in rat limbic forebrain and hypothalamus in relation to fasting, feeding and satiation: Stimulation of eating by 2-arachidonoyl glycerol. *Br J Pharmacol* 136:550–557.
4. Williams CM, Kirkham TC (1999) Anandamide induces overeating: Mediation by central cannabinoid (CB_1) receptors. *Psychopharmacology (Berl)* 143:315–317.
5. Higgs S, Williams CM, Kirkham TC (2003) Cannabinoid influences on palatability: Microstructural analysis of sucrose drinking after delta(9)-tetrahydrocannabinol, anandamide, 2-arachidonoyl glycerol and SR141716. *Psychopharmacology (Berl)* 165: 370–377.
6. Jarrett MM, Limebeer CL, Parker LA (2005) Effect of Delta9-tetrahydrocannabinol on sucrose palatability as measured by the taste reactivity test. *Physiol Behav* 86:475–479.
7. Mahler SV, Smith KS, Berridge KC (2007) Endocannabinoid hedonic hotspot for sensory pleasure: Anandamide in nucleus accumbens shell enhances 'liking' of a sweet reward. *Neuropsychopharmacology* 32:2267–2278.
8. Friedman JM (2004) Modern science versus the stigma of obesity. *Nat Med* 10: 563–569.
9. Kawai K, Sugimoto K, Nakashima K, Miura H, Ninomiya Y (2000) Leptin as a modulator of sweet taste sensitivities in mice. *Proc Natl Acad Sci USA* 97:11044–11049.
10. Shigemura N, et al. (2004) Leptin modulates behavioral responses to sweet substances by influencing peripheral taste structures. *Endocrinology* 145:839–847.
11. Nakamura Y, et al. (2008) Diurnal variation of human sweet taste recognition thresholds is correlated with plasma leptin levels. *Diabetes* 57:2661–2665.
12. Rehfeld JF (1998) The new biology of gastrointestinal hormones. *Physiol Rev* 78: 1087–1108.
13. Shin YK, et al. (2008) Modulation of taste sensitivity by GLP-1 signaling. *J Neurochem* 106:455–463.
14. Ledent C, et al. (1999) Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. *Science* 283:401–404.
15. Damak S, et al. (2003) Detection of sweet and umami taste in the absence of taste receptor T1r3. *Science* 301:850–853.
16. Talavera K, et al. (2005) Heat activation of TRPM5 underlies thermal sensitivity of sweet taste. *Nature* 438:1022–1025.
17. Luk T, et al. (2004) Identification of a potent and highly efficacious, yet slowly desensitizing CB_1 cannabinoid receptor agonist. *Br J Pharmacol* 142:495–500.
18. Yoshida R, et al. (2006) Taste responsiveness of fungiform taste cells with action potentials. *J Neurophysiol* 96:3088–3095.
19. Kitagawa M, Kusakabe Y, Miura H, Ninomiya Y, Hino A (2001) Molecular genetic identification of a candidate receptor gene for sweet taste. *Biochem Biophys Res Commun* 283:236–242.
20. Max M, et al. (2001) Tas1r3, encoding a new candidate taste receptor, is allelic to the sweet responsiveness locus Sac. *Nat Genet* 28:58–63.
21. Montmayeur JP, Liberles SD, Matsunami H, Buck LB (2001) A candidate taste receptor gene near a sweet taste locus. *Nat Neurosci* 4:492–498.
22. Nelson G, et al. (2001) Mammalian sweet taste receptors. *Cell* 106:381–390.
23. Nelson G, et al. (2002) An amino-acid taste receptor. *Nature* 416:199–202.
24. Damak S, Mosinger B, Margolskee RF (2008) Transsynaptic transport of wheat germ agglutinin expressed in a subset of type II taste cells of transgenic mice. *BMC Neurosci* 9:96.
25. Sugiura T, Kobayashi Y, Oka S, Waku K (2002) Biosynthesis and degradation of anandamide and 2-arachidonoylglycerol and their possible physiological significance. *Prostaglandins Leukot Essent Fatty Acids* 66:173–192.
26. McLaughlin SK, McKinnon PJ, Margolskee RF (1992) Gustducin is a taste-cell-specific G protein closely related to the transducins. *Nature* 357:563–569.
27. Wilson RI, Nicoll RA (2002) Endocannabinoid signaling in the brain. *Science* 296: 678–682.
28. DeFazio RA, et al. (2006) Separate populations of receptor cells and presynaptic cells in mouse taste buds. *J Neurosci* 26:3971–3980.
29. Huang YA, Maruyama Y, Stimac R, Roper SD (2008) Presynaptic (Type III) cells in mouse taste buds sense sour (acid) taste. *J Physiol* 586:2903–2912.
30. Yoshida R, et al. (2009) Discrimination of taste qualities among mouse fungiform taste bud cells. *J Physiol* 587:4425–4439.
31. Herness S, Zhao FL, Lu SG, Kaya N, Shen T (2002) Expression and physiological actions of cholecystokinin in rat taste receptor cells. *J Neurosci* 22:10018–10029.
32. Shen T, et al. (2005) Co-expression patterns of the neuropeptides vasoactive intestinal peptide and cholecystokinin with the transduction molecules alpha-gustducin and T1R2 in rat taste receptor cells. *Neuroscience* 130:229–238.
33. Zhao FL, et al. (2005) Expression, physiological action, and coexpression patterns of neuropeptide Y in rat taste-bud cells. *Proc Natl Acad Sci USA* 102:11100–11105.
34. Monteleone P, et al. (2005) Blood levels of the endocannabinoid anandamide are increased in anorexia nervosa and in binge-eating disorder, but not in bulimia nervosa. *Neuropsychopharmacology* 30:1216–1221.
35. Rowland NE, Mukherjee M, Robertson K (2001) Effects of the cannabinoid receptor antagonist SR 141716, alone and in combination with dexfenfluramine or naloxone, on food intake in rats. *Psychopharmacology (Berl)* 159:111–116.
36. Thornton-Jones ZD, Kennett GA, Vickers SP, Clifton PG (2007) A comparison of the effects of the $CB_{(1)}$ receptor antagonist SR141716A, pre-feeding and changed palatability on the microstructure of ingestive behaviour. *Psychopharmacology (Berl)* 193:1–9.
37. Simiand J, Keane M, Keane PE, Soubrié P (1998) SR 141716, a CB_1 cannabinoid receptor antagonist, selectively reduces sweet food intake in marmoset. *Behav Pharmacol* 9:179–181.
38. Arnone M, et al. (1997) Selective inhibition of sucrose and ethanol intake by SR 141716, an antagonist of central cannabinoid (CB_1) receptors. *Psychopharmacology (Berl)* 132:104–106.
39. DiPatrizio NV, Simansky KJ (2008) Activating parabrachial cannabinoid CB_1 receptors selectively stimulates feeding of palatable foods in rats. *J Neurosci* 28:9702–9709.

Neuroscience 165 (2010) 300–312

A TRYPTAMINE-DERIVED CATECHOLAMINERGIC ENHANCER, (−)-1-(BENZOFURAN-2-YL)-2-PROPYLAMINOPENTANE [(−)-BPAP], ATTENUATES REINSTATEMENT OF METHAMPHETAMINE-SEEKING BEHAVIOR IN RATS

T. HIRANITA,[a1,2] T. YAMAMOTO[b]* AND Y. NAWATA[b]

[a]*Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan*

[b]*Department of Pharmacology, Faculty of Pharmaceutical Sciences, Nagasaki International University, 2825-7 Huis Ten Bosch, Sasebo, Nagasaki, 859-3298, Japan*

Abstract—Relapse to drug craving is problematic in treatment for drug abuse. Evidence suggests inactivation of dopaminergic neurotransmission during drug withdrawal. Meanwhile, a tryptamine analogue, (−)-1-(benzofuran-2-yl)-2-propylaminopentane [(−)-BPAP], has been reported to enhance electrical stimulation of monoamine release. This study examined the effect of (−)-BPAP on reinstatement of methamphetamine-seeking behavior in an animal model of relapse to drug abuse. Rats were trained to i.v. self-administer methamphetamine paired with a light and tone (methamphetamine-associated cues) under a fixed-ratio 1 schedule of reinforcement for 10 days. After extinction session under saline infusions without cues, a reinstatement test under saline infusions was begun. Reinstatement induced by methamphetamine-associated cues or methamphetamine-priming injections was attenuated by repeated administration of (−)-BPAP during the extinction phase. Acute administration of (−)-BPAP on test day dose-dependently attenuated both reinstatements. Acute administration of (−)-BPAP neither reinstated methamphetamine-seeking behavior alone nor affected methamphetamine self-administration. Pretreatment with either *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SCH-23390), a dopamine D_1-like receptor antagonist, or amisulpride, a dopamine D_2-like receptor antagonist, did not appreciably affected the acute effect of (−)-BPAP on both reinstatements. Co-pretreatment with the dopamine receptor antagonists failed to alter the effects of (−)-BPAP. Meanwhile, pretreatment with a dopamine D_1-like receptor agonist, (+/−)-6-chloro-7,8-dihydroxy-l-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide (SKF-81297), dose-dependently attenuated reinstatement induced by the cues or methamphetamine-priming injections. In contrast to (−)-BPAP, pretreatment with SCH-23390 reversed the effects of SKF-81297. Our findings suggest activation of dopamine D_1-like receptors results in attenuation of the reinstatement of methamphetamine-seeking behavior. Additionally, our findings provide evidence to develop (−)-BPAP and dopamine D_1-like receptor agonists as an anti-relapse medication for methamphetamine abusers.

Key words: craving, dopamine, methamphetamine, reinstatement, relapse, self-administration.

Abuse of the psychostimulant methamphetamine is a growing problem worldwide. Due to the prevalence of its abuse and lack of effective treatment in methamphetamine abuse, a clear need exists in clarifying the mechanisms underlying methamphetamine dependence. Among symptoms in drug dependence, relapse to craving is a main hurdle of treatment. In human and animal models of relapse, three different kinds of stimuli are capable of eliciting relapse: stress, cues predicting drug availability, and re-exposure to a previously self-administered drug (Shalev et al., 2002). Medications that reduce the ability of these stimuli to induce relapse may be effective in treatment of drug dependence. So far, we have reported (1) important roles of cannabinoid CB_1, nicotinic acetylcholine, and opioid receptors in reinstatement of methamphetamine-seeking behavior and (2) brain regions responsible for the reinstatement in rats (Anggadiredja et al., 2004a,b; Hiranita et al., 2004, 2006, 2008). We have reported agonists for $\alpha4\beta2$ nicotinic acetylcholine receptors and antagonists for cannabinoid CB_1 and opioid receptors as anti-relapse agents.

Evidence suggests inactivation of central dopaminergic neurotransmission during methamphetamine withdrawal. For example, clinical studies demonstrated loss of dopamine transporters in methamphetamine abusers during withdrawal (Volkow et al., 2001a,b). A post-mortem study also found reduced levels of dopamine nerve terminal markers such as dopamine, dopamine transporter, and tyrosine hydroxylase, an enzyme responsible for dopamine synthesis, in the striatum of methamphetamine abusers (Wilson et al., 1996). Recently, we demonstrated involvement of the nucleus accumbens, one of the main terminals of dopaminergic neurons, in the reinstatement of methamphetamine-seeking behavior in rats (Hiranita et al., 2006, 2008). Available reports on psychostimulants other than methamphetamine also suggest the inactivation of dopaminergic neurotransmission during drug withdrawal.

[1] Present address: Psychobiology Section, Medications Discovery Research Branch, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Department of Health and Human Services, Baltimore, MD 21224, USA.

[2] JSPS Research Fellowship for Japanese Biomedical and Behavioral Researchers at NIH.

*Corresponding author. Tel: +81-956-20-5629; fax: +81-956-39-3111.
E-mail address: tyamamot@niu.ac.jp (T. Yamamoto).

Abbreviations: BD 1063, *N*-[2-(3, 4-dichlorophenyl) ethyl]-4-methylpiperazine; (−)-BPAP, (−)-1-(benzofuran-2-yl)-2-propylaminopentane; CHO cells, Chinese hamster ovary cells; SCH-23390, *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SKF-81297, (+/−)-6-chloro-7,8-dihydroxy-l-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide; σ1-R, σ1 receptor.

0306-4522/10 $ - see front matter © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2009.10.055

Thus, a decrease in basal extracellular dopamine levels in the nucleus accumbens during withdrawal from cocaine self-administration has been shown (Weiss et al., 1992). Furthermore, decreased striatal 6-fluorodopa uptake, an index of dopaminergic presynaptic activity, was associated with increased duration of cocaine withdrawal (Wu et al., 1997). In the reinstatement model, dopamine D_1-like receptor agonists have been reported to block reinstatement of cocaine-induced cocaine-seeking behavior, whereas dopamine D_2-like receptor agonists enhance this behavior in rats (Self et al., 1996, 2000). Additionally, high levels of cocaine use have been reported to be subsensitive to the ability of the dopamine D_1-like receptor agonist (+/−)-6-chloro-7,8-dihydroxy-l-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide (SKF-81297) to inhibit cocaine-induced reinstatement of cocaine-seeking behavior, but supersensitive to the dopamine D_2-like receptor agonist quinpirole-induced reinstatement of cocaine seeking behavior in rats (Edwards et al., 2007). These findings suggest that inactivation of dopaminergic neurotransmission through dopamine D_1-like receptors during drug withdrawal might be pre-requisite to reinstatement of drug-seeking behavior. However, the involvement of the dopaminergic inactivation in relapse to methamphetamine-seeking behavior is not well understood.

Endogenous biogenic amines such as β-phenylethylamine and tryptamine have been found to enhance electrically stimulated release of [^{3}H] monoamines from the rat brainstem (Knoll et al., 1996). Based on the structure of these amines, (−)-1-(benzofuran-2-yl)-2-propylaminopentane [(−)-BPAP] has been synthesized and reported as a highly potent enhancer (Yoneda et al., 2001) of the electrically stimulated monoamine release (Miklya and Knoll, 2003). Dissimilar to methamphetamine (Yoneda et al., 2001) and tyramine (Shimazu et al., 2003b), however, (−)-BPAP alone does not release catecholamines. Furthermore, (−)-BPAP also has been reported to inhibit monoamine uptake (IC_{50} values: [3H] dopamine, [3H] noradrenaline, and [3H] serotonin; 42, 52, and 640 nM, respectively) (Shimazu et al., 2003b). However, none of the standard monoamine uptake inhibitors has the enhancing effect of electrically stimulated monoamine release (Miklya and Knoll, 2003). In addition, (−)-BPAP blocked tyramine-induced monoamine release from rat brain synaptosomes (Shimazu et al., 2003b). These findings suggest (−)-BPAP as an atypical monoamine uptake inhibitor. Behavioral studies demonstrated that (−)-BPAP stimulated locomotor activity in rats (Shimazu et al., 2003a) and that (−)-BPAP-induced hyperlocomotion was attenuated by pre-treatment with *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SCH-23390), a dopamine D_1-like receptor antagonist (Shimazu et al., 2001). Additionally, chronic administration of (−)-BPAP ameliorated impairment of social interaction behavior following forced swimming and the ameliorating effect was blocked by pre-treatment with either SCH-23390 or sulpiride, a dopamine D_2-like receptor antagonist (Tsunekawa et al., 2008). These findings suggest that (−)-BPAP functions as the activator of dopaminergic neurotransmission. Considering reports on inhibitory action of dopamine D_1-like receptor agonists on reinstatement of cocaine-seeking behavior (Self et al., 1996, 2000), we examined whether pretreatment with (−)-BPAP would block reinstatement of methamphetamine-seeking behavior in rats in comparison with SKF-81297, a dopamine D_1-like receptor agonist.

EXPERIMENTAL PROCEDURES

Drugs

The drugs used were methamphetamine hydrochloride (Dainippon Pharmaceutical Co., LTD, Osaka, Japan), [(−)-BPAP] (gift from Fujimoto Pharmaceutical Corporation), R-(+)-SCH-23390 (a dopamine D_1-like receptor antagonist, Sigma, St. Louis, MO, USA), amisulpride (a dopamine D_2-like receptor antagonist, Sigma), and SKF-81297 (a dopamine D_1-like receptor agonist, Sigma). All of the drugs were dissolved in 0.9% saline. Methamphetamine was delivered i.v. for self-administration (0.02 mg/0.1 ml/infusion) and i.p. for priming injections (1.0 mg/kg) 30 min before test sessions. R-(+)-SCH-23390 and SKF-81297 were administered s.c. 30 and 15 min before test sessions, respectively, while (−)-BPAP and amisulpride were administered i.p. 30 min and 1 h before test sessions, respectively. Repeated administration of (−)-BPAP or saline was administered daily, i.p. 30 min after the extinction sessions for 5 days.

Subjects

At the beginning of this study, 192 male Wistar rats (250–350 g, 10 weeks old, Nippon SLC Co., Hamamatsu, Japan) were individually housed in a temperature- and humidity-controlled environment under a 12-h light/dark cycle (lights on at 7:00 AM). Food and water were available *ad libitum* in the home cage except when daily food intake was limited to 15–20 g after the catheter implantation to fix the distance between the proximal position of a catheter in the vein and the surface of the atrial auricle. Procedures for animal handling were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the Declaration of Helsinki and Faculty of Pharmaceutical Sciences, Kyushu University Publication, 1988.

Apparatus

The injector system contained a fluid swivel (Instech Lab., Inc., PA, USA) mounted on the top of each operant chamber (Neuroscience, Inc., Tokyo, Japan). One end of the swivel was connected via polyethylene tubing (Kaneka Medix Co., Osaka, Japan) encased in a protective stainless steel spring tether (Instech Laboratories, Inc., PA, USA) to the animal's catheter while the other end of the swivel was connected via polyethylene tubing to the infusion pump. The operant chambers were enclosed in ventilated, sound-attenuating cubicles and controlled by computer software (Med Associates Inc., VT, USA). The chamber's light was switched on throughout the session. Lever-pressing responses resulted in methamphetamine infusion (0.02 mg/infusion over 6 s) accompanied by light (mounted 4 cm above the lever, 200 lux) and tone (85 dB/2.9 kHz) for 26 s (methamphetamine associated-cues). The subsequent 20 s was a "time out" period during which lever presses were still recorded but not accompanied with infusions.

Surgery

Silicon catheters (Silascon; inner and outer diameter: 0.5 and 1.0 mm, respectively; Kaneka Medix Co., Osaka, Japan) were surgically implanted into the jugular vein under sodium pentobarbital (40 mg/kg i.p., Kyoritsu Seiyaku Co., Tokyo, Japan) anesthesia as

described previously (Hiranita et al., 2006). After the surgery, catheter patency was maintained by daily infusion of 0.15 ml saline solution containing heparin (30 U/ml) after each session.

Autoshaping

Autoshaping procedures to lever-press for food pellet reinforcement (45 mg; Bioserv, Holton Industries Co., Frenchtown, NJ, USA) in operant chambers under a fixed-ratio 1 schedule of reinforcement (each lever-pressing is reinforced) followed by the surgery for the self-administration training were used. Both the right and left levers were designated as active with a cue (light). The room lamp over the levers was illuminated to indicate that lever-pressing responses resulted in the immediate delivery of a food pellet and activation of the feedback tone for 0.5 s. Each session lasted for 20 min. Cessation of lever-pressing training occurred when the rat was able to deliver 30 pellets within 180 s for three consecutive sessions.

Methamphetamine self-administration, extinction, and reinstatement

Two days after surgery, rats were trained to self-administer methamphetamine under a fixed-ratio 1 schedule of reinforcement in a 2 h daily session for 10 days. Each injection was accompanied by a light and tone (methamphetamine-associated cues). During this time, inactive lever responses had no programmed consequences but were recorded. After the self-administration sessions, at least five extinction sessions (1 h) were conducted daily during which active lever responding resulted in an infusion of saline instead of methamphetamine without presentation of the methamphetamine-associated cues (and until the rats achieved the extinction criterion of less than 10 responses per session on the previously active lever). Reinstatement tests under saline infusions were carried out for 30 min from day six of extinction (or the day after rats achieved the extinction criterion) every 6 days under a fixed-ratio 1 schedule. In the cue-induced reinstatement test, immediately after the onset of the session, rats were re-exposed to the methamphetamine-associated cues and each press on the active lever resulted in presentation of the cues. In the methamphetamine-primed reinstatement test, methamphetamine (1.0 mg/kg i.p.) was injected 30 min before the test. Each response during the test session resulted in an infusion of saline but not the methamphetamine-associated cues. In the present study, subjects were mainly divided to the four following groups. The first group was used for repeated administration of (−)-BPAP (n=15) or saline (n=12) during extinction phase. The second group was used for pre-session treatment with (−)-BPAP before methamphetamine self-administration on the 10th tenth day of methamphetamine self-administration phase (n=6). The third group was used for pre-session treatment with (−)-BPAP, SKF-81297 or SCH-23390 alone after extinction phase (n=16, 18, or 18, respectively). The fourth group was used for pre-session treatment with (−)-BPAP or SKF-81297 on reinstatement test day (n=65 or 42, respectively). In the first group, subjects were further divided to sub-groups depending on treatment (eight for treatment with saline on cue presentation, six for treatment with (−)-BPAP on cue presentation, seven for treatment with saline on methamphetamine-priming injections, or six for treatment with (−)-BPAP on methamphetamine-priming injections). In the fourth group, subjects were also divided to sub-groups depending on reinstatement factors (methamphetamine-associated cues or methamphetamine-priming injections), treatment drugs, or the drug doses (see each figure legend for more detail). Each rat in the fourth group was used for two reinstatement tests first on methamphetamine-associated cues and then methamphetamine-priming injections. The sample sizes of methamphetamine-priming injections in each group were the same as or less than those of methamphetamine-associated cues because data from subjects with problems related to health or catheter issues have been removed. All of the tests were conducted with a mixed order schedule of drug doses. In order to minimize the overall number of subjects, control data were shared in the sub-groups pre-treated with (−)-BPAP or SKF-81297.

Operant task performance for food reinforcement

All subjects had sessions to lever-press for food-pellet reinforcement under a fixed-ratio 1 schedule 5 min after the self-administration or reinstatement session. Each test ended when rats had received 30 pellets or 1200 s had passed.

Data analysis

Data represent the mean±SEM of number of responses or methamphetamine infusions and were analyzed by ANOVA (a between-subjects design). The significance of effects on responding or methamphetamine infusions was assessed by ANOVA, with Dunn or Bonferroni t-test for *post-hoc* analyses as appropriate. To determine if there was a difference in effects of re-exposure to methamphetamine-associated cues or drug priming injections, a two-way (repeated administration of (−)-BPAP) and one-way (others) measures ANOVA was used. A one-way repeated measures ANOVA was used to assess the pre-session treatment effects of a single administration of (−)-BPAP on methamphetamine self-administration. A two-way repeated measures ANOVA was used to assess effects of repeated administration of (−)-BPAP on lever responses during the extinction phase. A two-way measures ANOVA was used to assess the effects of pre-session treatments of the test drugs on reinstatement of methamphetamine-seeking behavior, and food-maintained behavior (drug doses and reinstatement factors). Pearson's correlations were used to analyze correlation between total amount of methamphetamine-intake and number of responses at test sessions. Differences were considered significant at $P<0.05$.

RESULTS

On the first day of methamphetamine self-administration, the numbers of active and inactive lever responses per session were 73.8±7.3 and 70.7±38.4, respectively. During the second and third sessions, both numbers of active and inactive lever responses per session decreased from 46.5±4.0 and 17.7±10.1 to 29.1±2.1 and 4.5±0.8, respectively. During subsequent sessions, both the number of active and inactive lever responses did not alter (data not shown). Compared with lever responses, the total amount of daily methamphetamine intake was less vari-

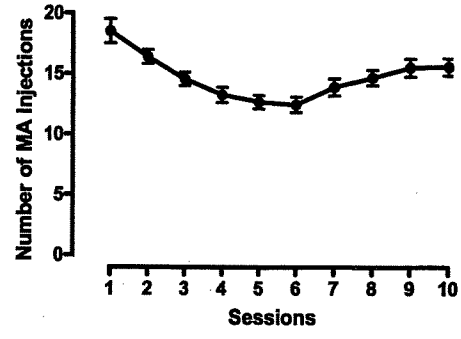

Fig. 1. Methamphetamine self-administration in rats. Rats were allowed to self-administer methamphetamine (0.02 mg/0.1 ml/injection) under a fixed ratio one schedule of reinforcement for a daily 2 h sessions for 10 days (n=180).

able (Fig. 1). On the first day of methamphetamine self-administration, the numbers of methamphetamine infusions per session were 18.5±1.0. In the subsequent sessions, the numbers of methamphetamine infusions per session decreased gradually until the sixth session (12.4±0.6). During the last four sessions, the numbers of methamphetamine infusions per session increased slightly. Total intake of methamphetamine over the course of the self-administration was 3.0±0.1 mg in rats excluding rats used for experiments on the effect of pre-session treatment with (−)-BPAP on methamphetamine self-administration. During the last three sessions of methamphetamine self-administration, both numbers of active and inactive lever responses per session were stable (23.5±1.5 and 3.1±0.1, respectively). On the first day of the extinction session, the numbers of active and inactive lever responses per session were 16.8±1.8 and 4.5±0.6, respectively. Through extinction sessions, the numbers of active lever responses per session decreased, whereas the numbers of inactive lever responses per session were relatively stable (data not shown). On the last day of extinction sessions, the numbers of active and inactive lever responses were 4.9±0.3 and 2.9±0.4, respectively in rats, excluding rats used for experiments on repeated administration of (−)-BPAP on reinstatement with methamphetamine-seeking behavior.

Effect of repeated administration of (−)-BPAP during extinction phase on reinstatement of methamphetamine-seeking behavior

During extinction sessions, the number of lever responses decreased gradually (data not shown). Two-way repeated measures ANOVA indicated significant effects of repeated administration of (−)-BPAP during extinction phase on extinction days ($F(4,100)=13.584$, $P<0.001$ and $F(4,100)=3.732$, $P=0.007$), but not drug treatment ($F(1,100)=3.265$, $P=0.083$ and $F(1,100)=0.0996$, $P=0.755$) or the interaction ($F(4,100)=0.728$, $P=0.575$ and $F(4,100)=0.0478$, $P=0.996$, active and inactive lever responses, respectively). In the saline-pretreated group as the control of (−)-BPAP administration, *post-hoc* comparison indicated significant effects on active lever responses on the first day of extinction compared with those of the third, fourth and fifth day ($t=3.269$, 3.269 and 3.897, $P=0.015$, 0.015 and 0.002, respectively). In the (−)-BPAP-pretreated group, *post-hoc* comparison indicated significant effects on active lever responses on the first day of extinction compared with those of the third, fourth and fifth day ($t=4.328$, 4.474 and 5.497, $P<0.001$, 0.001 and 0.001, respectively). In addition, *post-hoc* comparison indicated significant effects on active lever responses between saline- and (−)-BPAP groups on the first day of extinction ($t=2.036$, $P=0.044$). After extinction sessions, subsequent re-exposure to methamphetamine-associated cues and methamphetamine-priming injections increased active lever responses (Fig. 2). The increases in active lever responses in both were attenuated by repeated administration of (−)-BPAP during the extinction phase (Fig. 2). Two-way measures ANOVA indicated significant effect of (−)-BPAP treatment on the dose ($F(1,23)=122.507$, $P<0.001$), but not reinstatement factor (cues or methamphetamine-priming injections; $F(1,23)=0.133$, $P=0.179$), and significant effect on the interaction ($F(1,23)=16.203$, $P<0.001$). *Post hoc* analysis indicated significant effect of (−)-BPAP treatment on the increases in active lever responses induced by methamphetamine-associated cues ($t=5.056$, $P<0.001$) and methamphetamine-priming injections ($t=10.518$, $P<0.001$). On the other hand, methamphetamine-associated cues and methamphetamine-priming injections did not affect inactive lever responses (Fig. 2, $P\geq0.66$, both). However, two-way measures ANOVA indicated significant effect of (−)-BPAP treatment on the dose ($F(1,23)=7.562$, $P<0.011$), but not reinstatement factor ($F(1,23)=0.817$, $P=0.372$) or the interaction ($F(1,23)=0.817$, $P=0.372$). *Post hoc* analysis indicated significant effect of (−)-BPAP treatment on inactive lever responses induced by methamphetamine-associated cues ($t=2.627$, $P=0.015$) but not by methamphetamine-priming injections ($t=1.282$, $P=0.213$). In addition, the total amount of methamphetamine intake was not correlated with the increase in active lever responses induced by either methamphetamine-associated cues or methamphetamine-priming injections ($r=-0.382$ and -0.276, $P=0.350$ and 0.550, and $n=8$ and 7, respectively).

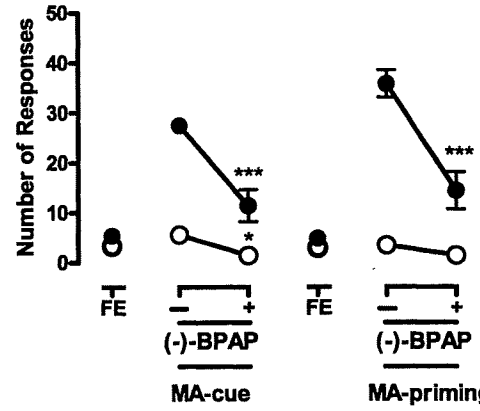

Fig. 2. Effects of repeated administration of (−)-BPAP during extinction phase (1.0 mg/kg i.p., 30 min after the extinction sessions daily for 5 days) on reinstatement of methamphetamine-seeking behavior induced by methamphetamine-associated cues or methamphetamine-priming injections. The reinstatement tests were performed 24 h after the last administration of (−)-BPAP. Closed and open circles indicate responding on active and inactive levers. * $P<0.05$, and *** $P<0.001$ versus a vehicle-treated group challenged with methamphetamine-associated cues or methamphetamine-priming injections. FE; final extinction. The order of test session was first cue presentation and then methamphetamine-priming injections. Data on FE consist of groups challenged with and without (−)-BPAP. In the test on methamphetamine-associated cues, the sample sizes of the FE session, vehicle, and (−)-BPAP pretreatment were 14, eight, and six, respectively. In the test on methamphetamine-priming injections, the sample sizes of the FE session vehicle, and (−)-BPAP pretreatment were 13, seven, and six, respectively.

Effect of acute administration of (−)-BPAP on reinstatement of methamphetamine-seeking behavior

In an experiment on controls, methamphetamine-associated cues and methamphetamine-priming injections rein-