Table 5. *Cont.*

Relation	Alcohol dependence	Methamphetamine dependence	*p*	
Aunt (maternal) (%)				
Alcohol	2.83	2.04	1.00	
Drug	0.41	2.08	0.30	
Psychiatric disorder	0.41	2.04	0.31	
Uncle (maternal) (%)				
Alcohol	14.45	6.00	0.17	
Drug	0.79	0.00	1.00	
Psychiatric disorder	1.19	3.92	0.20	
Sisters (%)				
Alcohol	3.73	2.44	1.00	
Drug	0.00	2.50	0.14	
Psychiatric disorder	2.93	2.50	1.00	
Grandmother (paternal) (%)				
Alcohol	1.47	0.00	1.00	
Drug	0.00	1.96	0.20	
Psychiatric disorder	0.98	3.85	0.18	
Grandfather (paternal) (%)				
Alcohol	18.37	7.84	0.09	
Drug	0.00	1.96	0.20	
Psychiatric disorder	0.50	1.96	0.37	
Father (%)				
Alcohol	36.33	16.67	0.005	*
Drug	1.02	0.00	1.00	
Psychiatric disorder	1.72	0.00	1.00	
Aunt (paternal) (%)				
Alcohol	3.45	0.00	0.36	
Drug	0.00	0.00	-	
Psychiatric disorder	0.87	2.08	0.44	
Uncle (paternal) (%)				
Alcohol	20.87	5.88	0.009	*
Drug	0.00	1.96	0.18	
Psychiatric disorder	0.44	0.00	1.00	
Brothers (%)				
Alcohol	25.94	4.88	0.002	*
Drug	0.00	9.52	0.0005	*
Psychiatric disorder	1.68	4.88	0.22	

* Significant difference.

3.4. Comparisons of Family Relationships between Patients with Alcohol Dependence and Patients with Methamphetamine Dependence

In the Family/Social relationship domain, patients answered "Yes," "No," or "Neither" about whether they had a close, long-lasting, personal relationship with family members, partners, or friends in their life. Participants who answered "Yes" were assigned to the "good relationships group," and participants who answered "No" were assigned to the "bad relationships group." In the comparison of

experience of good relationships with family members (Table 6), patients with alcohol dependence had a significantly higher ratio of experience of good relationships with their father ($z = 17.77$, $p < 0.0001$).

Table 6. Comparisons of the ratios of good family relationships between patients with alcohol dependence and patients with methamphetamine dependence.

	Alcohol dependence	Methamphetamine dependence	*p*	
Mother (%)	76.07	64.71	0.07	
Father (%)	70.55	43.08	< 0.0001	*
Brothers/sisters (%)	72.43	60.66	0.09	
Partner (%)	62.89	58.00	0.52	
Children (%)	72.29	53.33	0.14	
Friends (%)	77.74	69.49	0.18	

* Significant difference.

3.5. Comparison of Severity of Addiction between Good and Bad Family Relationships

Tables 7 and 8 show the comparisons of ASI CSs between good and bad family relationships. Patients with alcohol dependence who experienced bad relationships with their brothers and sisters ($t = 2.99$, $p = 0.003$) and partners ($t = 3.47$, $p = 0.0006$) had a higher CS of employment/support problems. Patients who experienced bad relationships with their partners had a higher CS of family/social problems ($t = 4.90$, $p < 0.0001$). Patients who experienced bad relationships with their mothers ($t = 2.73$, $p = 0.02$), fathers ($t = 2.84$, $p = 0.01$), brothers and sisters ($t = 2.82$, $p = 0.005$), and friends ($t = 2.99$, $p = 0.02$) had a higher CS of psychiatric problems. In patients with methamphetamine dependence, no significant difference was found between good and bad family relationships in ASI CSs.

Table 7. Comparison of severity of addiction between good and bad family relationships in patients with alcohol dependence.

	Mother				Father			
	Good relationship	Bad relationship	*p*		Good relationship	Bad relationship	*p*	
Medical	0.22 (0.29)	0.29 (0.33)	0.12		0.22 (0.28)	0.29 (0.34)	0.14	
Employment	0.53 (0.28)	0.55 (0.29)	0.64		0.53 (0.29)	0.55 (0.27)	0.60	
Alcohol use	0.55 (0.22)	0.54 (0.22)	0.63		0.55 (0.23)	0.55 (0.23)	0.78	
Drug use	0.01 (0.04)	0.01 (0.03)	0.80		0.01 (0.04)	0.01 (0.03)	0.66	
Legal	0.004 (0.03)	0.01 (0.05)	0.42		0.004 (0.03)	0.004 (0.04)	0.99	
Family/Social	0.23 (0.22)	0.25 (0.21)	0.57		0.23 (0.22)	0.23 (0.20)	0.88	
Psychiatric	0.13 (0.18)	0.20 (0.24)	0.01	*	0.12 (0.18)	0.20 (0.23)	0.01	*

Table 7. *Cont.*

	Brothers and Sisters				Partner			
	Good relationship	**Bad relationship**	***p***		**Good relationship**	**Bad relationship**	***p***	
Medical	0.22 (0.29)	0.26 (0.32)	0.30		0.26 (0.31)	0.27 (0.31)	0.77	
Employment	0.51 (0.27)	0.61 (0.30)	0.00	*	0.48 (0.29)	0.60 (0.27)	0.00	*
Alcohol use	0.53 (0.22)	0.57 (0.23)	0.18		0.55 (0.22)	0.53 (0.22)	0.46	
Drug use	0.01 (0.04)	0.01 (0.04)	0.60		0.01 (0.04)	0.01 (0.04)	0.26	
Legal	0.003 (0.03)	0.01 (0.05)	0.32		0.005 (0.04)	0.01 (0.04)	0.67	
Family/Social	0.22 (0.22)	0.26 (0.19)	0.17		0.19 (0.19)	0.33 (0.23)	0.00	*
Psychiatric	0.13 (0.17)	0.20 (0.25)	0.02	*	0.13 (0.18)	0.17 (0.22)	0.09	
	Children				**Friends**			
	Good relationship	**Bad relationship**	***p***		**Good relationship**	**Bad relationship**	***p***	
Medical	0.25 (0.31)	0.32 (0.32)	0.11		0.24 (0.30)	0.31 (0.34)	0.15	
Employment	0.50 (0.30)	0.56 (0.27)	0.13		0.51 (0.29)	0.59 (0.29)	0.07	
Alcohol use	0.54 (0.23)	0.55 (0.21)	0.81		0.55 (0.22)	0.55 (0.22)	1.00	
Drug use	0.01 (0.04)	0.004 (0.02)	0.38		0.01 (0.04)	0.01 (0.02)	0.43	
Legal	0.003 (0.02)	0.01 (0.04)	0.52		0.004 (0.03)	0.01 (0.05)	0.36	
Family/Social	0.22 (0.22)	0.27 (0.19)	0.14		0.23 (0.21)	0.23 (0.22)	0.82	
Psychiatric	0.13 (0.19)	0.15 (0.21)	0.70		0.13 (0.18)	0.22 (0.26)	0.02	*

* Significant difference.

Table 8. Comparison of severity of addiction between good and bad family relationships in patients with methamphetamine dependence.

	Mother			Father		
	Good relationship	**Bad relationship**	***p***	**Good relationship**	**Bad relationship**	***p***
Medical	0.05 (0.17)	0.13 (0.24)	0.21	0.04 (0.13)	0.12 (0.25)	0.08
Employment	0.67 (0.22)	0.74 (0.25)	0.28	0.70 (0.21)	0.70 (0.25)	0.99
Alcohol use	0.12 (0.19)	0.19 (0.27)	0.26	0.10 (0.20)	0.16 (0.23)	0.27
Drug use	0.09 (0.09)	0.12 (0.12)	0.27	0.08 (0.09)	0.12 (0.11)	0.19
Legal	0.02 (0.07)	0.04 (0.13)	0.29	0.01 (0.06)	0.03 (0.11)	0.32
Family/Social	0.14 (0.13)	0.23 (0.20)	0.05	0.13 (0.13)	0.21 (0.18)	0.06
Psychiatric	0.23 (0.24)	0.32 (0.27)	0.17	0.24 (0.23)	0.30 (0.27)	0.34
	Brothers/Sisters			**Partner**		
	Good relationship	**Bad relationship**	***p***	**Good relationship**	**Bad relationship**	***p***
Medical	0.05 (0.14)	0.12 (0.24)	0.18	0.08 (0.20)	0.05 (0.14)	0.54
Employment	0.66 (0.21)	0.77 (0.25)	0.08	0.62 (0.26)	0.73 (0.21)	0.10
Alcohol use	0.10 (0.19)	0.21 (0.26)	0.08	0.19 (0.24)	0.13 (0.24)	0.43
Drug use	0.09 (0.09)	0.12 (0.12)	0.31	0.09 (0.12)	0.09 (0.09)	0.93
Legal	0.02 (0.08)	0.04 (0.11)	0.51	0.04 (0.11)	0.002 (0.01)	0.09
Family/Social	0.15 (0.16)	0.23 (0.17)	0.07	0.18 (0.14)	0.17 (0.14)	0.80
Psychiatric	0.23 (0.26)	0.30 (0.24)	0.36	0.24 (0.26)	0.24 (0.23)	0.94

Table 8. *Cont.*

	Children			Friends		
	Good relationship	Bad relationship	*p*	Good relationship	Bad relationship	*p*
Medical	0.11 (0.20)	0.07 (0.19)	0.71	0.08 (0.21)	0.09 (0.20)	0.77
Employment	0.54 (0.34)	0.66 (0.27)	0.42	0.69 (0.24)	0.69 (0.23)	0.94
Alcohol use	0.16 (0.18)	0.34 (0.34)	0.24	0.13 (0.21)	0.17 (0.25)	0.55
Drug use	0.04 (0.06)	0.07 (0.11)	0.52	0.09 (0.10)	0.12 (0.12)	0.46
Legal	0.03 (0.09)	0.00 (0.00)	0.42	0.02 (0.09)	0.04 (0.11)	0.62
Family/Social	0.15 (0.13)	0.15 (0.12)	0.89	0.18 (0.15)	0.16 (0.16)	0.69
Psychiatric	0.14 (0.19)	0.14 (0.19)	0.97	0.28 (0.24)	0.69	

* Significant difference.

4. Discussion

With regard to the comparisons of family relationships between patients with alcohol dependence and patients with methamphetamine dependence, patients with methamphetamine dependence had difficulty developing good relationships with their father. With regard to the association between good relationships and the severity of substance dependence, in patients with alcohol dependence, bad relationships with parents, brothers and sisters, and friends were related to severe psychiatric problems. Bad relationships with brothers and sisters and partners were related to severe employment/support problems. Bad relationships with partners were related to severe family/social problems. In patients with methamphetamine dependence, no association was found between relationships and severity of substance dependence.

With regard to the associations between ASI CSs, psychiatric problems were related to drug use and family/social relationships in patients with alcohol dependence, and psychiatric problems were related to medical, employment/support, and family/social relationship problems in patients with methamphetamine dependence. In patients with alcohol dependence, relationships with various family members and friends were related to their mental condition, and bad relationships with their partners may be heavily involved in their difficult interpersonal relationships. Because problems with family/social relationships were related to psychiatric problems, bad relationships with their partners may be involved in psychiatric problems through their difficulties with interpersonal relationships. Additionally, the association between psychiatric problems and drug use in patients with alcohol dependence may be affected by the drugs prescribed for their psychiatric problems. Notably, some patients with alcohol dependence reported dependence on barbiturates or other analgesics/hypnotics/tranquilizers. Moreover, a deterioration of psychiatric problems may be involved in increased medical problems, employment/support problems, and drug use problems. These results suggest that although the ASI was developed to independently evaluate each of these seven problem areas [12], family relationships may be particularly related to psychiatric problems. Moreover, with regard to the associations between family/social relationships and specific symptoms, bad family/social relationships in alcohol dependence were related to the presence of serious depression, serious anxiety or tension, and serious thoughts of suicide, and bad family/social relationships in methamphetamine dependence were not related to the presence of specific psychiatric symptoms. Bad

family/social relationships in patients with alcohol dependence and patients with methamphetamine dependence may be differentially related to psychiatric problems. Investigating the association between family relationships and psychiatric disorders may be useful, based on the relationship between family/social relationships and psychiatric status found in the present study.

The average age of the patients with alcohol dependence was higher than the average age of the patients with methamphetamine dependence, suggesting that having a long-term residence may be attributable to the higher average age of the patients with alcohol dependence. With regard to educational background, the higher ratio of junior high school graduation in patients with alcohol dependence may be attributable to the age group of patients with alcohol dependence, which contained many older patients. The higher ratio of being a high school dropout in patients with methamphetamine dependence may reflect their difficulty maintaining their relationships or completing their schoolwork on school days. With regard to employment status, the higher ratio of retirement in patients with alcohol dependence may be attributable to their higher average age, and the higher ratios of part-time employment and unemployment in patients with methamphetamine dependence may reflect their difficulty retaining a job. With regard to abuse experience, the higher ratio of being a victim of physical abuse in patients with methamphetamine dependence may make developing a trusting relationship with someone difficult. Consistent with this possibility, a previous study suggested that male victims of physical and sexual abuse have difficulties seeking and retaining gainful employment, trusting others, developing intimate relationships, and regulating their anger and behavior [14]. The higher number of convictions in patients with methamphetamine dependence suggests that methamphetamine dependence is complicated by antisocial personality disorder.

With regard to family histories of alcohol dependence, drug dependence, and psychiatric disorders, patients with alcohol dependence had higher ratios of having a father, paternal uncle, and brother with alcohol-related problems. Patients with methamphetamine dependence had higher ratios of having a brother with drug-related problems. These significant results were found only with male relatives, and substance (alcohol or drug) use that became a problem for patients was common when substances were used by their male relatives. However, because these results may have been affected by the high prevalence of individuals with alcohol or drug dependence in the male population [15], these results should be interpreted with caution. Additionally, information about antisocial characteristics among not only the patients but also their families may be worth collecting in future studies to ascertain differences in the interactions between parents and children with substance dependence.

Based on the above results from patients with alcohol dependence, unestablished family relationships over time influenced a wide range of problems, especially the severity of psychiatric problems. This result suggests the usefulness of psychological therapy for treating family dysfunction and self-help group therapy. In patients with methamphetamine dependence, unestablished relationships with their father over the years may not have been linked to their present severity of substance dependence in ASI CSs. Moreover, not simply relationships with specific family members but overall family/social relationships may be related to severity in ASI CSs (e.g., Psychiatric, Medical, and Legal problems). Given the result that patients with methamphetamine dependence often lived with their parents, investigating the effect of bad relationships with their father on relationships with their brother with drug-related problems may be important. Furthermore, verifying the possibility that

patients with methamphetamine dependence may not often establish good relationships with their father because of their experiences of abuse by their parents may be meaningful in future studies.

A previous study suggested the importance of distinguishing between alcohol and drug dependence disorders and examining their differential etiological pathways [16]. The present study may also suggest the necessity of separately investigating the association between family relationships and various problems related to substance dependence in alcohol dependence and methamphetamine dependence. The results of the present study may provide support for the possibility that the results of the ASI as an intake instrument may be an indicator of early intervention for family and social problems, and personalized programs that augment usual interventions may be useful.

Although this study provided useful new insights, it has a few limitations. First, the sample did not contain female patients. Role differences in a family may exist between males and females. Future studies should assess female patients. Second, the uniformity of the participants in this study may be problematic, including differences in age and present status (*i.e.*, inpatient, outpatient, or recovering individual) between the alcohol dependence group and methamphetamine dependence group. Third, this study utilized a cross-sectional design, so we could not establish a causal relationship between family relationships and problems related to alcohol or drug dependence. However, the results of this study may be beneficial for future longitudinal studies.

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Inhibitory Role of Inducible cAMP Early Repressor (ICER) in Methamphetamine-Induced Locomotor Sensitization

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Abstract

Background: The inducible cyclic adenosine monophosphate (cAMP) early repressor (ICER) is highly expressed in the central nervous system and functions as a repressor of cAMP response element-binding protein (CREB) transcription. The present study sought to clarify the role of ICER in the effects of methamphetamine (METH).

Methods and Findings: We tested METH-induced locomotor sensitization in wildtype mice, ICER knockout mice, and ICER I-overexpressing mice. Both ICER wildtype mice and knockout mice displayed increased locomotor activity after continuous injections of METH. However, ICER knockout mice displayed a tendency toward higher locomotor activity compared with wildtype mice, although no significant difference was observed between the two genotypes. Moreover, compared with wildtype mice, ICER I-overexpressing mice displayed a significant decrease in METH-induced locomotor sensitization. Furthermore, Western blot analysis and quantitative real-time reverse transcription polymerase chain reaction demonstrated that ICER overexpression abolished the METH-induced increase in CREB expression and repressed cocaine- and amphetamine-regulated transcript (CART) and prodynorphin (Pdyn) expression in mice. The decreased CART and Pdyn mRNA expression levels *in vivo* may underlie the inhibitory role of ICER in METH-induced locomotor sensitization.

Conclusions: Our data suggest that ICER plays an inhibitory role in METH-induced locomotor sensitization.

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Introduction

The inducible cyclic adenosine monophosphate (cAMP) early repressor (ICER) is the collective name for a group of proteins produced from the cAMP response element modulator (CREM)/ICER gene driven by the P2 internal promoter located in an intron of the CREM gene [1]. Lacking the CREM *N*-terminus, ICER only contains two DNA binding domains (DBD I and DBD II) and lacks the activation and kinase-inducible domains. Consequently, ICER functions as an endogenous repressor of transcription of several cAMP response element (CRE)-containing genes [1–3]. The P2 promoter of the ICER gene contains two pairs of CRE sequences. The phosphorylated CRE-binding protein (CREB) can induce transcription of the ICER gene from the P2 promoter. The increased ICER competes with CREB in binding with the CRE sequence, blocking transcription from CRE-containing promoters, including ICER's own promoter, and functioning as a potent endogenous CREB antagonist [1,4].

Four ICER isoforms have been identified: ICER I, ICER Iγ, ICER II, and ICER IIγ. ICER I mRNA contains DBD I and DBD II, but DBD II is absent in the ICER I protein because a stop codon exists at the end of DBD I. The ICER II isoform contains only DBD II. ICER Iγ and ICER IIγ are characterized by a deficiency of exon γ from ICER I and ICER II, respectively [4].

Numerous reports have shown that CREB in the nucleus accumbens (NAc) is associated with responses to drugs of abuse and emotional responses. Chronic drug administration increases levels of CREB immunoreactivity and CRE-binding activity [5–6]. Overexpression of CREB by introducing herpes simplex virus-CREB into the NAc decreases behavioral responses to drug administration, whereas blockade of CREB transcription via introducing a dominant-negative CREB mutant or via genetic knockout increases behavioral responses to drug administration [7–10]. However, other studies showed that genetic ablation of CREB did not affect the rewarding effects of psychostimulants [11–13], indicating that the role of CREB in drug-induced responses is debatable. Recent findings suggest that ICER mRNA expression was threefold higher in the striatum after amphetamine injection [14], suggesting that the endogenous functional CREB antagonist ICER may participate in the mechanisms that underlie the effects of drugs of abuse.

The prodynorphin (Pdyn) peptide is an endogenous ligand of the κ opioid receptor. Cocaine- and amphetamine-regulated transcript (CART) was first sequenced as a peptide with unknown function [15], and previous studies revealed that the CART

peptide is co-localized with Pdyn in brain regions associated with drug reward, including the NAc and ventral tegmental area (VTA) [16–17]. Both CART and Pdyn play roles as psychostimulant neuromodulators [8,18–20]. CART and Pdyn mRNA are suggested to be CRE-mediated transcripts regulated by CREB *in vitro* and *in vivo* [8,21–23].

Kojima *et al.* [24] generated two types of ICER mutant mice—ICER knockout mice and ICER-overexpressing mice—and suggested a negative role for ICER in regulating long-term fear memory and kindling epileptogenesis. The present study used two types of transgenic mice with opposite genetic alterations of ICER gene expression (i.e., ICER knockout and ICER I-overexpressing mice) and investigated the role of ICER in methamphetamine (METH)-induced locomotor sensitization. Locomotor sensitization is characterized by the progressive enhancement of locomotor activity after repeated psychostimulant exposure [25–26]. The augmentation of this behavioral response can be maintained for several months after the cessation of drug treatment [27]. We observed an inhibitory effect of ICER on METH-induced locomotor sensitization. To identify the downstream components of ICER-mediated gene transcription *in vivo* and provide a possible mechanism that contributes to the inhibitory role of ICER in METH-induced locomotor sensitization, we determined METH-induced CREB and phosphorylated CREB (pCREB) levels using Western blot analysis and further determined CART and Pdyn mRNA expression levels in the striatum (caudate putamen [CPu], which mediates locomotor activity) but not in the NAc (which mainly mediates the rewarding effects of drugs of abuse) in ICER I-overexpressing mice and their littermates using real-time reverse transcription polymerase chain reaction (RT-PCR).

Results

METH-induced locomotor sensitization in ICER I-overexpressing mice

Consistent with a previous study [28], on Day 1, the initially elevated levels of locomotor activity in wildtype mice were reduced to near-zero levels after 180 min habituation. ICER I-overexpressing mice displayed a similar pattern of locomotor activity as wildtype mice (Fig. 1*a*). No significant difference in baseline locomotion was observed between genotypes ($n = 7$ for wildtype mice; $n = 9$ for ICER I-overexpressing mice; $F_{1,16} = 0.49$, $p = 0.49$; Fig. 1*a*). On Day 20, ICER I-overexpressing mice displayed decreased levels of spontaneous locomotor activity during the 180 min habituation period compared with wildtype mice ($F_{1,14} = 9.934$, $p = 0.007$; Fig. 1*b*). After a METH injection (1 mg/kg), a significant difference was observed between the two genotypes ($F_{1,14} = 14.566$, $p = 0.0019$; Fig. 1*b*). Repeated administration of METH (1 mg/kg) on Days 1, 3, 5, 7, 9, 11, 13, and 20 significantly increased locomotor activity in both wildtype and ICER I-overexpressing mice (Fig. 1*c*). A two-way, mixed-design analysis of variance (ANOVA; Genotype×Day) revealed a significant effect of Day ($F_{7,98} = 19.13$, $p<0.0001$), indicating the presence of METH-induced locomotor sensitization. METH-induced locomotor sensitization in ICER I-overexpressing mice significantly decreased compared with wildtype mice ($F_{1,14} = 12.54$, $p = 0.0033$; Fig. 1*c*), and a significant Genotype×Day interaction was observed ($F_{7,98} = 6.52$, $p<0.0001$; Fig. 1*c*). From Day 5, locomotor activity in ICER I-overexpressing mice was significantly lower than in wildtype mice (Student's *t*-test).

METH-induced locomotor sensitization in ICER knockout mice

On Day 1, the levels of locomotor activity in wildtype and ICER knockout mice were reduced to near-zero after 180 min habitua-

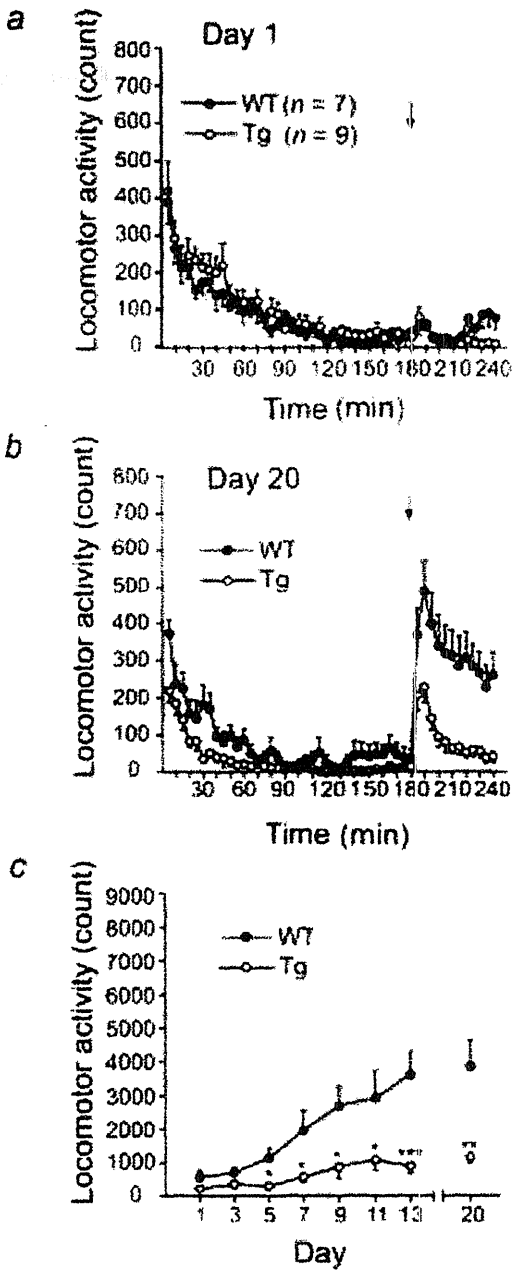

Figure 1. Spontaneous and METH-stimulated locomotor activity in wildtype mice (WT) and ICER I-overexpressing mice (Tg). METH (1 mg/kg) was administered once per day on Days 1, 3, 5, 7, 9, 13, and 20 in WT ($n = 7$) and Tg ($n = 9$) mice. *a.* Time-course of spontaneous locomotor activity before and after METH administration on Day 1. The data are expressed as mean ± SEM beam breaks in 5 min bins. The arrow indicates the start of a METH injection. *b.* Time-course of spontaneous locomotor activity before and after METH administration on Day 20. The data are expressed as mean ± SEM beam breaks in 5 min bins. The arrow indicates the start of a METH injection. *c.* METH-induced locomotor sensitization. The data are expressed as mean ± SEM beam breaks during the 60 min period after METH injection (1 mg/kg) on Days 1, 3, 5, 7, 9, 13, and 20. *$p<0.05$, **$p<0.01$, ***$p<0.001$, significant difference in locomotor activity scores between WT and Tg mice.

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tion. No significant difference in baseline locomotion was observed between genotypes ($n = 13$ for both wildtype and knockout mice; $F_{1,24} = 0.27$, $p = 0.61$; Fig. 2*a*). After repeated procedures on Days 1, 3, 5, 7, 9, 11, and 13 and a 7 day drug-free period, on Day 20, the levels of locomotor activity in the two genotypes were reduced but did not reach near-zero levels after 180 min habituation, which might have been caused by the repeated METH administration. No significant difference was detected between the two genotypes during the habituation period ($F_{1,24} = 2.731$, $p = 0.12$; Fig. 2*b*). After a METH injection (1 mg/kg), locomotor activity in both genotypes increased significantly. No significant difference was observed between the two genotypes ($F_{1,24} = 2.071$, $p = 0.16$; Fig. 2*b*). Repeated administration of METH (1 mg/kg) significantly increased locomotor activity in both wildtype and ICER knockout mice (Fig. 2*c*). A two-way, mixed-design ANOVA (Genotype×Day) revealed a significant effect of Day ($F_{7,168} = 25.88$, $p<0.0001$), indicating the presence of METH-induced locomotor sensitization. ICER knockout mice showed a tendency toward higher locomotor activity compared with their wildtype littermates ($F_{1,24} = 2.96$, $p = 0.098$). ICER knockout mice displayed greater locomotor activity on Day 3 and Day 11 compared with wildtype mice ($p<0.05$; Tukey-Kramer *post hoc* test). No significant Genotype× Day interaction was observed ($F_{7,168} = 0.62$, $p = 0.74$).

METH-induced CREB expression and phosphorylation in the CPu was abolished in ICER I-overexpressing mice

Two-way ANOVA revealed marginal differences between genotypes in CREB and pCREB protein levels in the CPu after repeated METH treatment (CREB: $F_{1,40} = 3.76$, $p = 0.06$; pCREB: $F_{1,40} = 3.51$, $p = 0.07$). No significant difference in the effect of METH was found (CREB: $F_{3,40} = 1.28$, $p = 0.29$; pCREB: $F_{3,40} = 1.38$, $p = 0.26$), and no Genotype×METH interaction was observed (CREB: $F_{3,40} = 1.90$, $p = 0.15$; pCREB: $F_{3,40} = 1.79$, $p = 0.16$). The Dunnett *post hoc* test revealed that repeated METH/saline challenge significantly increased CREB protein levels in wildtype mice compared with the saline group ($n = 6$ per group, $p<0.05$; Fig. 3*a*). The level of activated CREB protein (pCREB) in the repeated METH/saline challenge group also significantly increased in wildtype mice ($n = 6$ per group, $p<0.05$, Dunnett *post hoc* test; Fig. 3*b*). However, the levels of CREB and pCREB protein were not significantly altered after repeated METH injection in ICER I-overexpressing mice (Fig. 3).

ICER overexpression significantly reduced CART and Pdyn mRNA expression in the CPu

To identify the downstream components of CRE-mediated gene transcription that contribute to reduced METH-induced locomotor sensitization in ICER I-overexpressing mice, real-time RT-PCR was conducted. First, ICER mRNA levels were evaluated using ICER-specific primers. Significant effects were found for Genotype ($F_{1,24} = 1850.5$, $p<0.001$, two-way ANOVA; Fig. 4*a*). However, METH injection did not significantly affect ICER mRNA levels in wildtype mice ($n = 4$ per group, $p>0.05$, Dunnett *post hoc* test). Furthermore, we evaluated CART and Pdyn mRNA levels because they are suggested to be CRE-mediated transcripts and psychostimulant neuromodulators. Although METH did not alter CART or Pdyn mRNA expression in ICER I-overexpressing mice and their littermates (CART: $F_{3,24} = 0.31$, $p = 0.81$; Pdyn: $F_{3,24} = 0.38$, $p = 0.77$; two-way ANOVA), CART and Pdyn mRNA expression levels were significantly reduced in ICER I-overexpressing mice compared with their littermates (CART: $F_{1,24} = 17.25$, $p<0.01$; Pdyn: $F_{1,24} = 12.21$, $p<0.01$; two-way ANOVA; Fig. 4*b*, *c*). No significant Genotype×METH interaction was observed (CART: $F_{3,24} = 0.21$, $p = 0.89$; Pdyn: $F_{3,24} = 0.17$, $p = 0.92$).

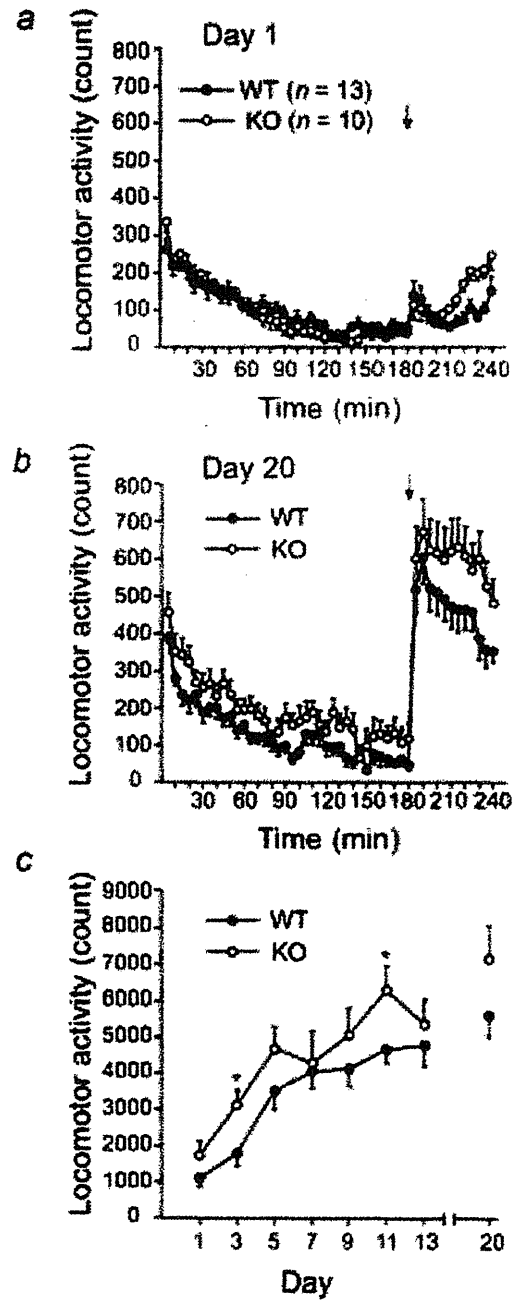

Figure 2. Spontaneous and METH-stimulated locomotor activity in wildtype (WT) and ICER knockout (KO) mice. METH (1 mg/kg) was administered once per day on Days 1, 3, 5, 7, 9, 13, and 20 in WT ($n = 13$) and ICER-KO ($n = 13$) mice. *a.* ime-course of spontaneous locomotor activity before and after METH administration on Day 1. The data are expressed as mean ± SEM beam breaks in 5 min bins. The arrow indicates the start of a METH injection. *b.* Time-course of spontaneous locomotor activity before and after METH administration on Day 20. The data are expressed as mean ± SEM beam breaks in 5 min bins. The arrow indicates the start of a METH injection. *c.* METH-induced locomotor sensitization. The data are expressed as mean ± SEM beam breaks during the 60 min period after METH injection (1 mg/kg). *$p<0.05$, significant difference in locomotor activity scores between WT and KO mice.
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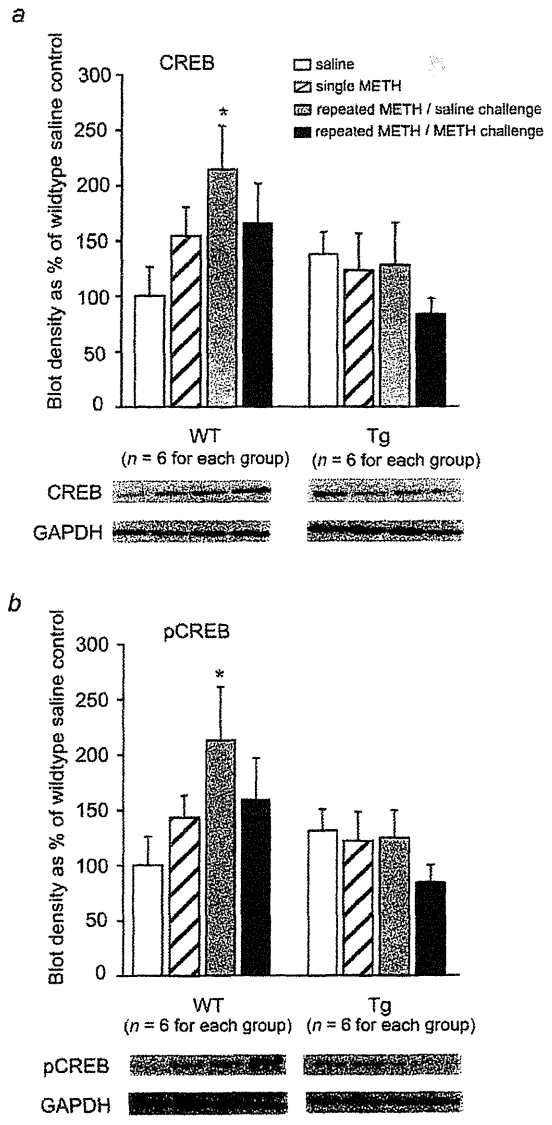

Figure 3. CREB expression and phosphorylation in the CPu after single and repeated METH treatment. The mice were administered METH (1 mg/kg, i.p.) or saline once or received METH (1 mg/kg, i.p.) once every other day from Day 1 to Day 13 and challenged with saline or METH (1 mg/kg, i.p.) on Day 20 after a 7 day drug-free period. The mice were decapitated 1 h after the last METH or saline treatment. The blot density of each group was normalized to that of the wildtype saline group and is expressed as mean ± SEM ($n = 6$). *a.* METH-induced CREB expression in the CPu in wildtype mice (WT) and ICER I-overexpressing mice (Tg). *$p<0.05$, significant difference in normalized CREB blot density compared with wildtype saline group. *b.* METH-induced CREB phosphorylation in the CPu in wildtype mice (WT) and ICER I-overexpressing mice (Tg). *$p<0.05$, significant difference in normalized pCREB blot density compared with wildtype saline group.
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Discussion

The present study investigated the role of ICER in long-lasting METH-induced behavioral alterations by evaluating METH-induced locomotor sensitization in ICER knockout and ICER-overexpressing mice. The major findings of the present study were that ICER I overexpression significantly inhibited METH-induced locomotor sensitization and blocked METH-induced increases in CREB and pCREB protein levels. Additionally, CART and Pdyn mRNA expression levels in the CPu were significantly reduced in ICER-overexpressing mice. ICER knockout mice displayed a tendency toward higher activity after repeated METH administration compared with their wildtype littermates, although no significant difference was detected between ICER knockout mice and their wildtype littermates. Considering the negative regulatory role of CREB in the effects of psychostimulants [18,29–30], the reduction in METH-induced locomotor sensitization in ICER-overexpressing mice may be attributable to reduced CART and Pdyn mRNA expression, rather than attributable to increased CREB and pCREB protein levels.

Inhibitory role of ICER in METH-induced locomotor sensitization

Although the mechanisms that underlie locomotor sensitization are not fully understood, it is hypothesized to reflect neuronal adaptations in several brain regions, including in dopamine neurons and the CPu [25]. In the present study, ICER I-overexpressing mice exhibited a significant reduction in METH-induced locomotor sensitization compared with wildtype mice (Fig. 1*c*), whereas ICER knockout mice showed a minimal enhancement of METH-induced locomotor sensitization compared with wildtype mice (Fig. 2*c*). Altogether, these results suggest that ICER plays an inhibitory role in METH-induced locomotor sensitization.

CREB overexpression in the NAc reportedly decreased cocaine- and morphine-induced conditioned place preference (CPP), and decreased CREB in the NAc increased cocaine- and morphine-induced CPP [7–8], suggesting that increased CREB in the NAc has an inhibitory effect on the induction of CPP. However, recent studies have reported conflicting results, in which genetic ablation of CREB did not affect the rewarding properties of psychostimulants [11–13]. Similarly, some studies demonstrated an inhibitory role of CREB in cocaine-induced sensitization [31–32], whereas other studies with CREB mutant mice suggested either minor effects [33] or no effects [11] of CREB on cocaine-induced sensitization. In the present study, overexpression of the endogenous CREB repressor ICER inhibited METH-induced locomotor sensitization. Thus, the inhibitory effect of CREB on the psychostimulant-induced response is debatable. A possible explanation for these discrepant results may include the different gene manipulations (i.e., forebrain- or NAc-specific gene manipulation), different drug types (i.e., METH or cocaine/morphine), and different targeted genes (i.e., ICER or CREB).

Enhanced pCREB in the striatum is a molecular marker of neuroadaptations to chronic psychostimulant-induced plasticity [8,21,29,34–35]. In the present study, both CREB and pCREB levels increased in wildtype mice after repeated METH injection. The increased CREB and subsequent pCREB induced by repeated METH might homeostatically oppose the effect of METH [29]. However, the repeated METH-induced increases in CREB levels were blocked by ICER I overexpression, suggesting that the negative regulation of the CREB pathway was absent in ICER I-overexpressing mice. Therefore, the CREB pathway may not be involved in the reduced locomotor sensitization observed in ICER I-overexpressing mice. Additionally, ICER expression was 60-fold greater in ICER overexpressing mice than in wildtype mice, which may not occur under physiological conditions. The 60-fold increase in expression may interfere with the CREB signaling pathway and homeostatic regulation of CREB.

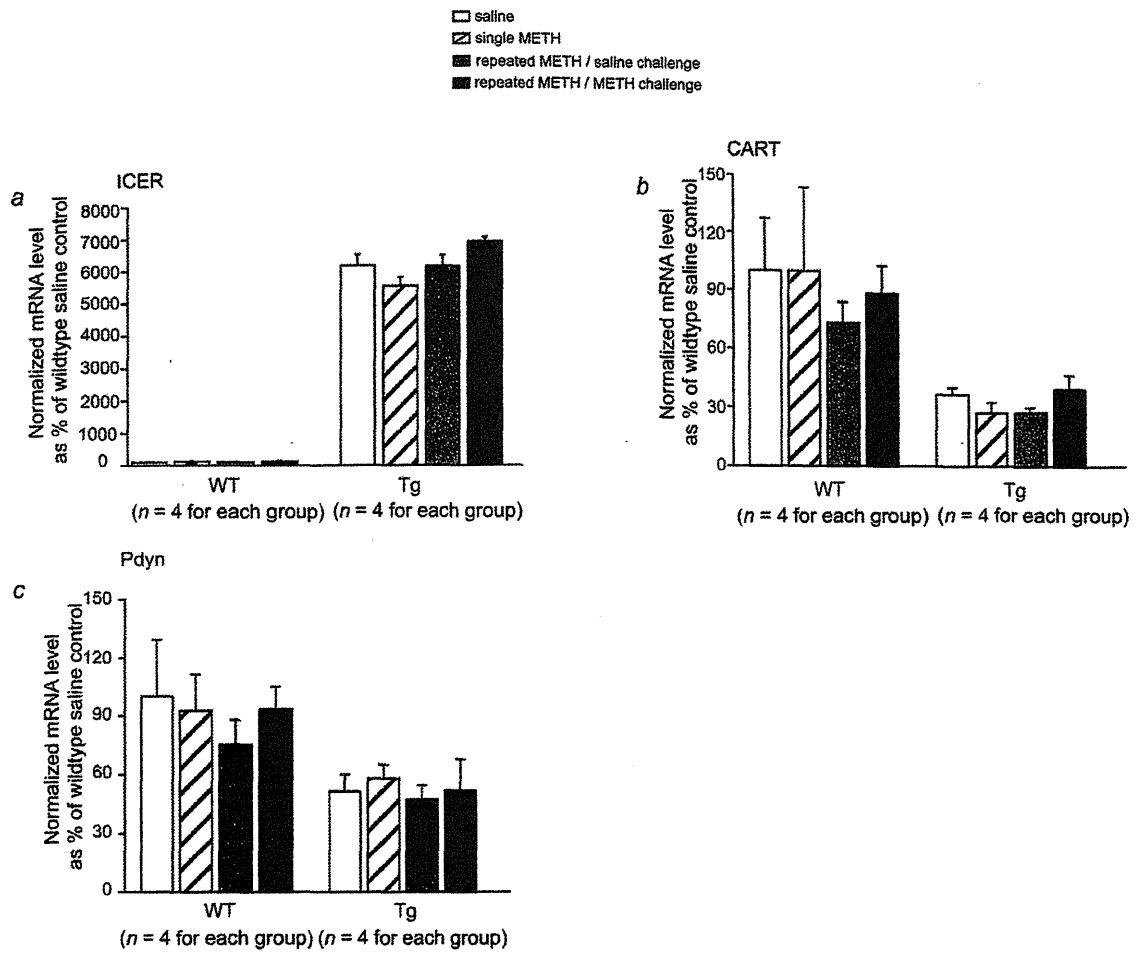

Figure 4. ICER, CART, and Pdyn mRNA levels in the CPu after single and repeated METH treatment. The mice were administered METH (1 mg/kg, i.p.) or saline once or received METH (1 mg/kg, i.p.) once every other day from Day 1 to Day 13 and challenged with saline or METH (1 mg/kg, i.p.) on Day 20 after a 7 day drug-free period. The mice were decapitated 1 h after the last METH or saline treatment. *a.* ICER mRNA expression in the CPu in wildtype (WT) and ICER I-overexpressing (Tg) mice. The data are expressed as mean ± SEM ($n=4$). *b.* CART mRNA expression in the CPu after single and repeated METH treatment. The data are expressed as mean ± SEM ($n=4$). *c.* Pdyn mRNA expression in the CPu after single and repeated METH treatment. The data are expressed as mean ± SEM ($n=4$).
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Inhibitory role of ICER in regulating CART and Pdyn mRNA expression

CART and dynorphin are peptidergic neurotransmitters expressed in the CPu and other brain regions and modulate the rewarding effects of drugs of abuse [17,26.36]. CART's involvement in the actions of psychostimulants was first noted in a study that demonstrate that acute cocaine and amphetamine upregulated CART mRNA in the rat brain [37]. However, this report has been controversial because this finding has been difficult to replicate [38–40]. Other studies found that binge cocaine exposure, rather than acute administration, reliably increases CART expression [38,41]. Additionally, Pdyn mRNA has been reported to increase or not change in response to binge cocaine administration [42,43]. In the present study, neither acute nor repeated administration of METH (1 mg/kg) altered CART and Pdyn mRNA expression in wildtype mice. Furthermore, METH administration (1 mg/kg) did not alter ICER mRNA expression in wildtype mice. A possible reason for this might be that the 1 mg/kg dose of METH may not have been sufficient to induce detectable alterations of ICER, CART, and Pdyn mRNA. However, CART and Pdyn mRNA expression levels significantly decreased as ICER mRNA levels significantly increased, suggesting an inhibitory role of ICER in CART and Pdyn expression. Both the CART and Pdyn genes contain a CRE site in their promoter regions [21–22], and CART and Pdyn mRNA levels are regulated by CREB *in vitro* [21,44] and *in vivo* [8,23]. Therefore, as a CRE-mediated gene transcription repressor, ICER may inhibit the expression of CART and Pdyn *in vivo*. Our studies using ICER I-overexpressing mice support this hypothesis.

CART and Pdyn as neuromodulators of the behavioral effects of psychostimulants

The CART and Pdyn peptides are neurotransmitters expressed in brain regions associated with drug reward, including the NAc and VTA [16–17]. Numerous studies have suggested that CART and Pdyn play a homeostatic role in the NAc to oppose the effects of cocaine. For example, pretreatment with Dyn A (1–17) is effective at decreasing striatal dopamine levels and attenuating

cocaine-induced CPP in mice [45]. Overexpression of CREB, with resulting increases in Pdyn gene expression, in the NAc has been shown to decrease the rewarding effects of cocaine [8]. Microinjection of CART peptide 55–102 into the NAc blocked the rewarding effects of cocaine and amphetamine [46–48]. CREB overexpression increases CART mRNA levels in the NAc and decreases the rewarding effects of drugs [23]. However, studies in knockout mice have reported conflicting results. CART knockout mice exhibited attenuated locomotor sensitization induced by amphetamine [18], and Pdyn knockout mice showed decreased locomotor activity evoked by cocaine [30]. ICER I-overexpressing mice with decreased CART and Pdyn expression levels displayed attenuated METH-induced locomotor sensitization in the present study. These discrepant results among CART and Pdyn studies may be attributable to differences between systemic and NAc-specific downregulation of CART or Pdyn. Further studies are needed to clarify the effects of CART and Pdyn in brain regions other than the NAc.

Conclusion

The present study suggests that ICER plays an inhibitory role in METH-induced locomotor sensitization. Our results support the modulatory effects of the ICER pathway in regulating the effects of drugs of abuse and provide an incentive for exploring the therapeutic potential of stimulating the ICER pathway in the treatment of drug abuse.

Materials and Methods

Ethics statement

The experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committee (Animal Experimentation Ethics Committee of Tokyo Metropolitan Institute of Medical Science, Approval ID: 11-029), and all animals were cared for and treated humanely in accordance with our institutional animal experimentation guidelines.

Animals

Wildtype, ICER knockout, and ICER I-overexpressing mice were produced by conventional gene targeting and transgenic methods [24]. Briefly, the P2 exon encoding the 5′ coding sequence of ICER was deleted to generate ICER-specific knockout mice. To generate ICER I-overexpressing mice, the entire coding sequence of cDNA was subcloned into a pNN265 vector, and the promoter for Ca^{2+}/calmodulin-dependent protein kinase II α (CaMKIIα) was used to express ICER I in the forebrain. The expression patterns of other CREB/CREM family members are not altered in either ICER knockout mice or ICER I-overexpressing mice. ICER knockout mice and their littermates were produced by heterozygote-heterozygote mating. ICER I-overexpressing mice and their wildtype littermates were produced by mating ICER I-overexpressing mice (line I-19) and C57BL/6 mice (CLEA Japan Inc., Shizuoka, Japan) because C57BL/6 is the genetic background strain of ICER I-overexpressing mice. Only naive male mice were used for the experiments. The mice were housed five per cage in a temperature- (22±2°C) and humidity-controlled (55±5%) environment on a 12 h/12 h light/dark cycle (lights on 8:00 a.m. to 8:00 p.m.). The mice had *ad libitum* access to a standard laboratory diet and water. All animal experiments were conducted during the light phase of the cycle, between 9:00 a.m. and 5:00 p.m.

Drugs

Methamphetamine hydrochloride (Dainippon-Sumitomo Pharmaceuticals, Osaka, Japan) was dissolved in saline (0.9% sodium chloride) and administered intraperitoneally (i.p.) in a volume of 10 ml/kg.

Locomotor activity

Locomotor activity corresponding to distance travelled was evaluated in a test chamber (25 cm diameter, 27 cm height) and measured in 5 min bins using digital counters with passive infrared sensors (Supermex system, Muromachi Kikai, Tokyo, Japan). Wildtype littermates of ICER knockout mice ($n = 13$), ICER knockout mice ($n = 13$), wildtype littermates of ICER I-overexpressing mice ($n = 7$), and ICER I-overexpressing mice ($n = 9$) were used. The mice were first habituated to the apparatus for 180 min and then injected with METH (1 mg/kg, i.p.). Locomotor activity was then measured for 60 min after the injection. The procedure was repeated seven times, once every other day from Day 1 to Day 13. After a 7 day drug-free period, locomotor activity was measured again after an injection of METH (1 mg/kg, i.p.) on Day 20.

Western blot analysis

The experiment involved four groups of ICER I-overexpressing mice and wildtype mice: Saline, Single METH, Repeated METH/Saline Challenge, and Repeated METH/METH Challenge. Saline and METH (1 mg/kg, i.p.) were administered once to Saline and Single METH mice, respectively, and the mice were decapitated 1 h after the injection. The Repeated METH/Saline Challenge and Repeated METH/METH Challenge groups received METH (1 mg/kg, i.p.) once every other day from Day 1 to Day 13 and were challenged with saline and METH (1 mg/kg, i.p.), respectively, on Day 20 after a 7 day drug-free period. The mice were decapitated 1 h after the last METH or saline treatment. The brains were removed in less than 45 s and cooled rapidly in ice-cold saline for 30 s. The CPu was then dissected. The tissue was quickly frozen on dry ice, stored at −80°C, and homogenized in 100 µl phosphate-buffered saline containing protease inhibitors (Roche Applied Science, Mannheim, Germany) and PhosStop phosphatase inhibitors (Roche Applied Science, Mannheim, Germany). The homogenate was diluted to 4 µg/µl with 2× Laemmli buffer, heated to 95°C for 2 min, and loaded (20 µg of protein) onto 5–20% gradient polyacrylamide gels. The proteins from eight groups were loaded onto the same gel and separated at 50 mA for approximately 1 h and then transferred onto polyvinylidene membranes in a semi-dry blotter. Nonspecific protein binding sites were blocked by incubating in Blocking One Solution (Nakalai Tesque Inc., Kyoto, Japan). The membranes were incubated overnight at 4°C with phosphor (Ser133) CREB (pCREB) antibody (1:2000; Millipore, Billerica, MA, USA). After incubation in secondary antibody (horseradish peroxidase-conjugated goat antibody to rabbit, 1:50,000; Zymed Labs, South San Francisco, CA, USA) for 1 h, the membrane was treated with chemiluminescent substrate (Millipore, Billerica, MA, USA) and visualized by exposure to Hyperfilm electrochemiluminescence film (GE Healthcare Bio-Sciences, Tokyo, Japan). pCREB blots were stripped with 10% acetic acid solution for 15 min at room temperature. The membranes were reprobed for CREB antibody (1:2000; Cell Signaling Technology, Tokyo, Japan). Finally, the blots were stripped and reprobed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA), and the sizes were compared with prestained molecular-weight standards. Individual CREB and pCREB values were divided by their respective sample GAPDH values to obtain CREB/GAPDH and pCREB/GAPDH ratio

values for each sample. The CREB/GAPDH and pCREB/GAPDH ratio values from the wildtype saline group were averaged, and the mean was used as a control value. Therefore, the CREB/GAPDH and pCREB/GAPDH ratio values of each sample were calculated as a percentage of this control.

Quantitative real-time reverse transcription polymerase chain reaction

The experiment involved four groups of ICER I-overexpressing mice and wildtype mice: Saline, Single METH, Repeated METH/Saline Challenge, and Repeated METH/METH Challenge. The saline and METH treatments, euthanasia, brain dissection, and storage of brain tissues were the same as described above for Western blot. Total RNA was isolated using Trizol reagent (Invitrogen Life Technology, Tokyo, Japan) and converted into cDNA using a SuperScript VILO cDNA Synthesis Kit (Invitrogen Life Technology, Tokyo, Japan). The real-time RT-PCR reaction was conducted using a LightCycler 480 Instrument (Roche Applied Science, Mannheim, Germany). The ICER, CART, Pdyn, and β-actin primers for real-time RT-PCR were the following: ICER (5′-GCTGAGGCTGATGAAAAACA-3′ and 5′-GCCACACGATTTTCAAGACA-3′), CART (5′-CGAGAAGAAGTACGGCCAAG-3′ and 5′-CACACAGCTTCCCGATCC-3′), Pdyn (5′-TTATGGCGGACTGCCTGT-3′ and 5′-CACTCCAGGGAGCAAATCAG3′), and β-actin (5′-CTAAGGCCAACCGTGAAAAG-3′ and 5′-ACCAGAGGCATACAGGACA-3′). Universal Probes #4, #108, #99, and #64 (Roche Applied Science, Mannheim, Germany) were used for ICER, CART, Pdyn, and β-actin, respectively. Amplification consisted of a preincubation step (95°C for 10 min), 45 cycles of denaturation for 10 s at 95°C, and annealing for 30 s at 60°C. Amplification curves were produced to calculate the crossing point at which the fluorescence of a sample rises above the initial lag phase. Absolute quantification analysis was performed using LightCycler 480 software (Roche Applied Science, Mannheim, Germany). Serial dilutions of an external standard with a predefined, known concentration were used to create a standard curve. The standard dilutions were amplified in separate wells but within the same instrument as the target samples. The crossing points of standards and unknown samples were then used to determine the concentration of the target mRNA. ICER, CART, and Pdyn mRNA levels were normalized according to β-actin mRNA levels. The ICER/β-actin, CART/β-actin, and Pdyn/β-actin values from the wildtype saline group were averaged, and the mean was used as a control value. Therefore, the relative expression levels of CART and Pdyn were calculated as a percentage of this control.

Statistical analysis

The data are expressed as mean ± SEM. The data for the Western blot, real-time RT-PCR, and locomotor sensitization experiments were analyzed by two-way, mixed-design ANOVA and repeated-measures ANOVA followed by the Dunnett *post hoc* test (for the Western blot analysis and real-time RT-PCR experiments) or Tukey-Kramer *post hoc* test (for the locomotor sensitization experiment). Values of $p<0.05$ were considered statistically significant.

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Author Contributions

Conceived and designed the experiments: NK KI. Performed the experiments: WH YT HY SK NK. Analyzed the data: WH YT KI. Contributed reagents/materials/analysis tools: SE TS NK KI. Wrote the paper: WH NK KI.

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The Selective Serotonin Reuptake Inhibitor Paroxetine, but not Fluvoxamine, Decreases Methamphetamine Conditioned Place Preference in Mice

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Abstract: Monoamine transporters are the main targets of methamphetamine (METH). Recently, we showed that fluoxetine, a selective serotonin reuptake inhibitor (SSRI), decreased METH conditioned place preference (CPP), suggesting that serotonin transporter (SERT) inhibition reduces the rewarding effects of METH. To further test this hypothesis, in the present study we investigated the effects of additional SSRIs, paroxetine and fluvoxamine, on METH CPP in C57BL/6J mice. In the CPP test, pretreatment with 20 mg/kg paroxetine abolished the CPP for METH, whereas pretreatment with 100 mg/kg fluvoxamine prior to administration of METH failed to inhibit METH CPP. These results suggest that paroxetine, a medication widely used to treat depression, may be a useful tool for treating METH dependence. Further, these data suggest that molecules other than the SERT [such as G protein-activated inwardly rectifying K^+ (GIRK) channels] whose activities are modulated by paroxetine and fluoxetine, but not by fluvoxamine, are involved in reducing METH CPP by paroxetine and fluoxetine.

Keywords: Conditioned place preference, Fluvoxamine, Methamphetamine, Mice, Paroxetine, Serotonin transporter.

INTRODUCTION

Methamphetamine (METH) is abused in worldwide [1]. In Japan, the number of people arrested for METH possession or use is approximately 100 times higher than those arrested for cocaine, opioids, or cannabis. Further, METH frequently induces psychotic states with symptoms similar to those seen in paranoid schizophrenia [2]. Such psychotic states are treated primarily in hospitals resulting in high medical costs. Thus, there is great need for the discovery of new medications for METH abuse [3] because the current treatments are mostly oriented toward the treatment of psychosis with no treatments available to prevent relapse to METH abuse.

The dopamine transporter (DAT) is the main target for METH and cocaine. However, mice lacking the DAT show conditioned place preference (CPP) to cocaine [4] and self-administer cocaine [5]. Interestingly, heterozygous and homozygous serotonin transporter (SERT) knockout mice that also have a homozygous knockout of the DAT do not exhibit cocaine CPP [6]. Cocaine administration leads to increases in extracellular dopamine concentration in the striatum of DAT knockout mice but not of DAT/SERT double knockout mice [7]. Taken together, these reports suggest that SERT inhibition may decrease METH and cocaine CPP.

Recently, we showed that fluoxetine, a selective serotonin reuptake inhibitor (SSRI), abolished METH CPP when METH was administered during both the development and expression phases of the CPP procedure, supporting the hypothesis that SERT inhibition decreased the rewarding effects of METH [8]. To further test this hypothesis, in the present study we investigated the effects of the SSRIs paroxetine (Paxil®) and fluvoxamine (Lubox® or Depromel®) on METH CPP.

MATERIALS AND METHODS

Mice

Male C57BL/6J mice (8-10 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan) and were housed for 1-2 weeks before the experiments began in an animal facility maintained at 22 ± 2°C and 55 ± 5% relative humidity under a 12/12 h light/dark cycle with lights on at 8:00 am. Food and water were available *ad libitum*. All behavioral testing was conducted during the light phase. The experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committee of the Tokyo Institute of Psychiatry, and all animals were cared for and treated humanely in accordance with our institutional animal experimentation guidelines.

Conditioned Place Preference (CPP) Test

The CPP test was performed according to the method of Hoffman and Beninger [9] with some modifications. We used a two-compartment Plexiglas chamber (Neuroscience Inc., Osaka, Japan). One compartment (17.5 × 15 × 17.5 cm: width × length × height) was black with a smooth floor, and the other compartment was of the same dimensions, but with a white textured floor. This two-compartment chamber was located in a sound- and light-attenuated box under conditions

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of dim illumination (approximately 40 lux) to reduce bias toward either compartment [10]. Mice were assigned randomly to the treatment groups (see below).

On Day 1, the mice (n = 14-26 per group) were allowed to freely explore the two compartments for 15 min. On Day 2, the mice again were allowed to explore the two compartments freely for 15 min, and the time spent in each compartment and the number of transitions between compartments were measured. Conditioning sessions then were conducted once daily for 4 consecutive days (Days 5-8). For the Day 5 conditioning session, mice were i.p. injected with saline or SSRI (20 mg/kg paroxetine or 100 mg/kg fluvoxamine) 60 min before injection with METH (2 mg/kg, i.p.). Immediately after METH administration, mice were confined to the black or white compartment for 50 min. On Day 6, the mice were pretreated with the same solution (saline or SSRI, i.p.) 60 min before a saline injection. Immediately after the saline injection, mice were confined to the opposite compartment for 50 min. On Days 7 and 8, the same conditioning as on Days 5 and 6 was repeated. On Day 9, the mice were pretreated with saline or SSRI (20 mg/kg paroxetine or 100 mg/kg fluvoxamine, i.p.), and 60 min later were allowed to freely explore the two compartments for 15 min without METH injection. The time spent in each compartment and the number of transitions between compartments were measured. In summary, there were a total of eight groups in this experiment corresponding to the four pretreatments (paroxetine, fluvoxamine, saline; there were two saline groups that were run concurrently with the paroxetine and fluvoxamine groups) and the two phases of the experiment during which they were pretreated with the drug (conditioning days 5-8 or test day 9). The CPP score was defined as the time spent in the drug-paired compartment during the CPP test phase (Day 9) minus the time spent in the same compartment during the preconditioning exploratory phase (Day 2). The transition score was defined as the number of transitions during the CPP test phase (Day 9) minus the number of transitions during the preconditioning exploratory phase (Day 2).

Drugs

Methamphetamine hydrochloride was purchased from Dainippon Pharmaceutical (Osaka, Japan). Paroxetine maleate and fluvoxamine maleate were purchased from Sigma (St. Louis, MO, USA) and TOCRIS (Hung Road, Bristol, UK), respectively. All drugs were dissolved in saline. Drugs and vehicle were administered i.p. in a volume of 0.1 ml/10 g body weight. All drug doses are reported as salt.

Statistical Analyses

The CPP and transition scores of mice pretreated with saline or SSRI during the conditioning and CPP test phases were subjected to a two-way analysis of variance (ANOVA). The ANOVA had two between-subjects factors, each with two levels (saline/SSRI pretreatment in the conditioning phase and saline/SSRI pretreatment in the CPP test phase). Two separate ANOVAs were conducted on the paroxetine and fluvoxamine data. Similar ANOVAs were conducted on the transition scores. The CPP scores from the paroxetine experiment were subjected to a one-way ANOVA followed by *post hoc* comparisons with the Scheffe test. In this ANOVA, there were four levels corresponding to the four treatment conditions (saline in both the conditioning and the CPP test phases, pretreatment with paroxetine only in the conditioning phase, pretreatment with paroxetine only in the CPP test phase, pretreatment with paroxetine in both the conditioning and the CPP test phases). For the CPP data, the durations of time that the mice spent in the METH-paired compartment before and after conditioning were compared using paired t-tests for each group. For the transition data, the number of transitions between the METH-paired compartment and the saline-paired compartment before and after conditioning were compared using paired t-tests for each group. The level of significance was set at 0.05.

RESULTS

Effects of Paroxetine on METH CPP

The two-way ANOVA revealed that mice treated with paroxetine during the test phase exhibited decreased CPP scores compared to mice treated with saline during the test phase ($F_{1,72}$ = 7.888, $P < 0.01$), whereas mice treated with paroxetine during the conditioning phase did not differ significantly from mice treated with saline during the test phase in the CPP score [$F_{1,72}$ = 1.704, not significant (n.s.); Fig. (**1A**)]. There was no statistically significant interaction between the factor saline/paroxetine during the conditioning phase and the factor saline/paroxetine during the CPP test phase ($F_{1,72}$ = 0.1690, n.s.), indicating that the important factor was treatment with paroxetine during the expression phase of the experiment. In addition, a one-way ANOVA on the CPP scores was conducted on data for all four groups. The ANOVA showed a significant difference in the CPP scores among these four groups ($F_{3,72}$ = 3.940, $P < 0.05$). The Scheffe *post hoc* test showed that the CPP score of the paroxetine/paroxetine group was significantly lower than that of the saline/saline group ($P < 0.05$). Paired t-tests were conducted to compare the duration of time before and after conditioning for each of the four groups (Fig. (**1B**)). Whereas the saline/saline and paroxetine/saline groups spent significantly more time in the METH-paired compartment after conditioning than before conditioning (saline/saline: n = 23, df = 22, t = -6.050, $P < 0.001$; paroxetine/saline: n = 15, df = 14, t = -2.884, $P < 0.05$), the saline/paroxetine and paroxetine/paroxetine groups did not show METH CPP (saline/paroxetine: n = 15, df = 14, t = -2.033, n.s.; paroxetine/paroxetine: n = 23, df = 22, t = -0.908, n.s.). Paroxetine pretreatment had no significant effects on the transition scores compared to the saline/saline treatment group (data not shown).

Effects of Fluvoxamine on the METH CPP

The two-way ANOVA revealed that both the factor saline/fluvoxamine pretreatment during the conditioning phase and the factor saline/fluvoxamine pretreatment during the CPP test phase had no effects on CPP scores (conditioning phase: $F_{1,68}$ = 0.045, n.s.; CPP test phase: $F_{1,68}$ = 3.016, n.s.; Fig. (**2A**)). There was no statistically significant interaction between the two factors ($F_{1,68}$ = 0.066, n.s.). Paired t-tests were conducted to compare the duration of time before and after conditioning for each of the four groups. All four groups spent significantly more time in the METH-paired

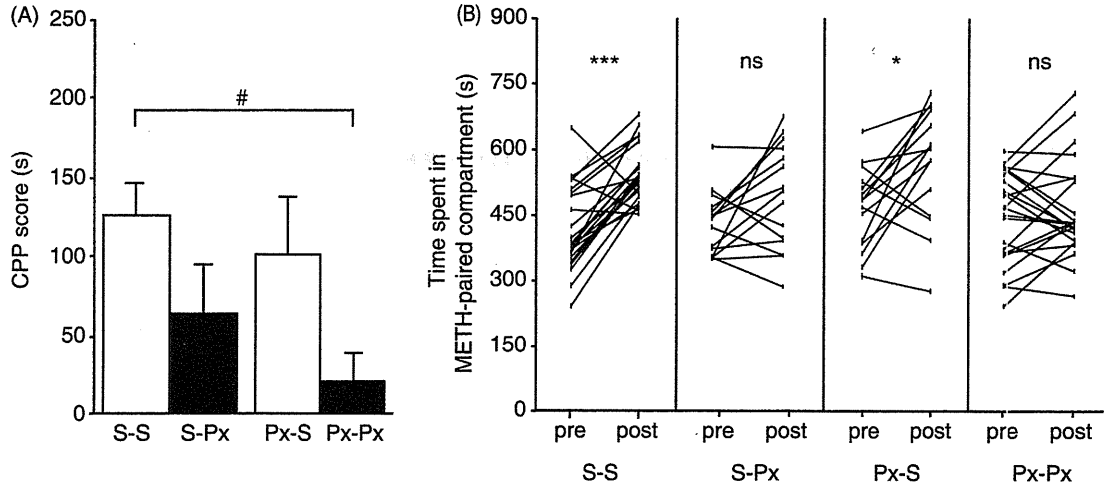

Fig. (1). Effects of paroxetine on CPP for METH in mice. (**A**) Reduction of METH CPP by paroxetine (Px) pretreatment. Mice were pretreated with saline (S) in both the conditioning and CPP test phases (S-S), paroxetine only in the CPP test phase (S-Px), paroxetine only in the conditioning phase (Px-S), and paroxetine in both the conditioning and the CPP test phases (Px-Px). The CPP score was defined as the time spent in the drug-paired compartment during the CPP test phase (Day 9) minus the time spent in the same compartment during the preconditioning phase (Day 2). The CPP score of the Px-Px group was significantly lower than that of the S-S group ($^{\#}P < 0.05$). (**B**) Comparison of time spent in the conditioned compartment before and after conditioning in the four groups. There was a significant CPP in the S-S and Px-S groups, but not in the S-Px and Px-Px groups (when paroxetine was administered in the CPP test phase). $^{***}P < 0.001$, $^{*}P < 0.05$, ns: not significant ($P > 0.05$).

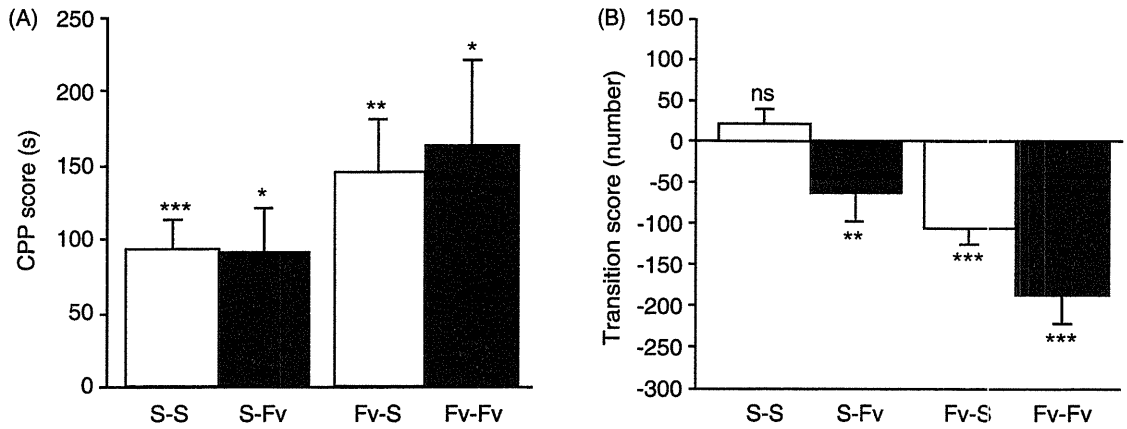

Fig. (2). Effects of fluvoxamine on CPP for METH and on transitions between compartments. (**A**) Lack of a significant effect of fluvoxamine (Fv) on METH CPP. Mice were pretreated with saline in both the conditioning and the CPP test phases (S-S), fluvoxamine only in the CPP test phase (S-Fv), fluvoxamine only in the conditioning phase (Fv-S), and fluvoxamine in both the conditioning and the CPP test phases (Fv-Fv). There was a significant CPP in all groups. Fluvoxamine pretreatment in the conditioning phase and/or the CPP test phase failed to inhibit METH CPP (pre- and post-conditioning preference test results were analyzed with paired *t*-tests, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$). (**B**) Decreases in transitions between the compartments by fluvoxamine pretreatment. There were significant decreases in transitions in the S-Fv, Fv-S, and Fv-Fv groups, but not in the S-S group [number of transitions in the pre- and post-conditioning phases was analyzed with paired *t*-tests, $^{***}P < 0.001$, $^{**}P < 0.01$, ns: not significant ($P > 0.05$)]. The transition score was defined as the number of transitions during the CPP test phase (Day 9) minus the number of transitions during the preconditioning phase (Day 2).

compartment after conditioning than before conditioning (saline/saline: $n = 26$, $df = 25$, $t = -4.541$, $P < 0.001$; saline/fluvoxamine: $n = 14$, $df = 13$, $t = -2.983$, $P < 0.05$; fluvoxamine/saline: $n = 18$, $df = 17$, $t = -3.949$, $P < 0.01$; fluvoxamine/fluvoxamine: $n = 14$, $df = 13$, $t = -2.757$, $P < 0.05$).

The two-way ANOVA revealed that both fluvoxamine pretreatment during the conditioning phase and during the CPP test phase significantly decreased transition scores (conditioning phase: $F_{1,68} = 24.321$, $P < 0.001$; CPP test phase: $F_{1,68} = 10.292$, $P < 0.01$; Fig. (**2B**)). There was no statistically significant interaction between the two factors ($F_{1,68} = 0.007$, n.s.). Paired *t*-tests were conducted to compare the number of transitions before and after conditioning for each of the four groups. The S-S group showed no significant differences in the number of transitions before and after conditioning ($n = 26$, $df = 25$, $t = -1.213$, n.s.). However, mice pretreated with fluvoxamine (saline/fluvoxamine, fluvoxamine/saline, fluvoxamine/fluvoxamine) showed significant decreases in the number of transitions after conditioning (saline/fluvoxamine: $n = 14$, $df = 13$, $t = 3.829$, $P < 0.01$; fluvoxamine/saline: $n = 18$, $df = 17$, $t = 5.520$, $P < 0.001$; fluvoxamine/fluvoxamine: $n = 14$, $df = 13$, $t = 6.025$, $P < 0.001$).

DISCUSSION

In the present study, we showed that paroxetine, a widely used medication for treating depression, inhibited METH CPP in mice, similar to the results we reported previously with fluoxetine [8]. No significant effects of paroxetine on transition scores suggest that the effects of paroxetine on METH CPP are not due to changes in locomotor activity but due to reduction of METH reward and conditioned reward by paroxetine. Based on these findings, it appears worthwhile to investigate the clinical effects of paroxetine on METH abuse. By contrast, the other SSRI tested here, fluvoxamine, did not affect METH CPP. These data demonstrate that there are differences in the effects of SSRIs on METH CPP, suggesting the possibility that molecules other than the SERT are involved in the inhibition of METH CPP by paroxetine and fluoxetine reported here and in our previous study [8].

In addition to SERT inhibition, paroxetine inhibits the function of muscarinic cholinergic receptors [11], nicotinic acetylcholine receptors [12], volume-related anion channels [13], membrane steroid transporters [14], and nitric oxide synthase [15]. Recently, Kobayashi and colleagues [16] reported that paroxetine also inhibits the function of G protein-activated inwardly rectifying K^+ (GIRK) channels. It is intriguing that paroxetine and fluoxetine, but not fluvoxamine, inhibit GIRK channels [16-18]. Various G protein-coupled receptors (such as M2 muscarinic, α2 adrenergic, D_2 dopaminergic, $5\text{-}HT_{1A}$, opioid, nociceptin/orphanin FQ, and A_1 adenosine) activate GIRK channels [19-22] through the direct action of G protein subunits [23]. In addition, GIRK channels are activated by ethanol independently of G protein-coupled signaling pathways [24, 25]. Activation of GIRK channels leads to membrane hyperpolarization [22]. These channels play an important role in the inhibitory regulation of neuronal excitability. Thus, modulators of GIRK channel activity may affect many brain functions. Kobayashi and colleagues [26] also have reported that ifenprodil, a cerebral vasodilator which inhibits morphine CPP [27], also inhibits the function of GIRK channels. Morgan and colleagues [28] demonstrated that GIRK channel knockout mice exhibited dramatically reduced intravenous self-administration of cocaine. In the present study, we found that paroxetine and fluoxetine, but not fluvoxamine, inhibited METH CPP. These findings, together with the previous findings, suggest that the inhibition of GIRK channels by paroxetine or fluoxetine may be involved in the inhibition of METH CPP by these drugs.

Fluvoxamine administration (60 mg/kg) leads to a significant decrease in spontaneous locomotor activity [29]. Consistent with this observation, significant decreases in transition scores were observed in all of the 100 mg/kg fluvoxamine-treated groups compared to the saline/saline-treated group in the present study. The number of transitions of the fluvoxamine/fluvoxamine treated group during the CPP test phase (101.4 ± 85.3, mean ± SEM) was the smallest among the four groups in this experiment, but more than 100 transitions indicated adequate locomotion to reveal potential differences in CPP. The lack of effect of fluvoxamine on CPP for methamphetamine is likely to reflect a lack of effect of fluvoxamine on the rewarding effects of METH rather than being a nonspecific effect of fluvoxamine.

In conclusion, we found that paroxetine, but not fluvoxamine, inhibited METH CPP in mice. Although further preclinical studies are needed to elucidate the mechanisms underlying these inhibitory effects of paroxetine on processes relating to METH dependence, it appears worthwhile to investigate the clinical effects of paroxetine on METH abuse. The present results suggest that molecules other than the SERT (such as GIRK channels) are involved in the inhibition of METH CPP by paroxetine and fluoxetine.

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ABBREVIATIONS

ANOVA	=	Analysis of variance
CPP	=	Conditioned place preference
DAT	=	Dopamine transporter
GIRK	=	G protein-activated inwardly rectifying K^+
METH	=	Methamphetamine
n.s.	=	Not significant
SERT	=	Serotonin transporter
SSRI	=	Selective serotonin reuptake inhibitor

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