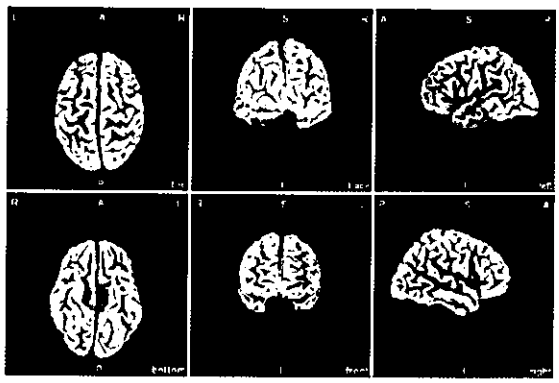
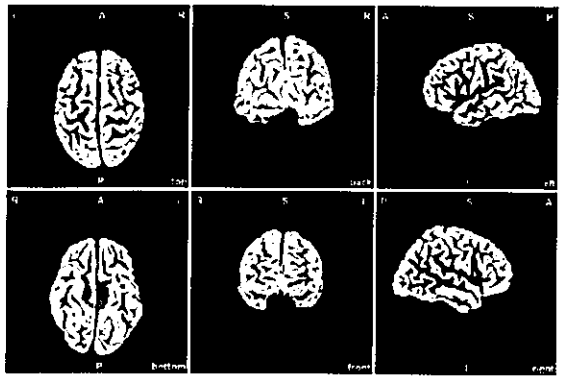
(a) 健常者で活躍した部位



(b) 総合失調症患者で活躍した部位



(c) 総合失調症患者で有意に活躍が低下した部位

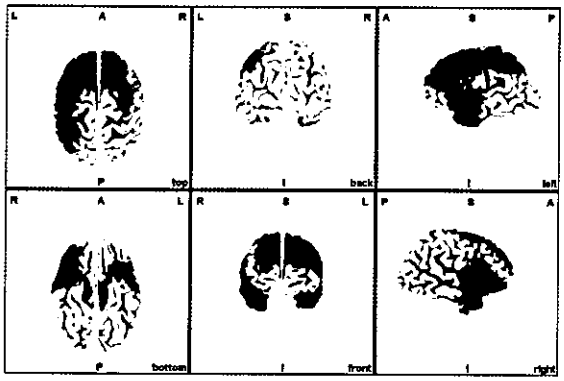


図 1. 単語課題遂行中の活動部位と SnPM 解析結果

刺激提示後 270～500 ms にかけて電位活動が認められた部位を LORETA によって推定し，SnPM により両群の活動部位について統計検定をおこなった．

健常者も統合失調症患者も左優位に両側側頭葉と左前頭前野に電位活動が認められ (a)(b)，統合失調症患者は健常者と比較し，両側側頭葉と両側前頭葉，および左頭頂葉で電位活動が有意に低下していた (c)．

(筆者作成)

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Psychostimulant Alters Expression of DNA Methyltransferase mRNA in the Rat Brain

YOHTARO NUMACHI,[a,c] SUMIKO YOSHIDA,[b] MOTOYASU YAMASHITA,[a] KO FUJIYAMA,[c] MAKI NAKA,[a] HIROO MATSUOKA,[c] MITSUMOTO SATO,[d] AND ICHIRO SORA[a]

[a]*Department of Psychobiology and Department of Psychiatry, Tohoku University Graduate School of Medicine, Sendai, 980-8574, Japan*

[b]*Department of Health and Welfare, Sendai College, Miyagi, 989-1693, Japan*

[c]*Department of Psychiatry, Tohoku University Graduate School of Medicine, Sendai, 980-8574, Japan*

[d]*Department of Psychiatry, Tohoku Fukushi University Graduate School, Sendai, 981-8522, Japan*

ABSTRACT: **Methamphetamine (MAP), the most frequently abused substance in Japan, causes severe drug dependence and psychosis, similar to schizophrenia. It is suggested that long-term alterations in gene expression is related to MAP-induced brain dysfunction, including dependence and psychosis. DNA (cytosine-5) methyltransferase (Dnmt), a methylating enzyme of cytosine residues on CpG-dinucleotides, plays an important role in X chromosome inactivation, genomic imprinting, and gene expression. Reelin is an extracellular matrix protein secreted by GABAergic interneurons. Heterozygous reeler mice that exhibit a 50% downregulation of reelin expression replicate the dendritic spine and GABAergic defects described in schizophrenia. DNA methylation plays an important role in the epigenetic modification of reelin expression. We previously found that MAP could alter expression of Dnmt1 mRNA in the rat brain. In this study, we examined the brain mRNA for Dnmt2 and reelin in MAP-treated Wistar rats. Acute MAP (4 mg/kg) treatment significantly decreased Dnmt2 mRNA by 27% to 39% in hippocampus dentate gyrus, CA1, and CA3 24 h after treatment, and significantly decreased reelin mRNA by 28% in frontal cortex 3 h after treatment. These results suggest that (1) MAP can alter DNA methylation as well as expression of genes in these brain regions, and (2) decrease in reelin mRNA in the frontal cortex is similar to heterozygous reeler mice, which might be related to schizophrenia-like psychotic symptoms of MAP psychosis.**

KEYWORDS: **psychostimulant; DNA methyltransferase; reelin; psychosis; gene expression**

Address for correspondence: Yohtaro Numachi, M.D., Ph.D., Division of Psychobiology, Tohoku University Graduate School of Medicine, Sendai, 980-8574, Japan. Voice: +81-22-717-7262; fax: +81-22-717-7266.
ynumachi-thk@umin.ac.jp

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INTRODUCTION

Methamphetamine (MAP) is a potent psychostimulant that has been widely abused in Japan. MAP is an indirect dopamine agonist and causes paranoid psychotic state (stimulant-induced psychosis) almost identical to schizophrenia. Clinical features of stimulant-induced psychosis are: (1) the psychotic state may continue after MAP withdrawal; (2) vulnerability to relapse by reuse of MAP or psychological stress persists for a long time; and (3) neuroleptics prevent relapse by MAP reuse. These features suggest that MAP can cause long-term changes in brain function as well as gene expression,[7] which would underlie the relapse of psychotic state in stimulant-induced psychosis as well as schizophrenia.[5]

Recently, it was reported that reelin and glutamate decarboxylase$_{67}$ (GAD$_{67}$: GABA synthesizing enzyme) mRNA and protein levels are reduced by 50% in postmortem brain from schizophrenic patients.[2] Reelin is an extracellular matrix protein that regulates positioning of cortical pyramidal neurons, interneuron, and Purkinje cell during development.[2] In adult brain, reelin is secreted by specific GABAergic interneurons from the telencephalon and hippocampus. The defective gene in the reeler mouse, characterized by locomotor defects and ataxia caused by defective neuronal migration in the developing brain, was identified as the reelin gene. The reelin haploinsufficient heterozygous reeler mouse does not express the phenotype of the reelin null mutant. But the heterozygous reeler mouse shows similar neuroanatomical and neurochemical abnormalities with schizophrenic patients, including downregulation of GAD$_{67}$ and the neuropil density with reduced pyramidal dendritic spines in the telencephalon. Collectively, reelin and GABAergic function downregulation might be related to neurodevelopmental abnormalities for vulnerability to schizophrenia.[2]

It is suggested that epigenetic regulation through DNA methylation is responsible for reelin downregulation in the schizophrenic brain. DNA (cytosine-5) methyltransferase (Dnmt), a methylating enzyme of cytosine residues on CpG-dinucleotides, plays an important role in X chromosome inactivation, genomic imprinting, and gene expression.[3] Experiments using cultured human cell showed that methylation of the CpG island in the promoter of human reelin gene acts as an epigenetic switch to silence reelin gene expression.[2] There are four mammarian Dnmts: Dnmt1, 2, 3a, and 3b. We previously found that MAP can alter mRNA expression of Dnmt1, which maintains methylation pattern of DNA, in the rat brain. In this study, we examined the brain mRNA for Dnmt2 and reelin in MAP-treated Wistar rats.

MATERIALS AND METHODS

Animals and Tissue Preparation

All experiments were conducted in accordance with the guidelines of the animal ethics committee at Tohoku University Graduate School of Medicine (Sendai, Japan). Male Wistar rats (Funabashi Farm, Japan, 7 wks old, 150–200 g) were sacrificed 0, 1, 3, 9, and 24 h after injection of 4 mg/kg MAP hydrochloride (Philopon, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) in saline solution. Brains were quickly frozen, from which coronal brain sections of 14-mm thickness were cut.

Preparation of RNA Probe and Northern Hybridization

Rat Dnmt2 and reelin cDNA fragment (Genbank accession NM_010067, n.t. 102–547, 446 bp, Genbank accession NM_080394, n.t. 8726–9147, 422 bp, respectively) was subcloned into pGEM-T Easy Vector. Northern hybridization was performed using a DIG RNA labeling kit and the signal was detected by the luminescent system.

In Situ *Hybridization and Quantification of Radioactive Signals*

The brain sections were hybridized with ^{35}S-labeled anti-sense cRNA probe overnight at 55°C. Sections were exposed to a Kodak XAR film for 6 days after the posthybridization process. A sense cRNA probe was used as a negative control. Optical density measurements were quantified from the X-ray film using MCID (Imaging Research Inc.) software and an NIH image. The signals were corrected by background.

Statistical Analysis

Statistical analyses were performed using ANOVA followed by the Fischer or Scheffe *post hoc* test. The criterion of statistical significance was less than .05.

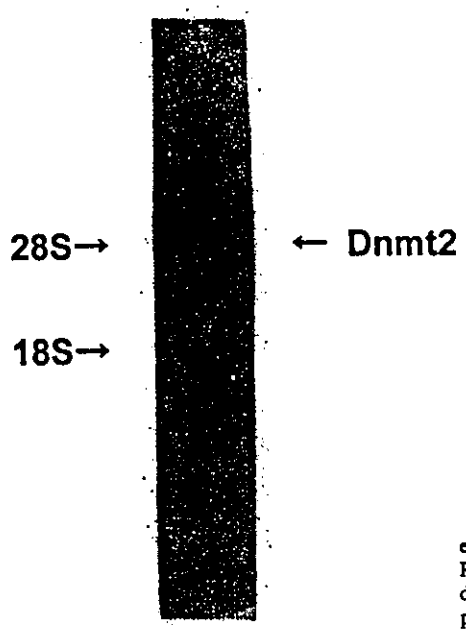

FIGURE 1. Representative Northern image of Dnmt2. Using antisense RNA probe, single signal of 4.4 kb was detected, which is consistent with a previous report.[4]

RESULTS

Northern Blot Hybridization

As shown in FIGURE 1, a single band of 4.4 kb for Dnmt2 was detected with antisense riboprobe. Our result was consistent with a previous report for the cloning and functional analysis of murine Dnmt2, which detected three transcripts of 1.6, 2.6, and 4.4 kb.[4]

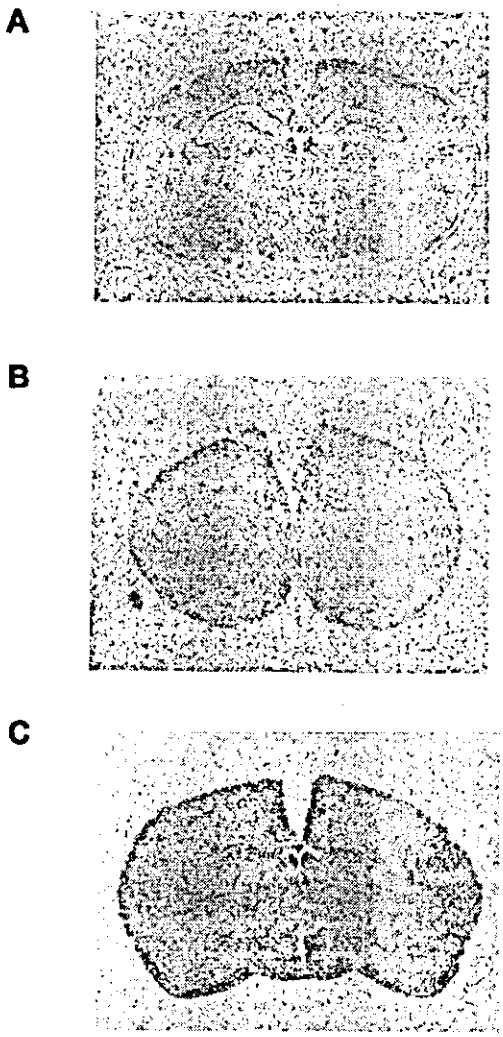

FIGURE 2. Representative *in situ* image of Dnmt2 and reelin. (A) Dnmt2 mRNA in rostral slice, including hippocampus; (B) reelin mRNA in caudal slice, including striatum; and (C) reelin mRNA in rostral slice, including hippocampus.

In Situ *Hybridization*

Representative *in situ* images are shown in FIGURE 2. Strong hybridization signals of Dnmt2 and reelin were observed in brain regions: piriform cortex, hippocampus, and habenular nucleus. FIGURE 3 shows changes in Dnmt2 mRNA after MAP treatment, which significantly decreased by 27% to 39% in hippocampus dentate gyrus (DG), CA1, and CA3 24 h after MAP. No change was observed in habenular nucleus. Changes in reelin mRNA are shown in FIGURE 4, which significantly decreased by 28% in frontal cortex 3 h after MAP. Reelin mRNA was not changed by MAP in piriform cortex, striatum, hippocampus, and habenular nucleus.

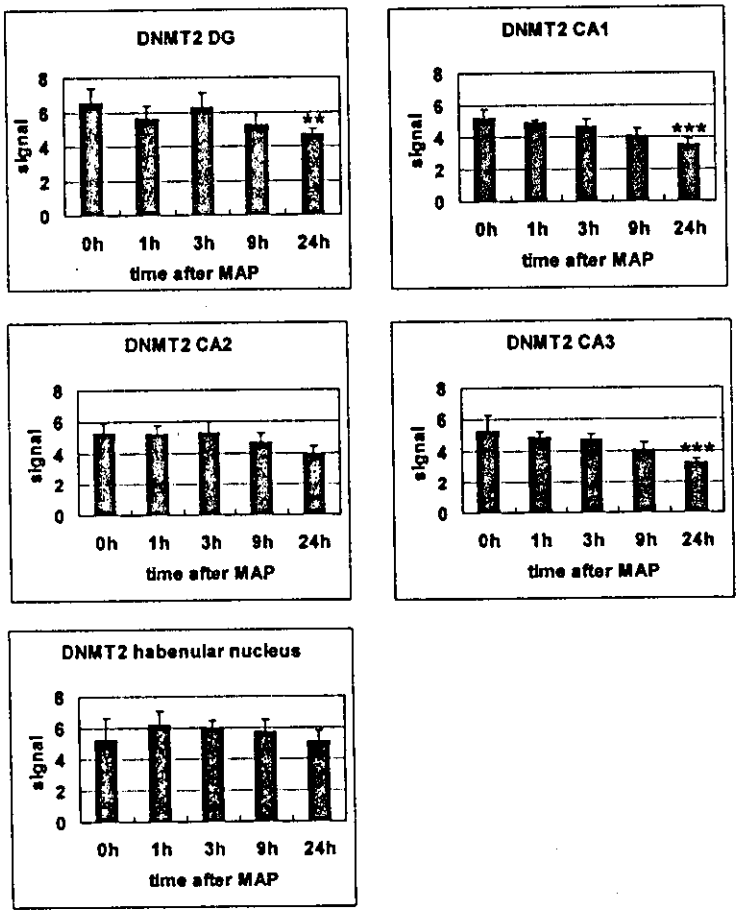

FIGURE 3. Dnmt2 mRNA in the rat brain: time course after single MAP treatment. Values are expressed as mean ± S.D. $^{**}P < .01$; $^{***}P < .0001$; analyzed by ANOVA.

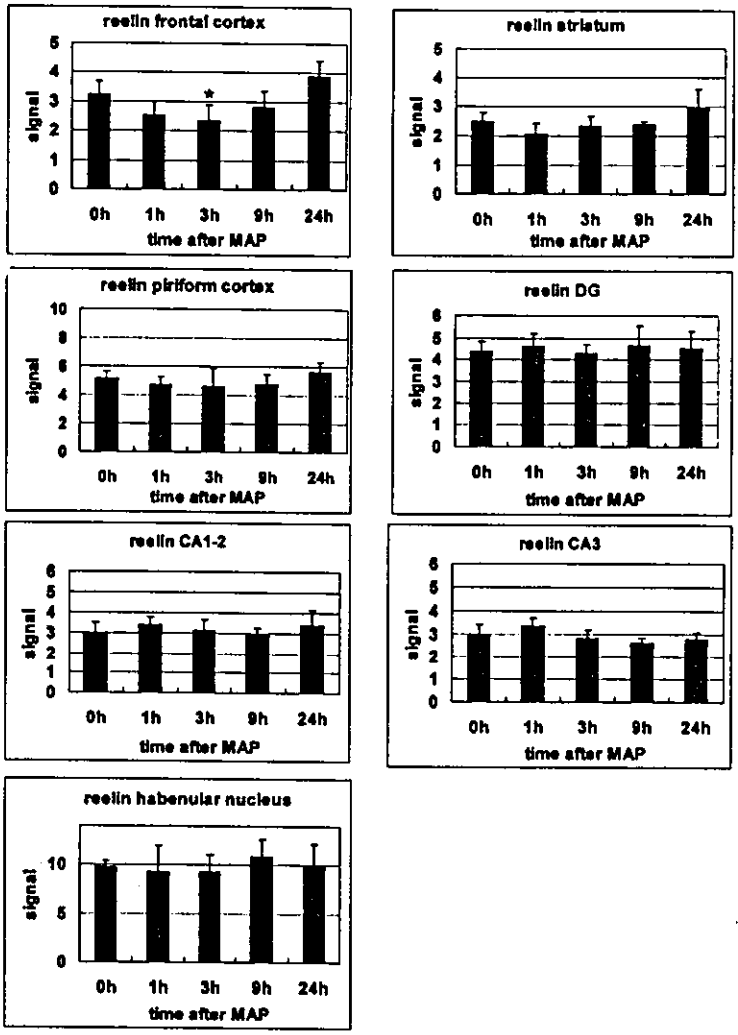

FIGURE 4. Reelin mRNA in the rat brain: time course after single MAP treatment. Values are expressed as mean ± S.D. $*P < .03$; analyzed by ANOVA.

DISCUSSION

Thus far, four mammarian Dnmts (Dnmt1, 2, 3a, and 3b) have been identified. While Dnmt1 is a maintenance methyltransferase to preserve the preexisting methylation pattern of DNA, Dnmt3a and 3b are *de novo* methyltransferase. Dnmt2, despite having all the conserved DNA methyltransferase motifs, may be involved in

cellular processes other than DNA methylation, such as DNA repair, DNA recombination, and carcinogenesis.[4] We observed decreased Dnmt2 mRNA in the hippocampus 24 h after MAP treatment, but not in the habenular nucleus. In our previous experiment, we also observed decreased Dnmt1 mRNA in the hippocampus of Fischer 344 rats, 3 h after acute MAP treatment. Hippocampus plays an important role in adaptive behavior, including drug dependence and psychosis.[1] Decreased hippocampal Dnmt2 mRNA by MAP might reflect long-term alterations in gene expression, which is responsible for the persistence of MAP-induced mental disorders.

Epigenetic differences (i.e., differences in DNA methylation status) in genomic DNA might explain the discordance for schizophrenia in monozygotic twins.[6] So far, we only have data on MAP-induced changes in Dnmt1 and Dnmt2 for mRNA. Further studies will be necessary to discover whether MAP can affect the protein expression and function of Dnmts, and whether MAP can alter DNA methylation of genes related to the pathogenesis of schizophrenia, including reelin.

We observed changes in reelin mRNA only in the frontal cortex, which was decreased by 30% 3 h after MAP treatment, but returned to baseline by 24 h after MAP. Because the MAP-induced decrease in reelin mRNA was temporary, the functional significance of this alteration remains unclear. However, our results are partially consistent with a previous report, which found a significant decrease in reelin mRNA in the frontal cortex, temporal cortex, cerebellum, caudate nucleus, and hippocampus in schizophrenic patients.[2] Our results suggest that MAP can cause a decrease in reelin mRNA exclusively in the frontal cortex, which is similar to changes observed in schizophrenic patients and a possible animal model for schizophrenia, the heterozygous reeler mouse. Because the expression of reelin is regulated by DNA methylation, it should be elucidated if MAP can alter the methylation status of the CpG island in the promoter of the mouse reelin gene, as well as its mRNA and protein expression in the rat frontal cortex. If this is the case, a MAP-induced decrease in reelin mRNA might be related to the pathogenesis of schizophrenia-like symptoms in MAP psychosis.

ACKNOWLEDGMENT

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Regional Differences in Extracellular Dopamine and Serotonin Assessed by *In Vivo* Microdialysis in Mice Lacking Dopamine and/or Serotonin Transporters

Hao-wei Shen[1], Yoko Hagino[1], Hideaki Kobayashi[1], Keiko Shinohara-Tanaka[1], Kazutaka Ikeda[1], Hideko Yamamoto[1], Toshifumi Yamamoto[2], Klaus-Peter Lesch[3], Dennis L Murphy[4], F Scott Hall[5], George R Uhl[5] and Ichiro Sora*[,1,5,6]

[1]Department of Molecular Psychiatry, Tokyo Institute of Psychiatry, Japan; [2]Laboratory of Molecular Recognition, Graduate School of Integrated Science, Yokohama City University, Japan; [3]Department of Psychiatry and Psychotherapy, University of Wurzburg, Germany; [4]Laboratory of Clinical Science, Intramural Research Program, National Institute of Mental Health, USA; [5]Molecular Neurobiology Branch, Intramural Research Program, National Institute on Drug Abuse, USA; [6]Division of Psychobiology, Department of Neuroscience, Tohoku University Graduate School of Medicine, Japan

Cocaine conditioned place preference (CPP) is intact in dopamine transporter (DAT) knockout (KO) mice and enhanced in serotonin transporter (SERT) KO mice. However, cocaine CPP is eliminated in double-KO mice with no DAT and either no or one SERT gene copy. To help determine mechanisms underlying these effects, we now report examination of baselines and drug-induced changes of extracellular dopamine (DA_{ex}) and serotonin ($5\text{-}HT_{ex}$) levels in microdialysates from nucleus accumbens (NAc), caudate putamen (CPu), and prefrontal cortex (PFc) of wild-type, homozygous DAT- or SERT-KO and heterozygous or homozygous DAT/SERT double-KO mice, which are differentially rewarded by cocaine. Cocaine fails to increase DA_{ex} in NAc of DAT-KO mice. By contrast, systemic cocaine enhances DA_{ex} in both CPu and PFc of DAT-KO mice though local cocaine fails to affect DA_{ex} in CPu. Adding SERT to DAT deletion attenuates the cocaine-induced DA_{ex} increases found in CPu, but not those found in PFc. The selective SERT blocker fluoxetine increases DA_{ex} in CPu of DAT-KO mice, while cocaine and the selective DAT blocker GBR12909 increase $5\text{-}HT_{ex}$ in CPu of SERT-KO mice. These data provide evidence that (a) cocaine increases DA_{ex} in PFc independently of DAT and that (b), in the absence of SERT, CPu levels of $5\text{-}HT_{ex}$ can be increased by blocking DAT. Cocaine-induced alterations in CPu DA levels in DAT-, SERT-, and DAT/SERT double-KO mice appear to provide better correlations with cocaine CPP than cocaine-induced DA level alterations in NAc or PFc.
Neuropsychopharmacology advance online publication, 30 June 2004; doi:10.1038/sj.npp.1300476

Keywords: dopamine; serotonin; monoamine transporter; cocaine reward; knockout mice; *in vivo* microdialysis

INTRODUCTION

Cocaine increases extracellular levels of dopamine (DA), serotonin (5-HT) and norepinephrine (NE) by blocking the neural plasma membrane transporters for those neurotransmitters. Increased extracellular DA (DA_{ex}) levels in mesocorticolimbic DA systems have been postulated to mediate cocaine reward (Kuhar *et al*, 1991; Koob and Nestler, 1997; Bardo, 1998; Kelley and Berridge, 2002). However, homozygous dopamine transporter (DAT) knockout (KO) mice (DAT−/− mice) express intact cocaine reward in conditioned place preference (CPP) (Sora *et al*, 1998) and drug self-administration paradigms (Rocha *et al*, 1998). Cocaine reward is eliminated in double-KO mice with no DAT gene copies and either no or one copy of the SERT gene (Sora *et al*, 2001), but not in double-KO mice with neither DAT nor NET gene copies (Hall *et al*, 2002). Further, serotonin transporter (SERT) blockade with fluoxetine or norepinephrine transporter (NET) blockade with nisoxetine can yield rewarding effects in DAT-KO mice, which are never seen in wild-type animals (Hall *et al*, 2002).

We and others have postulated that the retention of cocaine reward in DAT-KO mice may be due to (a) roles for non-DA systems in normal cocaine reward and (b) adaptations to the lifelong loss of DAT found in DAT-KO mice (Kirkpatrick, 2001; Sora *et al*, 2001; Uhl *et al*, 2002). Some of these adaptive changes could come from involvement of redundant monoaminergic systems in cocaine

*Correspondence: Dr I Sora, Division of Psychobiology, Department of Neuroscience, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Sendai 980-8574, Japan, Tel: +81 22 717 7808, Fax: +81 22 717 7809, E-mail: isora@mail.cc.tohoku.ac.jp
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reward. Since each transporter displays significant affinities for each monoamine (Faraj *et al*, 1994; Giros *et al*, 1994; Gu *et al*, 1994; Eshleman *et al*, 1999), the absence of its cognate transporter might allow a monoamine to diffuse further from its site of release and be accumulated by another transporter.

Cocaine and selective norepinephrine transporter (NET) blockers (eg reboxetine) are each reported to increase DA_{ex} in NAc of DAT-KO mice, suggesting that NET could act as an alternative uptake site for DA in such animals and that NET blockade might be a mechanism for both the cocaine- and nisoxetine-induced rewards found in DAT-KO mice (Carboni *et al*, 2001; Hall *et al*, 2002). However, *in vitro* data fail to identify cocaine influences on CPu or NAc DA uptake in DAT-KO mice (Budygin *et al*, 2002; Moron *et al*, 2002). The simple idea that NET mediates cocaine reward in the absence of DAT is also incompatible with observations that cocaine reward is ablated in DAT/SERT double-KO mice that express normal levels of NET (Sora *et al*, 2001).

Roles for 5-HT systems in cocaine reward (or aversion) are also less than clear from current data (Cunningham and Callahan, 1991; Kleven *et al*, 1995; Rocha *et al*, 1997; Kleven and Koek, 1998; Lee and Kornetsky, 1998; Parsons *et al*, 1998; Shippenberg *et al*, 2000; Baker *et al*, 2001; Sasaki-Adams and Kelley, 2001). Homozygous SERT-KO mice display enhanced cocaine CPP that is increased even more in combined SERT/NET double-KO mice (Sora *et al*, 1998; Hall *et al*, 2002). SERT-KO mice, in themselves and in combination with DAT-KOs, thus provide interesting models in which to investigate 5-HT, DA, and 5-HT/DA interactions important for psychostimulant reward.

In this present study, we have therefore examined baselines and drug-induced changes of DA_{ex} and $5\text{-}HT_{ex}$ in several brain regions implicated in psychostimulant effects, the NAc, CPu and prefrontal cortex (PFc) in DAT-KO, SERT-KO, and both heterozygous and homozygous DAT/SERT double-KO mice. We have studied the effects of both the nonselective blocker cocaine and the selective SERT and DAT blockers fluoxetine and GBR12909. These investigations provide insights into adaptive processes found in these mice and into 5-HT, DA, and 5-HT/DA interactions of the possible importance for cocaine reward.

MATERIALS AND METHODS

Animals

Mutant mice lacking DAT, SERT, and littermate wild-type mice were obtained from heterozygote crosses on 129/C57 mixed genetic backgrounds. DAT/SERT double-KO mice were obtained by intercrossing single KO lines as described previously (Sora *et al*, 2001). DNA extracted from tail biopsies was genotyped using PCR. Mice were group-housed (two to four per cage) with food and water *ad libitum* in a room maintained at 22 ± 2°C and 65 ± 5% humidity under a 12 h light–dark cycle. Male and female mice from 10–24 weeks old of each genotype group (*n*equals;4–8) were used in each experiment equally. All animal experiments were performed in accordance with the Guidelines for the Care of Laboratory Animals of the Tokyo Institute of Psychiatry.

For the CPu cocaine study, all the nine DAT × SERT genotypes were examined (DAT+/+ SERT+/+, DAT+/+ SERT+/−, DAT+/+SERT−/−, DAT+/−SERT+/+, DAT+/−SERT+/−, DAT+/−SERT−/−, DAT−/− SERT+/+, DAT−/−SERT+/−, and DAT−/−SERT−/−). For NAc and PFc cocaine studies and for fluoxetine CPu and NAc studies, the four homozygous genotypes were examined (wildtype, DAT−/−SERT+/+, DAT+/+SERT−/−, DAT−/−SERT−/−). GBR12909 effects on CPu $5\text{-}HT_{ex}$ levels were examined in wild-type and DAT+/+SERT−/− mice.

Surgery

Mice were stereotaxically implanted with microdialysis probes under sodium pentobarbital anesthesia (50 mg/kg) in CPu (anterior +0.6 mm, lateral +1.8 mm ventral −4.0 mm from bregma), NAc (anterior +1.2 mm, lateral +1.0 mm ventral −5.0 mm from bregma) or PFc (anterior +2.0 mm, lateral +0.5 mm ventral −3.0 mm from bregma) according to the atlas of Franklin and Paxinos (1997). Probe tips were constructed with regenerated cellulose membranes that provided 50 kDa molecular weight cutoffs, outer diameters of 0.22 mm, and membrane lengths of either 1 mm (NAc) or 2 mm (CPu and PFc) (Eicom, Kyoto, Japan). Dialysis probe placements were verified histologically at the ends of each experiment (Figure 1), and experimental data were excluded if the membrane portions of the dialysis probes lay outside the central CPu, medial PFc or NAc core or shell regions, respectively.

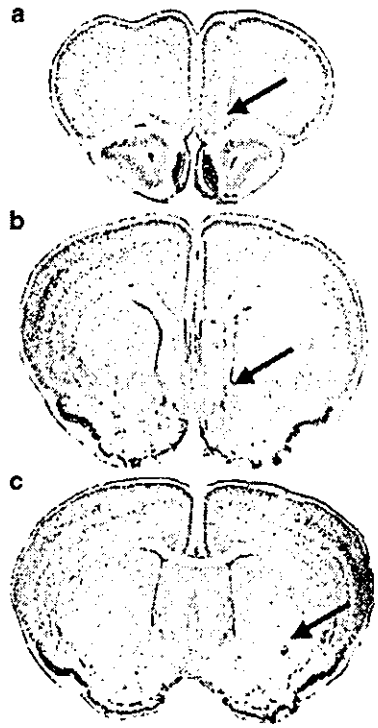

Figure 1 Location of dialysis probes in coronal sections of PFc (a), NAc (b), and CPu (c). The arrows illustrate the implantation sites of dialysis probes.

Microdialysis and Analytical Procedure

At 24 h after implantation, probes in freely moving mice were perfused with Ringer's solution (147 mM Na^+, 4 mM K^+, 1.26 mM Ca^{2+}, 1 mM Mg^{2+}, and 152.5 mM Cl^-, pH 6.5) at 1 µl/min for 180 min. DA_{ex} and 5-HT_{ex} baselines were obtained from average concentrations of three consecutive 10 min, 10 µl samples. These and subsequent 10 min, 10 µl dialysate fractions were analyzed using an AS-10 auto-injector (Eicom), high-performance liquid chromatography (HPLC), with a PPS-ODS reverse-phase column (Eicom) and a ECD-100 graphite electrode detector (Eicom). The mobile phase consisted of 0.1 M phosphate buffer (pH 5.5) containing sodium decanesulfonate (500 mg/l), EDTA (50 mg/l), and 1% methanol. Detection limits for DA and 5-HT were 1 fmol/sample with signal-to-noise ratios of at least 2. *In vitro* recoveries from the 1- and 2-mm membrane length probes were 10 and 15%, respectively.

Drugs

Test drugs were dissolved in saline for systemic administration or in Ringer's solution for local infusion via microdialysis probes. After establishment of stable baselines, cocaine HCl (10 mg/kg for subcutaneous injection or 100 µM for local infusion; Dainippon, Osaka, Japan), fluoxetine (20 mg/kg, Sigma, Tokyo, Japan), GBR12909 (10 mg/kg, Sigma) or saline (10 ml/kg) was administered subcutaneously (s.c.) and dialysates collected for 3 or 2 h, respectively.

Statistics

Baselines of DA_{ex} and 5-HT_{ex} were compared across genotype groups using two-way ANOVAs (DAT genotype, and SERT genotype). DA and 5-HT responses to drugs were expressed as percentages of baselines. Effects of drugs on DA_{ex} and 5-HT_{ex} were assessed by calculating the areas under time-response curves (AUC) for the first 120 or 180 min after drug administration. AUCs were analyzed using two-way ANOVAs (Drug, Genotype). Least significant difference tests were applied for multiple comparisons and *P*-values less than 0.05 were considered statistically significant. Statistical analyses used STATISTICA (StatSoft Inc., Tulsa, OK).

RESULTS

Baselines of DA_{ex} and 5-HT_{ex} in CPu, NAc, and PFc

The mean (±SEM) baselines of DA_{ex} and 5-HT_{ex} in dialysates from the CPu, NAc, and PFc in mice, who were subsequently treated with either vehicle or test drugs, are shown in Table 1. Two-way ANOVA of DA_{ex} baselines confirmed that DAT-KO had significant effects on DA_{ex} baselines in CPu ($F(1,91)=299.77$, $P<0.00001$) and NAc ($F(1,55)=101.49$, $P<0.00001$), but not PFc ($F(1,33)=0.07$, $P=0.79$). Dialysate DA in homozygous DAT-KO mouse CPu and NAc was approximately 10-fold higher than that in mice with either one or two copies of the DAT gene. 5-HT_{ex} baselines were unaffected by DAT-KO in any region.

SERT-KO exerted significant effects on 5-HT_{ex} baselines in each of these three regions ($F(2,91)=87.06$, $P<0.00001$; $F(1,55)=29.95$, $P<0.00001$; $F(1,33)=80.37$, $P<0.00001$, respectively). In CPu, NAc, and PFc, 5-HT_{ex} baselines in mice with no SERT gene were six to ten times as large as that found in mice with one or two copies of SERT gene. DA_{ex} baselines were unaffected by SERT-KO in any region.

Interestingly, there was a significant interaction between DAT and SERT genotype effects on basal NAc dialysate DA levels ($F(12,55)=4.33$, $P<0.05$). DA_{ex} levels in NAc of mice with no DAT or SERT genes (DAT−/−SERT−/−) were higher than those of mice with no DAT genes but two SERT genes (DAT−/−SERT+/+).

Systemic Cocaine Effects on DA_{ex} in CPu, NAc, and PFc

DA_{ex} level changes in CPu, NAc, and PFc following systemic cocaine administration are shown in Figure 2a, c, and e. DA responses to cocaine in the CPu of wild-type and DAT+/− mice peak at 40–60 min (Figure 2a). Cocaine also induces a slower DA response curve in the CPu of homozygous

Table 1 The Baselines (fmol/10 min) of DA_{ex} and 5-HT_{ex} in CPu, NAc and PFc

Genotype		CPu			NAc			PFc		
DAT	SERT	n	DA	5-HT	n	DA	5-HT	n	DA	5-HT
+/+	+/+	18	73.88±4.97	1.82±0.15	16	17.79±1.69	1.19±0.08	10	2.11±0.11	3.05±0.36
+/+	+/−	8	82.28±13.76	2.39±0.29	—	—	—	—	—	—
+/+	−/−	13	69.82±7.12	16.33±2.58*,+	15	16.23±2.23	15.50±4.20*	9	2.52±0.35	25.55±3.18*
+/−	+/+	9	79.69±12.32	1.28±0.13	—	—	—	—	—	—
+/−	+/−	10	105.12±10.70	2.90±0.33	—	—	—	—	—	—
+/−	−/−	9	93.04±14.09	17.73±3.30*,+	—	—	—	—	—	—
−/−	+/+	15	687.18±58.16*,#	1.61±0.10	14	188.17±23.00*	1.19±0.43	9	2.32±0.32	3.28±0.47
−/−	+/−	9	667.67±42.39*,#	2.38±0.31	—	—	—	—	—	—
−/−	−/−	9	548.78±31.42*,#	12.78±1.46*,+	14	275.34±38.47*,Δ	13.42±2.36*	9	2.22±0.15	17.06±2.61*

DA_{ex} or 5-HT_{ex} baselines were obtained from average concentrations (fmol/10 min) of three consecutive stable samples before injections. Values are the mean (±SEM) of baselines. *$P<0.0001$ compared to wild-type mice; #$P<0.0001$ compared to mice with one copy of DAT gene; +$P<0.0001$ compared to mice with one copy of SERT gene; Δ$P<0.001$ compared to DAT−/− mice.

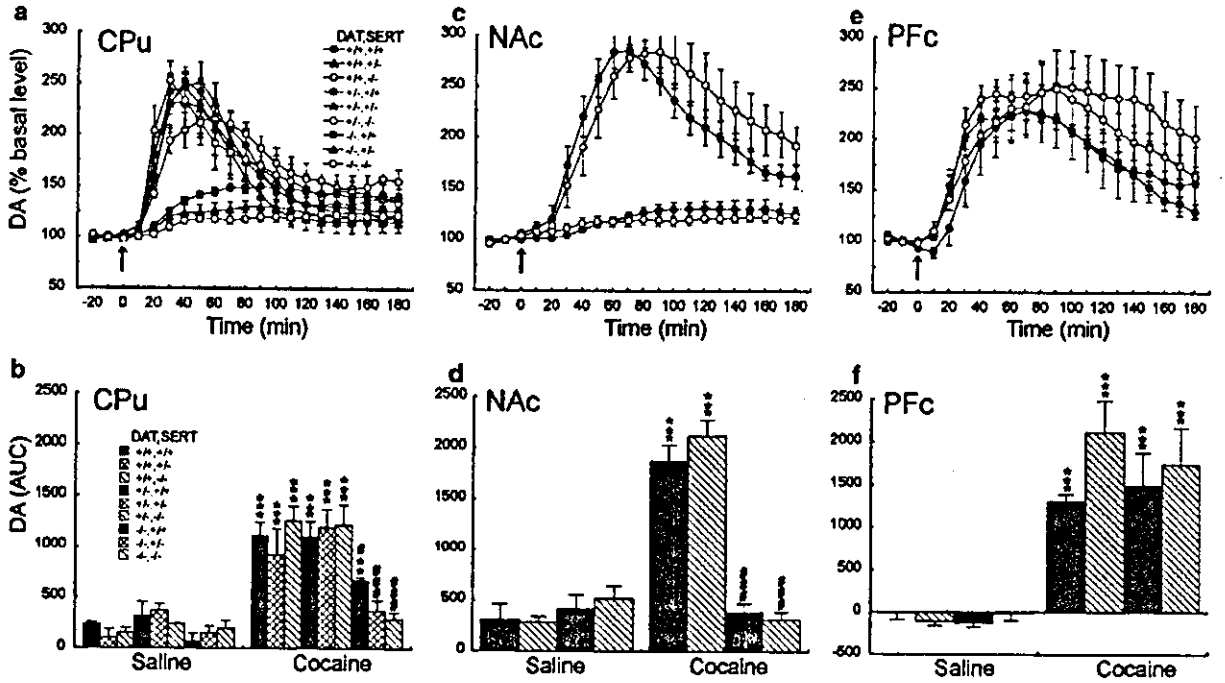

Figure 2 (a, c, and e) Temporal pattern of DA response to cocaine (10 mg/kg, s.c.) in CPu, NAc, and PFc, respectively. Each point represents the mean (±SEM) of the percentage of DA_{ex} baselines. The time of injections is indicated with an arrow. (b, d, and f) Histogram represents the mean AUC (±SEM) of DA response to saline or cocaine in CPu, NAc, and PFc during 180 min interval after injection. $^{**}P<0.01$, $^{***}P<0.001$ compared to the saline group of the same genotype; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ compared to the cocaine-treated wild-type group.

DAT-KO mice (DAT−/−SERT+/+), peaking at about 90 min (Figure 2a). This pattern is not observed in NAc, where DAT−/−SERT+/+ mice do not exhibit any larger increments in DA_{ex} levels (Figure 2c). In further contrast, wild-type, DAT−/−SERT+/+, and DAT−/−SERT−/− mice each exhibit indistinguishable cocaine-induced DA responses in PFc (Figure 2e).

Drug effects on DA_{ex} levels can be assessed by studying AUCs (Figure 2b, d, and f). ANOVAs of mean AUC (±SEM) for drug effects on DA_{ex} levels reveal that drugs have significant effects on DA AUC in CPu ($F(1,62)=132.32$, $P<0.0001$), NAc ($F(1,34)=80.60$, $P<0.0001$), and PFc ($F(1,28)=67.59$, $P<0.0001$). Genotype and drug × genotype interactions were significant for DA AUC in CPu ($F(8,62)=5.45$, $P<0.0001$; $F(8,62)=3.41$, $P<0.01$; respectively) and NAc ($F(3,34)=23.82$, $P<0.0001$; $F(3,34)=36.09$, $P<0.0001$; respectively), but not in PFc ($F(3,28)=0.89$, $P=0.46$; $F(3,28)=0.94$, $P=0.43$; respectively). In CPu (Figure 2b), DAT-KO mice exhibit statistically significant cocaine-induced increments in DA_{ex} levels, although these increases are less than those found in wild-type mice. By contrast, in DAT−/−SERT+/− and DAT−/−SERT−/− mice, the same genotypes that do not exhibit rewarding effects of cocaine also do not exhibit cocaine-induced increases in DA_{ex} in CPu. No significant differences are observed in cocaine-induced DA AUC increases in CPu between the DAT+/+ and DAT+/− mice. In NAc (Figure 2d), cocaine fails to increase DA_{ex} in DAT−/− SERT+/+ or in DAT−/−SERT−/− mice. There are no significant differences in cocaine-induced DA increases in NAc between wild-type and DAT+/+ SERT−/− mice. In PFc (Figure 2f), cocaine produces significant increases in DA_{ex} in all genotypes.

Systemic Cocaine Effects on 5-HT_{ex} in CPu, NAc, and PFc

The temporal patterns of 5HT responses to cocaine in CPu, NAc, and PFc are shown in Figure 3a, c, and e. DAT+/+ SERT−/− and DAT+/−SERT−/− mice show gradual 5HT responses to cocaine in CPu (Figure 3a) and NAc (Figure 3c), but not in PFc (Figure 3e). 5-HT response curves produced by cocaine are observed in CPu (Figure 3a) and NAc (Figure 3c) in all genotypes except DAT−/− SERT−/− mice. The peak of 5-HT response are smaller for SERT−/− mice than for either SERT+/+ or SERT+/− mice. SERT−/− mice exhibit no 5-HT response to cocaine in PFc (Figure 3c), while wild-type mice exhibit robust increases.

Drug effects on 5-HT_{ex} levels are expressed as mean AUC (±SEM) in Figure 3b, d, and f. Two-way ANOVAs of the AUC for 5-HT responses to cocaine show significant effects of Drug, Genotype, and Drug × Genotype interactions in CPu ($F(1,62)=181.49$, $P<0.0001$; $F(8,62)=5.01$, $P<0.0001$; $F(8,62)=4.88$, $P<0.0001$; respectively), NAc ($F(1,34)=31.57$, $P<0.0001$; $F(3,34)=6.44$, $P<0.0001$; $F(3,34)=8.41$, $P<0.0001$; respectively) and PFc ($F(1,28)=57.74$, $P<0.0001$; $F(3,28)=11.55$, $P<0.0001$; $F(3,28)=15.59$, $P<0.0001$; respectively). In CPu (Figure 3b) and NAc (Figure 3d), multiple AUC comparisons reveal that cocaine significantly increases 5-HT_{ex} in DAT+/+SERT−/− and DAT+/−SERT−/− mice, but

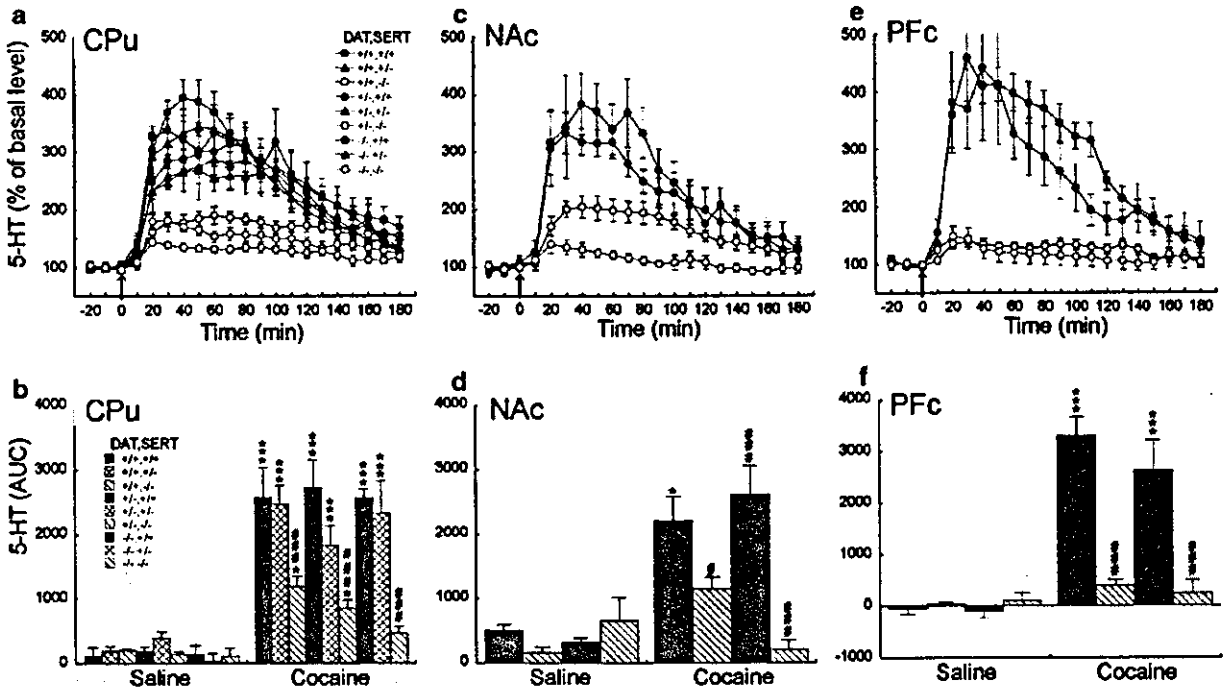

Figure 3 (a, c, and e) Temporal pattern of 5-HT response to cocaine (10 mg/kg, s.c.) in CPu, NAc, and PFc, respectively. The time of injections is indicated with an arrow. (b) Histogram represents the mean AUC (±SEM) of 5-HT response to saline or cocaine in CPu, NAc, and PFc during the 180 min interval after injection. $^{*}P<0.05$, $^{***}P<0.001$ compared to the saline group of the same genotype; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ compared to the cocaine-treated wild-type group.

not in DAT−/−SERT−/− mice. SERT+/− mice display cocaine-induced increases in 5-HT_{ex} in CPu that are similar to those found in wild-type values. PFc 5-HT_{ex} levels are not altered significantly by cocaine in SERT−/− mice (Figure 3f).

Systemic Fluoxetine Effects on DA_{ex} in CPu and NAc

The temporal patterns of DA response to fluoxetine in CPu and NAc of DAT+/+ SERT+/+, DAT−/−SERT+/+, DAT+/+SERT−/−, and DAT−/−SERT−/− mice are shown in Figure 4a and c. In CPu (Figure 4a), DAT−/−SERT+/+ mice exhibit gradual DA responses to fluoxetine that display time courses similar to those of cocaine and persist for at least 3 h (Figure 4a). Two-way ANOVAs of DA AUC responses show significant effects of Drug ($F(1,33)=9.62$, $P<0.01$) and Drug × Genotype interactions ($F(1,33)=4.94$, $P<0.01$). Multiple comparisons reveal that fluoxetine significantly increases DA AUC only in the CPu of DAT−/−SERT+/+ mice (Figure 4b). In NAc, DA responses to fluoxetine display no significant effects of either Drug ($F(1,29)=0.0076$, $P=0.93$), Genotype ($F(1,29)=0.49$, $P=0.69$), genotype ($F(1,29)=0.69$, $P=0.41$) or Drug × Genotype interaction ($F(3,29)=1.55$, $P=0.22$) (Figure 4d).

Systemic GBR12909 Effects on 5-HT_{ex} in CPu

The temporal pattern of CPu 5-HT response to GBR12909 is shown in Figure 5a. DAT+/+SERT−/− mice exhibit remarkable 5-HT_{ex} increases after administration of GBR12909, which are not seen in WT mice. These SERT-KO mice continue to display elevated CPu 5-HT_{ex} levels for at least 3 h. Two-way ANOVA of the AUC of the DA response to GBR 12909 shows significant effects of Drug ($F(1,13)=14.43$, $P<0.01$), Genotype ($F(1,13)=7.63$, $P<0.05$), and Drug × Genotype interactions ($F(1,13)=5.74$, $P<0.05$). Multiple comparisons show that GBR12909 administration significantly increases CPu 5-HT_{ex} in DAT+/+SERT−/−, but not in wild-type mice (Figure 5b).

Local Cocaine Effects on DA_{ex} and 5-HT_{ex} in CPu

DA_{ex} and 5-HT_{ex} level changes in CPu following local cocaine infusion are shown in Figure 6a and c. Local cocaine cannot induce DA response curve in CPu of DAT−/−SERT+/+ and DAT−/−SERT−/− mice, but produces gradual 5-HT response curve in DAT+/+SERT−/− mice.

ANOVAs of mean AUC (±SEM) for DA responses reveal significant effects of Drug, Genotype, and Drug × Genotype interactions in CPu ($F(1,24)=161.46$, $P<0.0001$; $F(3,24)=48.20$, $P<0.0001$; $F(3,24)=47.30$, $P<0.0001$; respectively). Multiple AUC comparisons show that local cocaine fails to increase DA_{ex} in CPu of DAT−/−SERT+/+ or in DAT−/−SERT−/− mice (Figure 6b). ANOVAs of mean AUC (±SEM) for 5-HT responses also reveal significant effects of Drug, Genotype, and Drug × Genotype interactions in CPu ($F(1,24)=43.26$, $P<0.0001$; $F(3,24)=9.55$, $P<0.0001$; $F(3,24)=9.70$, $P<0.0001$; respectively). Multiple comparisons reveal that local cocaine significantly increases 5-HT_{ex} in wild-type, DAT+/+SERT−/− and DAT−/−SERT+/+ mice, but not in DAT−/−SERT−/− mice (Figure 6d). Moreover, there were no significant changes in NAc DA_{ex} in DAT−/−

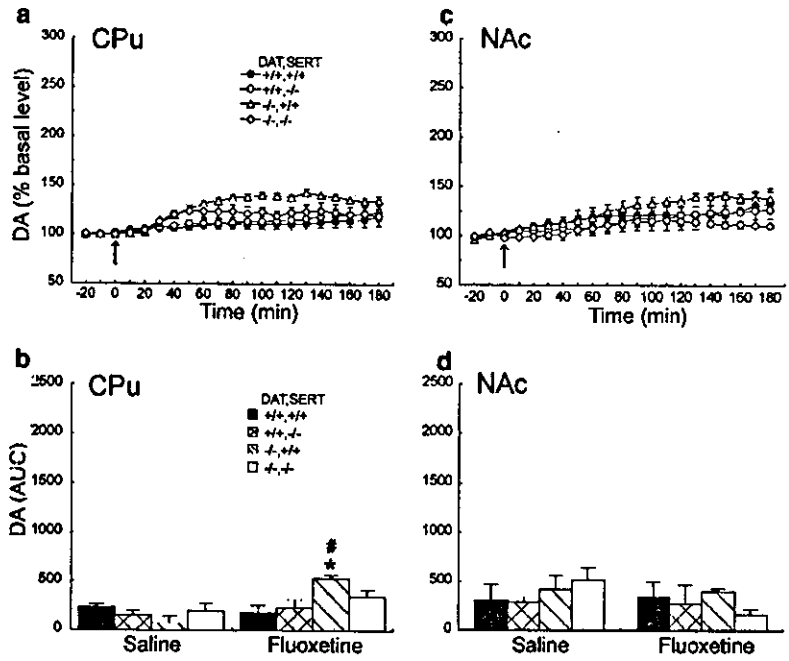

Figure 4 (a and c) Temporal pattern of DA response to fluoxetine (20 mg/kg, s.c.) in CPu and NAc, respectively. The time of injections is indicated with an arrow. (b and d) The histogram represents the mean AUC (±SEM) of DA response to saline or fluoxetine in CPu and NAc during the 180 min interval after injection. $^{*}P<0.05$ compared to the saline group of the same genotype; $^{\#}P<0.05$ compared to the fluoxetine-treated wild-type group.

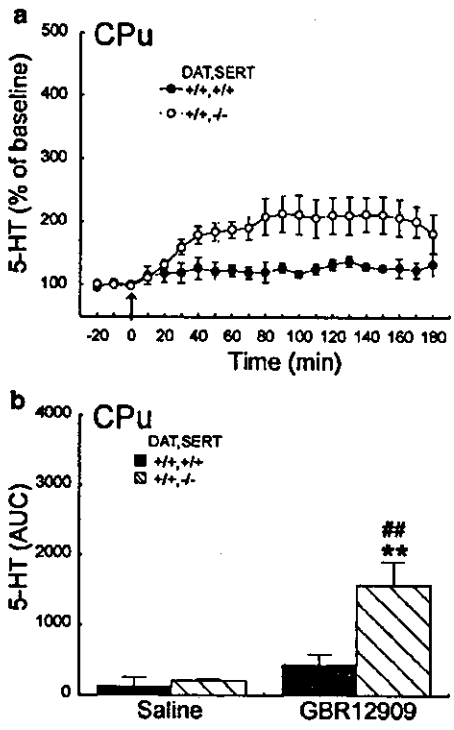

Figure 5 (a) Temporal pattern of 5-HT response to GBR12909 (10 mg/kg, s.c.) in CPu. The time of injections is indicated with an arrow. (b) The histogram represents the mean AUC (±SEM) of 5-HT response to saline or GBR12909 in CPu during 180 min interval after injection. $^{**}P<0.01$ compared to the saline group of the same genotype; $^{\#\#}P<0.01$ compared to the GBR12909-treated wild-type group.

SERT+/+ and DAT−/−SERT−/− mice after local cocaine infusions (data not shown).

DISCUSSION

These microdialysis results reveal parallels with and differences from the patterns of KO effects on reward elicited by cocaine and fluoxetine that we have previously reported in these mouse strains. We can thus evaluate hypotheses about the pharmacological profiles and brain localization of processes hypothesized to mediate cocaine reward with regard to their convergence or divergence with this microdialysis data.

Differential DA Responses in to Cocaine in CPu, NAc, and PFc and Correlations with Assessments of Cocaine Reward

The current data do not provide simple correlations with models that postulate that enhanced NAc DA_{ex} levels alone are necessary and sufficient for cocaine reward. Although this hypothesis has been supported by data from microinjection and lesion studies (Kuhar *et al*, 1991; Koob and Nestler, 1997; Bardo, 1998; Kelley and Berridge, 2002), many results from gene KO studies fail to support the simple hypothesis that DA alone mediates the rewarding effects of cocaine. Our current observations that cocaine does not increase DA_{ex} in NAc of homozygous DAT-KO mice contrasts with the nearly-intact cocaine reward found in these animals (Rocha *et al*, 1998; Sora *et al*, 1998). These *in vivo* microdialysis data are also consistent with studies which document failure of cocaine to block DA uptake in

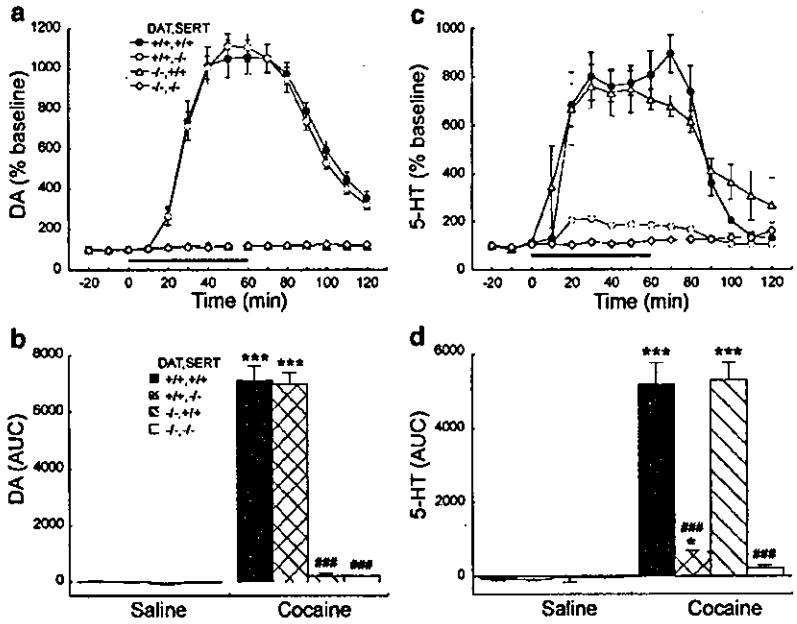

Figure 6 (a and c) Temporal pattern of DA and 5-HT response to local cocaine infusion (100 μM) in CPu, respectively. Horizontal bar indicates the time of infusions. (b and d) The histogram represents the mean AUC (±SEM) of DA and 5-HT response to saline or cocaine in CPu during 120 min interval after injection. $^{*}P<0.05$, $^{***}P<0.001$ compared to the saline group of the same genotype; $^{\#\#\#}P<0.001$ compared to the cocaine-treated wild-type group.

NAc samples taken from DAT homozygous mice in *in vitro* experiments (Budygin *et al*, 2002; Moron *et al*, 2002).

The current data also fail to provide simple correlations with models that postulate that enhanced PFc DA levels are necessary and sufficient for cocaine reward. This hypothesis has also been supported by a substantial body of lesion and microinjection data (Goeders and Smith, 1983; Goeders *et al*, 1986; Bardo, 1998; Tzschentke, 2001). Cocaine increases DA_{ex} in PFc of both wild-type and homozygous DAT-KO mice that exhibit cocaine reward and DAT/SERT double homozygous KO mice that do not display cocaine reward.

Intriguingly, the current results for DA in CPu appear to provide the best fit with studies of cocaine-induced place preferences. Although intra-CPu cocaine does not affect DA_{ex} levels in DAT-KO mice, systemic cocaine causes about 1.5-fold increase in peak DA_{ex} concentrations in CPu dialysate from DAT-KO mice that are rewarded by cocaine, but not from DAT/SERT double homozygous KO mice that lack cocaine CPP. Systemic fluoxetine also increases CPu DA_{ex} levels in homozygous DAT-KO mice in which this compound is rewarding, but not in wild-type mice or homozygous SERT-KO mice in which fluoxetine does not produce a place preference.

Differential 5-HT Responses to Cocaine in CPu, NAc, and PFc and Correlations with Assessments of Cocaine Reward

Although cocaine-induced increases in CPu and NAc $5\text{-}HT_{ex}$ are found in SERT-KO mice that exhibit enhanced cocaine CPP, the magnitude of the increases in $5\text{-}HT_{ex}$ after cocaine administration is attenuated when it is compared with wild-type mice. Interestingly, chronic SERT blockade with fluoxetine can also potentiate cocaine reward (Cunningham and Callahan, 1991; Kleven and Koek, 1998). It is conceivable that the attenuation of cocaine-induced $5\text{-}HT_{ex}$ rise may lead mice more sensitive to the reward effect of cocaine. These sorts of data, and the current results, continue to point to possible roles for 5-HT in cocaine reward, especially in light of the more complex hypotheses of the basis of cocaine reward discussed below.

$5\text{-}HT_{ex}$ Clearance by DAT, DA_{ex} Clearance by NET, and opportunities for 'Promiscuous Uptake'

Removal of a transporter that usually provides inactivation, re-accumulation, and recycling of a released monoamine neurotransmitter provides opportunities for greater diffusion of the monoamine, documented by higher extracellular dialysate concentrations noted here. Removal of a cognate transporter also enhances the opportunities for transmitter uptake by a transporter that normally recognizes another monoamine. The presence of the same vesicular transporter in DAT-, SERT-, and NET-expressing neurons provides the opportunity for the monoamine that has been taken up by a non-cognate plasma membrane transporter to be accumulated into vesicles, and to be re-released as a 'false transmitter' (Liu and Edwards, 1997; Uhl *et al*, 2000). DA accumulation by NET-expressing neurons also provides the opportunity for DA to be subjected to β-hydroxylation to produce norepinephrine, providing a 'true' transmitter for noradrenergic neurons. It is interesting to note that elimination of monoamine transporters has different effects on basal monoamine levels in different brain regions, supporting ideas that factors that mediate DA and 5-HT clearance from synaptic clefts may differ substantially from one terminal field to another.

Many of the present and previously reported results appear to provide evidence for uptake by non-cognate transporters, and even for possible 'false transmission' in these transporter-KO mice. Cocaine and the selective DAT blocker GBR12909 produces a substantial increase in dialysate 5-HT in SERT-KO mice that is not found in wild-type animals. These findings were supported by previous reports that have documented 5-HT uptake by cultured neurons from SERT-KO mice that could be blocked by selective DAT blockers (Pan *et al*, 2001), and 5-HT-like immunoreactivity in substantia nigra and ventral tegmental area dopaminergic neurons (Zhou *et al*, 2002). False transmission may be region-dependent, with differences in the relative densities of DAT- SERT- and NET-expressing neural elements providing differential opportunities for such processes.

Moreover, our observations of virtually identical PFc DA_{ex} baselines in each of these KO strains appear to support a relatively reduced prominence of DAT-mediated DA uptake in this region even in wild-type mice. These observations are compatible with the relatively sparse distribution of PFc DAT in several species (Freed *et al*, 1995; Sesack *et al*, 1998), in contrast with more prominent NET and SERT expression. They are also in accord with pharmacological and other evidence for significant NET-mediated DA uptake in rodent PFc (Di Chiara *et al*, 1992; Tanda *et al*, 1997; Yamamoto and Novotney, 1998). DA may thus be accumulated by NET in PFc of both wild-type and DAT-KO mice.

The current observations in DA response to cocaine and fluoxetine in CPu of DAT-KO mice may provide a different picture. Although systemic cocaine and fluoxetine increase significantly CPu DA_{ex} in DAT-KO mice, local cocaine fails to change it. These results demonstrate that SERT does nt play a role of 'promiscuous uptake' in DA clearance. Systemic cocaine- or fluoxetine-induced DA increase in CPu of DAT-KO mice may result from DA release from activated DA neuron rather than local clearance by SERT.

Comparisons with Other Results

Observations that CPu dialysate monoamine levels apparently provide the best parallel with the loss of cocaine CPP found in current results could be consistent with a previously underappreciated role for CPu structures in mediating some of the 'learned' features of cocaine reward that are manifest in conditioned place preference testing (White and McDonald, 2002). These structures can be critical for stimulus-response 'habit' learning, including that related to reward (Jog *et al*, 1999; Reynolds *et al*, 2001). It is conceivable that this structure may play an even greater role in DAT-KO mice that lack cocaine-induced DA_{ex} elevations in NAc.

The failure of dialysis results for DA alone in NAc or PFc to parallel cocaine reward effects of various KOs and the apparent parallel in CPu should not prevent further consideration of: (a) multiple compensating contributions of monoamines to the rewarding effects of cocaine; (b) contributions of cocaine effects on monoamines in other brain regions, for example, ventral pallidum (Gong *et al*, 1996, 1997), ventral tegmental area (Roberts and Koob, 1982; Ranaldi and Wise, 2001) for cocaine reward; (c) effects of nonmonoaminergic adaptations to the retained cocaine reward in the transporter KO mouse strains that retain such reward. Monoamine actions in brain regions such as the ventral tegmental area have been postulated to be central to the rewarding actions of major drug classes, such as opiates (Wise, 1989; Garzon and Pickel, 2001) and stimulants. It is quite conceivable that monoamine actions in areas not sampled in the current studies could play roles in normal cocaine reward mechanisms, and in adaptations that may underlie the retention of cocaine reward in DAT- and in SERT-KO mice. Mice with single or multiple transporter deletions display many adaptive alterations, as assessed through behavioral, neurochemical, per- or post-synaptic receptor binding, gene expression, and other analytical approaches. None of the current data should hinder attempts to add more explanatory power for the remarkable behavioral pharmacological profiles displayed by these KO mice through use of any or all of these alternative approaches.

The current results in NAc and CPu DA response to cocaine in DAT-KO mice produced in our laboratory, while highly reproducible in our hands, differ from those obtained in reports from another line of DAT-KO mice that which showed that systemic cocaine and reboxetine (NET blocker) increased DA_{ex} remarkably in NAc of DAT-KO mice (Carboni *et al*, 2001). The different DA response to cocaine in NAc and CPu between Carboni's and our DAT KOs may be due to the different DNA construction which was used to disrupt DAT gene. Moreover, our findings are consistent with other reports which demonstrated that cocaine could not affect DA clearance in NAc of DAT-KO mice via *in vitro* experiments. It is noteworthy that (1) DA_{ex} baseline in NAc of DAT-KO mice is about 10 times greater than that in wild-type mice, and that (2) the capacity for DA uptake of NET is far weaker than that of DAT (Giros *et al*, 1994; Gu *et al*, 1994). These may be the reasons why NET cannot show redundancy for DAT in NAc.

In summary, the present work adds to previous data concerning the behavioral consequences of DAT and SERT deletion, by suggesting that cocaine CPP does not necessarily correlate with simple elevations of DA the NAc or PFc. It points out unanticipated correlations with DA_{ex} elevations in CPu. It is interesting that the CPu findings parallel behavioral observations of the rewarding profiles of not only cocaine but also of fluoxetine in these varying mouse strains. While these correlations do not prove causation, the data support careful re-examination of CPu roles in psychostimulant reward (or reward learning) in both wild-type and DAT-KO mice, including both the dorsal and ventral CPu regions likely to be sampled with our microdialysis approaches. Another view of the current results is that the double homozygous DAT/SERT combined KO mice that failed to display either cocaine-induced DA_{ex} or 5-HT_{ex} elevations in NAc also failed to exhibit cocaine CPP, suggesting perhaps that either DA_{ex} or 5-HT_{ex} elevation can mediate cocaine reward and that the absence of both effects is required to eliminates the cocaine CPP. The current data also add to the growing body of evidence that may indicate uptake of released monoamines by non-cognate transporters when their cognate transporters are deleted, and provide evidence for the brain-region specificity of these processes in wild-type and in transporter KO

mice. Each of these findings adds pieces to the complex puzzle of the mediation of cocaine reward by monoaminergic brain systems.

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●第55回日本自律神経学会総会/シンポジウム2/カテコラミントランスポーター
司会：曽良一郎・伊豫雅臣

カテコラミントランスポーターと高次神経機能

曽良一郎[a,b]　小林秀昭[b]

キーワード：モノアミントランスポーター，コカイン，ドーパミン，遺伝子欠損マウス，抗うつ薬
monoamine transporter, cocaine, dopamine, knockout mouse, antidepressant

抄録：モノアミンは運動調節や情動など高次神経機能に幅広く関与することから，さまざまな神経精神疾患の病態に深く関わっていると考えられている．モノアミントランスポーターは細胞膜やシナプス小胞に存在する．細胞膜トランスポーターは抗うつ剤や覚醒剤の標的分子である．モノアミン受容体には多数のサブタイプが存在するものの細胞膜トランスポーターは各モノアミンに一種類しかなく，さらにシナプス小胞モノアミントランスポーターはすべてのモノアミンを基質としている．このことから，モノアミントランスポーターはモノアミン神経伝達の制御には極めて重要な役割を果たすと考えられる．神経精神疾患の動物モデルは複雑な臨床症状の病態解明，治療法の開発に欠かすことができない．我々の作製したモノアミントランスポーター遺伝子ノックアウトマウスを中心に高次神経機能の解明あるいは神経精神疾患の動物モデルとしての有用性を紹介する．
（自律神経，40：238～243，2003）

はじめに

カテコラミンを含むモノアミンは運動調節や情動など高次神経機能に幅広く関与することから，さまざまな神経精神疾患の病態に深く関わっていると考えられている．モノアミンは合成後に神経終末内のシナプス小胞に蓄えられて神経終末から放出され，後シナプス受容体に情報を伝達する．細胞間隙に放出されたモノアミンは速やかに神経終末に回収される．このモノアミンの放出，再取り込みに関与するトランスポーターがモノアミントランスポーターであり，細胞膜トランスポーターとシナプス小胞トランスポーターの2種類が知られている．モノアミントランスポーターは覚醒剤，抗うつ薬の標的分子であることから，精神疾患の病態における役割を明らかにする上で，精神薬理学的に詳細に研究されてきた経緯がある．神経精神疾患の動物モデルは複雑な臨床症状を解析可能とするために病態解明，治療法の開発に欠かすことができない．しかし，従来の精神薬理学的方法では，作動薬および拮抗薬が必ずしも特異的にモノアミントランスポーターに作用しないことから限界があった．そこで我々はジーン・ターゲティング法を用いてトランスポーター遺伝子のノックアウトマウス（以下KOマウス）を作製し，精神神経疾患病態モデルを開発するため行動薬理学的，神経化学的手法を用いて解析を行ってきた．本論文では，我々が作製したモノアミントランスポーター遺伝子ノックアウトマウスを中心に高次神経機能の解明あるいは神経精神疾患の動物モデルとしての有用性を紹介したい．

[a]東北大学大学院医学系研究科・精神神経生物学分野
〒980-8574　宮城県仙台市青葉区星陵町1—1
[b](財)東京都医学研究機構・東京都精神医学総合研究所・分子精神医学研究部門
〒156-8585　東京都世田谷区上北沢2—1—8

1. モノアミントランスポーター

モノアミントランスポーターは，神経終末から放出されたモノアミンを素早く神経終末に再取り込みし，