The role of organic cation transporter-3 in methamphetamine disposition and its behavioral response in rats

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Abbreviations: OCT3, organic cation transporter-3; METH, methamphetamine; OCT3-AS, antisense against OCT3; OCT3-SCR, scrambled antisense against OCT3; PFC, prefrontal cortex; NAcc, nucleus accumbens; MPP^+, 1-methyl-4-phenylpyridinium; CP, choroid plexus; CSF, cerebrospinal fluid; HPLC, high performance liquid chromatography; AUC, area under the blood concentration time curve; DA, dopamine.

Abstract

Organic cation transporter-3 (OCT3) is expressed in several tissues including the brain. We have previously demonstrated that rats with behavioral sensitization to methamphetamine (METH) increased the brain penetration of METH with decreased expression of OCT3 in brain. Considering the earlier *in vitro* studies demonstrating that 1) OCT3 could transport dopamine (DA) and 2) the specific transport via OCT3 could be inhibited by METH, these results suggest that decreased OCT3 might decrease the efflux of METH and/or DA from brain, subsequently causing the development of behavioral sensitization. Thus, in the present study, behavioral task related to DA and pharmacokinetic experiment were performed using rats treated with antisense against OCT3 (OCT3-AS) since no specific ligands for OCT3 are still available. The continuous infusion of OCT3-AS into the third ventricle significantly decreased the expression of OCT3 in choroid plexus (CP) epithelial cells. Both METH-induced hyperlocomotion and METH-induced extracellular DA levels in nucleus accumbens and prefrontal cortex were significantly increased in OCT3-AS-treated rats. Moreover, the concentrations of METH were significantly increased in cerebrospinal fluid as well as extracellular areas at the nucleus accumbens in OCT3-AS-treated rats. These results suggested that decreased OCT3 elevated the concentration of METH and/or DA in brain, subsequently enhancing dopaminergic neuronal transmission and increasing METH-induced

hyperlocomotion. In summary, OCT3 at the CP could regulate the effect of METH by controlling the levels of METH and/or DA in brain. Thus, these results suggest that OCT3 may be a new molecular target to treat METH-related disorders such as drug abuse and schizophrenia.

1. Introduction

Behavioral sensitization is characterized by the enhancement of locomotor responses to psychostimulant drugs, such as amphetamine (Paulson et al., 1995; Mendrek et al., 1998) methamphetamine (METH) (Szumlinski et al., 2000; Davidson et al., 2001) and cocaine (Pierce et al., 1998; Beurrier et al., 2002) when those drugs are repeatedly administrated. Several researchers have reported that behavioral sensitization to psychostimulant drugs was accompanied by neuronal adaptation in mesocorticolimbic regions, including the prefrontal cortex (PFC), ventral tegmental area and nucleus accumbens (NAcc) (Wolf et al., 1994; Cador et al., 1999; Brady et al., 2005). However, recently, we have found that rats with behavioral sensitization to METH have increased the brain penetration of METH together with the decreased expression of organic cation transporters-3 (OCT3) in the brain (Kitaichi et al., 2003; Fujimoto et al., 2007). Further study revealed that the polymorphisms of *SLC22A3*-coded OCT3 are related to the development of polysubstance use in Japanese patients with METH dependence (Aoyama et al., 2006). Thus, OCT3 might be an important molecule revealing behavioral sensitization to METH. However, the relationship between OCT3 and neuronal functions, including dopaminergic transmission, remains unknown.

OCT3 is expressed in several tissues including the placenta, intestine, heart and brain, and

could transport several cations including dopamine (DA) as a sodium- and chloride-independent, transporter (Grundemann et al., 1997, 1998; Kekuda et al., 1998). It has been reported that specific transport of [^{3}H]MPP$^+$ could be inhibited by amphetamine and METH in OCT3-expressed cells, suggesting OCT3 could uptake METH instead of [^{3}H]MPP$^+$ (Wu et al., 1998) although there is no report demonstrating that amphetamine and METH are transported by OCT3. In the brain, OCT3 is highly expressed in the choroid plexus (CP) epithelial cells and circumventricular organs (Haag et al., 2004; Vialou et al., 2004; Amphoux et al., 2006; Gasser et al., 2006). It is well documented that CP plays an important role in detoxifying xenobiotics and endogenous waste by metabolic enzymes and efflux transport systems, which provide a barrier function between cerebrospinal fluid (CSF) and the blood circulation (blood-CSF barrier) to maintain the CNS condition (Ogawa et al., 1994; Kusuhara et al., 2004). Additionally, circumventricular areas are implicated in the changes in blood osmolarity and ingestion of salt and water (Vialou et al., 2004), circadian pacemaker (Aston-Jones et al., 2001), and stress-related signal to the hypothalamic-pituitary-adrenal axis (Lowry et al., 2003). These reports suggest that OCT3 might regulate the influx/efflux of cationic compounds between CSF and blood circulation, similar to other influx/efflux transporters at CP epithelial cells (Kusuhara et al., 2004), and/or by uptake into cells from extracellular areas, subsequently maintaining the brain functions, especially monoaminergic neuronal functions (Wu et al., 1998;

Takeda et al., 2002; Graff et al., 2004; Schildkraut et al., 2004). This assumption is partially supported by recent reports. That is, Vialou et al. showed that salt-intake behavior was altered in OCT3-null mice and Gasser et al. showed that OCT3 regulated the transport of monoamines in dorsomedial hypothalamus (Vialou et al., 2004; Gasser et al., 2006). Moreover, our preliminary studies in mice demonstrated that antisense treatment against OCT3 (OCT3-AS) significantly increased METH-induced hyperlocomotion (Kitaichi et al., 2005).

The hypothesis that OCT3 in CP epithelial cells might play an important role in controlling METH-induced extracellular DA levels in NAcc and PFC is supported by our previous study demonstrating that infusion of OCT3-AS increased METH-induced hyperlocomotion (Kitaichi et al., 2005). Moreover, we expect to alter the distribution of METH in the brain by decrease in OCT3 expression with OCT3-AS, since the brain penetration of METH increased in behavioral sensitization to METH rats together with decreased expression of OCT3 in the brain (Kitaichi et al., 2005). Then, we estimated the pharmacokinetic properties of METH in rats treated with OCT3-AS.

2. Result

2.1. Histochemical analysis

In immunostaining analysis for vehicle rats, OCT3-positive cells were strongly observed in CP epithelial cells and ependymal cells in lateral ventricle (LV) (Fig. 1A,B). The OCT3 positive signals were eliminated by incubation of anti-OCT3 antibody with OCT3 blocking peptide (Fig. 1C,D).

As shown in Fig. 2B, the intensity of OCT3-immunoreactivity in CP epithelial cells was significantly different ($F_{(2,9)} = 8.369$; $p < 0.0088$). Scheffe's post-hoc test indicated that OCT3-AS significantly decreased the expression of OCT3 in CP epithelial cells by 34% compared to vehicles; however, there were no significant differences in PFC ($F_{(2,6)} = 0.084$; $p = 0.9201$) and NAcc ($F_{(2,6)} = 0.291$; $p = 0.7574$) (data not shown). The efficacy of OCT3-AS was coincident with our previous study (Kitaichi et al., 2005).

2.2. Methamphetamine-induced hyperlocomotion

Repeated-measures ANOVA on spontaneous locomotor activity revealed no between-group differences (vehicle: 272 ± 42; OCT3-AS: 629 ± 235; OCT3-SCR: 526 ± 158; $F_{(2,73)} = 2.383$; $p = 0.099$); however, repeated-measures ANOVA also revealed that METH-induced hyperlocomotion was significantly different among these groups ($F_{(2,111)} = 26.799$, $p < 0.001$). Scheffe's post-hoc test

indicated that METH-induced hyperlocomotion in OCT3-AS-treated rats was significantly increased compared to vehicle and OCT3-SCR-treated rats at 0-30 and 30-60 min after METH injection (Fig. 3).

2.3. METH-induced extracellular DA level in NAcc and PFC

Repeated-measures ANOVA showed no between-group differences in the basal level of extracellular DA in NAcc (vehicle: 3.04 ± 0.19; OCT3-AS: 3.80 ± 0.10; OCT3-SCR: 4.10 ± 0.20 (pg/40 μl); $F_{(2,45)} = 1.046$, $p = 0.3597$) and PFC (vehicle: 0.30 ± 0.013; OCT3-AS: 0.32 ± 0.017; OCT3-SCR: 0.23 ± 0.026 (pg/40 μl); $F_{(2,51)} = 0.667$, $p = 0.5176$). Repeated-measures ANOVA also revealed that the METH-induced extracellular level of DA in NAcc ($F_{(2,183)} = 11.976$, $p < 0.001$) and PFC ($F_{(2,223)} = 15.746$, $p < 0.001$) was significantly different. Scheffe's post-hoc test indicated that the METH-induced extracellular level of DA in NAcc and PFC of OCT3-AS-treated rats was significantly increased compared to vehicle and OCT3-SCR-treated rats (Fig. 4).

2.4. Plasma concentration-time course of METH and pharmacokinetic parameters of METH

As seen in Fig. 5A-C, we detected METH by HPLC. No significant difference in the plasma concentration-time profile of METH was found among these groups ($F_{(2,45)} = 0.043$, $p = 0.9578$)

(Fig. 6A). Moreover, there were no significant differences in the pharmacokinetic parameters of METH. $t_{1/2}$ (vehicle: 0.81 ± 0.12; OCT3-AS: 0.70 ± 0.009; OCT3-SCR: 0.75 ± 0.029 (h); $F_{(2,9)}$ = 0.729, p = 0.5087) $AUC_{0\text{-}2}$ (vehicle: 748.6 ± 98.46; OCT3-AS: 798.1 ± 118.28; OCT3-SCR: 752.21 ± 142.27 (ng/ml h); $F_{(2,9)}$ = 0.069, p = 0.9335).

2.5. Concentration of METH in brain dialysate and CSF

As shown in Fig. 6B, repeated-measures ANOVA revealed that the METH concentration in the brain dialysate of NAcc was significantly different ($F_{(2,25)}$ = 18.186, p < 0.001); however, there were no significant differences in PFC (data not shown; $F_{(2,25)}$ = 0.140, p = 0.8699). Scheffe's post-hoc test indicated that METH concentration in NAcc was significantly increased in OCT3-AS-treated rats at all time points. Moreover, Scheffe's post-hoc test showed that METH concentration in CSF was significantly increased in OCT3-AS-treated rats ($F_{(2,9)}$ = 8.063, p = 0.0099) (Fig. 6C).

The CSF/plasma ratio was significantly different among these groups (vehicle: 0.59 ± 0.05; OCT3-AS: 0.95 ± 0.08; OCT3-SCR: 0.44 ± 0.06; $F_{(2,9)}$ = 22.200, p = 0.003). Scheffe's post-hoc test indicated that the CSF/plasma ratio was significantly increased in OCT3-AS-treated rats; however, there were no differences in the amphetamine/METH ratio (vehicle: 0.53 ± 0.11; OCT3-AS: 0.46 ± 0.07; OCT3-SCR: 0.58 ± 0.03; $F_{(2,9)}$ = 0.794, p = 0.4814) and Tissue/plasma ratio of METH (vehicle: 9.03 ± 1.17; OCT3-AS: 8.51 ± 0.95; OCT3-SCR: 8.00 ± 1.04; $F_{(2,18)}$ = 0.258, p = 0.7753).

3. Discussion

We previously demonstrated that the brain penetration of METH increased in rats having behavioral sensitization to METH together with decreased expression of OCT3 in the brain (Kitaichi et al., 2003; Fujimoto et al., 2007). Moreover, we have reported that mice treated with OCT3-AS enhanced METH-induced hyperlocomotion (Kitaichi et al., 2005). Considering the earlier *in vitro* studies demonstrating that 1) OCT3 could transport DA and 2) the specific transport via OCT3 could be inhibited by METH (Wu et al., 1998), these results suggest that decreased OCT3 might decrease the efflux of METH and/or DA from brain, subsequently causing the development of behavioral sensitization. Thus, in order to understand the function of brain OCT3, we performed behavioral and neurochemical analysis and pharmacokinetic experiments using OCT3-AS-treated rats. Here we found that intraventricular injection of OCT3-AS elevated the concentration of METH in the brain, subsequently enhancing dopaminergic neuronal transmission.

DA is one of the most important neurotransmitters showing the effects of METH since METH stimulates the release of DA (Wolf et al., 1994; Cador et al., 1999; Di Chiara et al., 2004; Brady et al., 2005). Thus, measuring METH-induced DA levels at dopaminergic neuronal terminals such as NAcc (Wolf et al., 1994; Cador et al., 1999; Di Chiara et al., 2004; Brady et al., 2005) and PFC (Shoblock et al., 2003, 2004) is ideal to understand the METH-induced behavioral responses.

Moreover, it has been reported that NAcc and PFC are important sites for the reinforcing effects (Banks et al., 1995) and psychostimulant effects (Beyer et al., 1999; Shoblock et al., 2003) of amphetamines. Thus, microdialysis analysis study to measure DA was performed in NAcc and PFC. The METH-induced increases in extracellular DA concentrations in NAcc were significantly increased in OCT3-AS-treated rats. Similarly, METH-induced DA levels in PFC were significantly increased in OCT3-AS-treated rats. These results suggest that the enhancement of METH-induced locomotor responses in OCT3-AS-treated animals would be caused by the increase of DA levels in NAcc and/or PFC by the decreased expression of OCT3.

In order to comprehend METH-induced neurochemical and behavioral responses, it is likely to be important to monitor METH levels in the brain since the degree of METH-induced hyperlocomotion was in proportion to extracellular levels of METH (Riviere et al., 2000) and METH-induced DA in NAcc (Gentry et al., 2004; Wallace et al., 1999) when administered METH systemically. Alternatively, there is a case in which METH levels in the striatum were altered even though the systemic dosage of METH was the same (Kitaichi et al., 2003). Thus, we measured METH concentration in NAcc and PFC in OCT3-AS-treated rats; moreover, considering that OCT3-AS inhibited OCT3 expression in circumventricular areas, METH levels in CSF were also monitored. The results revealed that the concentrations of METH in NAcc were significantly

increased in OCT3-AS-treated rats; moreover, METH in CSF was significantly increased in OCT3-AS-treated rats. These results suggest that the accumulation of METH in CSF could be caused by the decline in the elimination of METH from CSF to blood circulation due to decreased OCT3 in CP epithelial cells by OCT3-AS, and that decreased OCT3 elevated concentration of METH in NAcc, subsequently causing the enhancement of DA transmission.

It is interesting to note that earlier studies by Ogawa et al. (Ogawa et al., 1994) using a β-lactam antibiotic, benzylpenicillin, were partially similar to our present findings. According to their report, the efflux transport of benzylpenicillin through the CP was the major part of the total elimination clearance of benzylpenicillin (64%), while the remainder was accounted for by CSF convective flow (12%) and elimination via the brain parenchyma followed by efflux across the brain capillaries (24%). Considering their assumptions, it is expected that the efflux of METH to brain parenchyma was increased due to the decreased elimination of METH across the CP, subsequently increasing METH levels in NAcc.

Moreover, an earlier study by Riviere et al. (Riviere et al., 2000) showed that the METH concentration-time course curve was not parallel between the brain and serum, and the brain to serum concentration ratio was raised during the first 2 h after systemic METH administration. These data suggested that the brain distribution of METH was not only dependent on cerebral blood

flow, but also on METH lipid solubility. The factor(s) that affect METH distribution in the brain are not yet well understood; however, considering the present results, OCT3 in CP epithelial cells might play an important role in eliminating METH from the brain and regulate the distribution of METH in the brain.

In contrast to NAcc, the levels of METH in PFC were unchanged in OCT3-AS-treated rats (data not shown). One possibility is that PFC is anatomically away from ventricular areas if compared to NAcc. Thus, passively-diffused METH from ventricular areas to NAcc is likely critical to modulate METH levels in NAcc rather than in PFC. Additionally, we expect the possibility that the observed effect of OCT3-AS on increases METH-induced DA levels in NAcc and PFC is due to combination of increased DA release by METH and decreased clearance of DA from the two areas, since it has been reported that OCT3 can transport DA (Wu et al., 1998). And our previous data showed the treatment of neither antidepressant drug, imipramine (IMI) (4 mg/kg, i.p.) nor OCT3-AS (0.075 μg/0.25μl/h) reduced immobility time in forced swimming test, whereas the combination of IMI (4 mg/kg, i.p.) and OCT3-AS (0.075 μg/0.25μl/h) or single treatment of OCT3-AS (0.25 μg/0.25μl/h) significantly decreased immobility time, suggesting the blockage of OCT3 effectively increased extracellular levels of monoamine (Kitaichi et al., 2005). Therefore, it is plausible that METH-induced increases of DA levels in NAcc and PFC was due to the decreased

OCT3-mediated clearance of DA via CP.

In summary, we confirmed that OCT3-AS could enhance METH-induced hyperlocomotion in rats, similar to mice (Kitaichi et al., 2005). The measurement of DA and METH in brain extracellular areas revealed that OCT3 expressed in CP epithelial cells could regulate the brain distribution of METH and/or DA probably as an efflux transporter for METH from the brain to blood circulation and for DA from DA-rich brain extracellular areas to OCT3-expressed cells, subsequently elevating METH-induced DA in NAcc. Thus, it would be expected that OCT3 is an important molecule regulating METH-induced dopaminergic neuronal functions in the brain. Considering the recent report that other abusing drug/psychotomimetics such as 3,4-methylenedioxymetamphetamine and phencyclidine could be inhibited by OCT3 (Amphoux et al., 2006), selective ligands for OCT3 would be expected to treat neuronal disorders related to monoaminergic neuronal transmission such as depression, drug abuse and schizophrenia.

4. Experimental procedures

4.1. Reagents

Methamphetamine hydrochloride (METH) was purchased from Dainippon Pharmaceutical (Osaka, Japan). Anti-rat OCT3 antibody was purchased from Transgenic Inc. (Kumamoto, Japan). The OCT3 antibody was generated against an extracellular domain peptide sequence (KGIALPETVEDVEK) by Dr. Masato Inazu (Tokyo Medical Univ., Tokyo, Japan) and Transgenic Inc. (Kumamoto, Japan). All other chemicals were obtained commercially and used without further purification.

4.2. Animals

The animals were male Wistar rats (Nippon SLC, Hamamatsu, Japan), weighing between 280 and 300 g. The rats were housed under controlled environmental conditions (temperature of 23°C ± 1°C and humidity of 55% ± 5%) with a commercial food diet and water freely available. All animal experiments were performed according to the guidelines of Nagoya University School of Medicine for the care of laboratory animals.

4.3. Antisense Oligonucleotides

The 21-mer phosphorothioate-modified antisense oligodeoxynucleotides against OCT3 (OCT3-AS) were designed from the cDNA sequences of OCT3 (nucleotides -3 to +18, GenBank Database accession **NM019230**) targeting the area spanning the initiation codon (5' TGGTCGAACGTGGGCATGGTG 3'). Phosphorothioate-modified oligodeoxynucleotides with the same proportion of DNA bases as OCT3-AS but in a scrambled order (5' TAGTCGGAGGTGAGCGGCGTT 3', OCT3-SCR) were also prepared as controls. The selected sequences had little or no homology to any other known cDNA sequences registered in the GenBank Database (NIH, Bethesda, MD). OCT3-AS and OCT3-SCR were synthesized, purified and lyophilized by Sigma-Aldrich Japan K.K. Genosys Division (Ishikari, Hokkaido, Japan). OCT3-AS and OCT3-SCR were dissolved in filtered sterile buffered Ringer solution (140 mM NaCl, 3.0 mM KCl, 1.5 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, pH 7.4). Rats that were continuously infused with filtered sterile buffered Ringer solution by osmotic minipumps were used as a vehicle-treated control group (Kitaichi et al., 2005).

4.4. Surgery

Under pentobarbital (25 mg/kg, i.p.) anesthesia, all groups of rats were implanted an infusion cannula (Alza, Palo Alto, CA) with osmotic minipumps (model 1003D, Alza, Palo Alto, CA) containing Ringer, OCT3-AS or OCT3-SCR (1 μg/μl) solutions into the third ventricle: A -1.8 mm (anterior to bregma), V -4.3 mm (from the skull) according to the atlas of Paxinos and Watson (Paxinos et al., 1997) (Fig. 1A). The osmotic minipump was continuously infused at a rate of 1 μg/μl/h until the end of the experiments. The dose and infusion period of OCT3-AS and OCT3-SCR did not produce any abnormal behavior or weight loss.

At the same time of implantation for infusion cannula, the surgery for implanting guide was undertaken. For *in vivo* microdialysis experiment of DA or METH, a guide cannula (AG-8, EICOM, Kyoto, Japan) was implanted in the nucleus accumbens (NAcc) (A +1.5 mm, L +1.5 mm (lateral from the midsagittal suture), V -6.2 mm) or prefrontal cortex (PFC) (A +3.2 mm, L +0.6 mm, V -2.7 mm), according to the stereotaxic atlas (Fig. 7B,C). The guide cannula was fixed to the skull with cranioplastic cement (Narita et al., 2004; Ago et al., 2005). For METH pharmacokinetic experiment, the right jugular vein of each rat was also cannulated with a polyethylene tube for blood sampling. The implantation of cannulas in NAcc and PFC was done in separate animals.

4.5. Immunohistochemistry

Rats under light anesthesia with pentobarbital (25 mg/kg) were killed by exsanguination 3 days after infusion. The brain was immediately removed, and a tissue sample was fixed in 10% formaldehyde in PBS (pH 7.2). Fixed tissue specimens were embedded in paraffin wax, and 3 μm thick sections were cut with a microtome. For immunostaining, paraffin was removed, and endogenous peroxidase was blocked with methanol-H_2O_2, and nonspecific binding sites with 10% normal goat serum (Nichirei, Tokyo, Japan) for 20 min. Sections were washed and visualized by peroxidase activity using diaminobenzidine (DAB substrate kit, Vector Laboratories, Burlingame, CA). The specificity of the OCT3 immunostaining was confirmed by the disappearance of OCT3 positive signals with incubation of anti-OCT3 antibody and control peptide (KGIALPETVEDVEK) overnight at 4°C.

For image analysis of OCT3 immunostaining, digitized images were captured at a resolution of 1360 × 1024 pixels with digital camera at a magnification of × 400, and the mean density of OCT3-positive signals was measured by NIH Image. The upper and lower threshold density ranges were adjusted to encompass and match the immunoreactivity to provide an image with immunoreactive material appearing in black pixels, and non-immunoreactive material as white pixels. Non-immunoreactive density points were eliminated for each image. The area and density of

the standard area within the same threshold representing immunoreactivity were calculated and the integrated density was the product of the area and density (Narita et al., 2005).

4.6. Methamphetamine-induced hyperlocomotion

Psychostimulant-induced hyperlocomotion was evaluated as reported elsewhere (Vanderschuren et al., 1999; Kitaichi et al., 2003). Briefly, rats were administered continuously with vehicle, OCT3-AS or OCT3-SCR, as described above. After three days of infusion, rats were individually habituated to plastic cages (30 cm × 45 cm × 30 cm) for 2 h. After habituation, spontaneous locomotor activity was measured for 2 h before METH administration. METH (1 mg/kg, s.c.) was administered and locomotor activity was monitored for 3 h at 30 min intervals. Locomotor activity was automatically measured using electrical digital counters with infrared cell sensors on the walls (SCANET SV-20, Melquest Inc., Toyama, Japan) before and after giving METH.

4.7. In vivo microdialysis

At 3 days after surgery, a dialysis probe (AI-8-2; 2 mm membrane length, EICOM, Kyoto, Japan) was inserted through a guide cannula in NAcc or PFC and perfused at a flow rate of 2 μl/min with artificial cerebrospinal fluid (aCSF) containing 148 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 0.85 mM $MgCl_2$. After a 2 h equilibration period, sample collection began. Outflow fractions were

collected every 20 min. After three baseline fractions were collected, rats were injected with METH (1 mg/kg, s.c.). Sampling continued for 240 min after METH injection. Dialysis fractions were then analyzed using high performance liquid chromatography with an electrochemical detection system (HPLC-ECD) (HTEC-500, EICOM) and Power Chrom (EICOM). Briefly, HPLC-ECD consisted of 2.1 × 150 mm SC-5ODS column (EICOM) maintained at 25°C. The mobile phase was 0.1 M acetate-citric acid buffer (pH 3.9) containing 17% methanol, 140 mg/L sodium octyl sulfate and 5 mg/L EDTA-2Na, delivered at 0.23 ml/min by the HPLC-ECD system (Narita et al., 2004).

4.8. Pharmacokinetic analysis of Methamphetamine

A dialysis probe (AI-8-2) was inserted into rats through a guide cannula in NAcc and PFC, and perfused at a flow rate of 2 μl/min with aCSF. After a 2 h equilibration period, rats were treated with METH (5 mg/kg, s.c.), and dialysis fractions were collected for 2 h at 1 h intervals. Blood samples were collected at 30 min intervals after METH injection. Two hours after METH injection, rats were anesthetized with diethyl ether, and 100 μl of CSF was withdrawn from the cisterna magna through sterilized polyethylene tubes. Blood was then withdrawn from the abdominal aorta and the brain was removed immediately. Samples were stored at -80°C until analyzed. The dosage of METH and schedule for collecting of CSF samples were decided due to the measurement

limitation of METH concentration by HPLC analysis.

METH concentration was determined by HPLC, and then 100 μl of samples were vortexed with 100 μl of 10% sodium hydroxide, 50 μl of water containing β-phenylethylamine (2.5 μg/ml) as an internal standard and 1.2 ml of ethyl acetate. After deproteinization by centrifugation at 12,000g for 5 min, the upper layer was collected and evaporated to dryness under a stream of nitrogen gas at 45°C. The dried residues were reconstituted with 100 μl of 10 mM sodium carbonate-sodium bicarbonate buffer (pH 9.0) and 100 μl of 2 mM dansyl chloride. Finally, samples were prepared for HPLC by heating to 45°C for 1 h in the dark to derivatize METH and β-phenylethylamine to dansyl-METH and dansyl-β-phenylethylamine, respectively. Standard curves for measuring METH in plasma, brain tissues, dialysates and CSF samples proved to be linear for concentrations ranging from 0.0625 to 2 μg/ml of METH dissolved in pooled plasma, brain tissue homogenates derived from vehicle rats or aCSF for dialysates and CSF with a correlation coefficient of 0.999. It should be noted that endogenous β-phenylethylamine in samples is unlikely to affect the HPLC measurement of METH since the amount of β-phenylethylamine added as an internal standard is over 100-fold higher than that found in the brain.

The apparatus used for HPLC consisted of an LC-10A system (Shimadzu, Kyoto, Japan) equipped with a fluorescence detector (RF-10AXL; Shimadzu) (emission wavelength: 530 nm;

excitation wavelength: 343 nm), consisting of an LC-10A liquid pump and an SIL-10A autoinjector. The condition was as follows: column, a Cosmocil $5C_{18}$ column (4.6 by 150 mm; Nacalai Tesque, Kyoto, Japan); column temperature (CTO-10AC thermometer; Shimadzu), 40°C; mobile phase, water/acetonitrile (40:60 v/v) containing 1 mM imidazole; flow rate, 1 ml/min. The assay was shown to be linear for the concentrations measured, with correlation coefficients of more than 0.99 (Zametkin et al., 1984; Paterson et al., 1990; Kitaichi et al., 2003).

4.9. Statistical analysis

All data are expressed as the means ± S.E.M. Two-way analysis of variance (two-way ANOVA) with repeated measures was used to test for a drug-over-time effect on METH-induced locomotor activity and *in vivo* microdialysis experiment. One-way ANOVA was used to test for differences between groups in other experiments. When the ANOVA F ratios were significant ($p<0.05$), post-hoc analysis was done using Scheffe's test. All statistics reported in these experiments were generated using StatView (version 4.5, Abacus Concepts, Berkeley, CA), and *p*-values less than 0.05 were considered statistically significant.

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Figure legends

Figure 1. Immunohistochemical analysis of OCT3 in rat lateral ventricle (LV). The OCT3 positive signals in LV were detected in CP epithelial cells (white arrowheads) and ependymal cells (filled arrowheads) (A,B). The insert images indicate the higher magnification in black line box (B,D). The OCT3 positive signals were eliminated by incubation of anti-OCT3 antibody with OCT3 blocking peptide (C,D). Scale bars represent 200 μm (A,C), 20 μm (B,D).

Figure 2. (A) The effect of OCT3-AS on the expression of OCT3 in CP epithelial cells. Three days after the continuous infusion of vehicle, OCT3-AS, OCT3-SCR (1 μg/μl/h) into the third ventricle (3V), brain were removed and an immunohistochemical study was performed. Scale bars represent 50 μm. (B) The relative density of OCT3-positive signals was measured by NIH Image. Data represent the mean ± S.E.M. *$p<0.05$ vs. corresponding vehicle, †$p<0.05$ vs. corresponding OCT3-SCR (Scheffe's post-hoc test). Number in parentheses represent the number of animals in each group.

Figure 3. (A) Time-course effect of OCT3-AS on spontaneous and METH-induced

hyperlocomotion in rats. Three days after the continuous infusion of vehicle, OCT3-AS, and OCT3-SCR (1 μg/μl/h) into the third ventricle, locomotor activity was assessed. Spontaneous locomotor activity was measured for 120 min prior to METH injection. METH (1 mg/kg, s.c.)-induced hyperlocomotion was measured for a further 180 min. The arrow at 0 min indicates METH administration. Data represent the mean ± S.E.M. of locomotor activity at 30 min intervals. *p<0.05, **p<0.01 vs. corresponding vehicle, †p<0.05, ††p<0.01 vs. corresponding OCT3-SCR (Scheffe's post-hoc test). (B) Sum of spontaneous and METH-induced hyperlocomotion. Data in (A) were summed as spontaneous locomotor activity (from −120 to 0 min) and METH-induced hyperlocomotion (from 0 to 180 min). Data represent the mean ± S.E.M. *p<0.001 vs. corresponding vehicle, †p<0.01 vs. corresponding OCT3-SCR (Scheffe's post hoc test). Numbers in parentheses represent the number of animals in each group.

Figure 4. Time course effect of OCT3-AS on spontaneous and METH-induced increases in extracellular DA concentrations in NAcc (A) and PFC (C). Three days after the continuous infusion of vehicle, OCT3-AS, OCT3-SCR (1 μg/μl/h) into the third ventricle, *in vivo* microdialysis was performed. Following the subsequent collection of three baseline fractions, rats were treated with METH (1 mg/kg, s.c.). METH-induced levels of DA was measured for another 180 min. The arrow

at 0 min indicates the administration of METH. Data represent the mean ± S.E.M. of DA levels with a 20 min interval. Sum of spontaneous (from -80 to 0 min) and METH-induced (from 0 to 240 min) levels of DA in NAcc (B) and PFC (D). Each column represents the mean ± S.E.M. *p<0.05 vs. corresponding vehicle, †p<0.05 vs. corresponding SCR (Scheffe's post-hoc test). Numbers in parentheses represent the number of animals in each group.

Figure 5. Typical chromatographic charts of METH in blank plasma of rats (A), blank plasma of rats spiked with 20 ng of METH (B) and plasma of rats treated with METH (5 mg/kg) (C). Closed circle: internal standard (β-phenylethylamine); closed triangle: METH. Scale Bar indicates 10 min.

Figure 6. The plasma (A) and brain dialysate (B) concentration-time profile of METH in vehicle, OCT3-SCR and OCT3-AS rats. After METH administration (5 mg/kg, s.c.), blood samples were collected every 30 min and brain dialysate fractions were collected every 1 h. (C) METH concentration in CSF in rats treated with vehicle, OCT3-AS, OCT3-SCR 2 h after METH administration (5 mg/kg, s.c.). Data represent the mean ± S.E.M. *p<0.05, **p<0.01 vs. vehicle, †p<0.05, ††p<0.01 vs. OCT3-SCR (Scheffe's post-hoc test). Numbers in parentheses represents the number of animals in each group.

Figure 7. Localization of continuous infusion cannula and microdialysis probe sites in the NAcc and PFC in rats. The shaded area represents (A) continuous infusion cannula and (B,C) microdialysis probe-inserted regions in the rat brain. The schematic brain sections are from the atlas of Paxinos and Watson (1997).