Contribution of Tissue Plasminogen Activator on the Methamphetamine-induced Modulation of Dopamine Neuron Focused on Sensitization and Neurotoxicity

A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy in Biological Science
(Doctoral Program in Biological Sciences)

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Abbreviations

aCSF:	artificial cerebrospinal fluid
ANOVA:	analysis of variance
BDNF:	brain-derived neurotrophic factor
CNS:	central nervous system
CPP:	conditioned place preference
DAT:	dopamine transporter
ECM:	extracellular matrix
GDNF:	glial-derived neurotrophic factor
LTP:	long-term potentiation
METH:	methamphetamine
NAc:	nucleus accumbens
NMDA:	N-methyl-D-aspartate
PAR-1:	protease-activated receptor-1
PAR-1-/-:	PAR-1-deficient
PBS:	phosphate-buffered saline
TBS-T:	Tris-buffered saline-Tween20
TH:	tyrosine hydroxylase
TNF-α:	tumor necrosis factor-alpha
TNF-α-/-	TNF-α-deficient
tPA:	tissue plasminogen activator
tPA-/-:	tPA-deficient
VTA:	ventral tegmental area

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Abstract

Methamphetamine (METH) is a widely used addictive drug and its abuse is a serious problem in many countries. It is well-known that METH increases dopamine release in the nucleus accumbens. METH induces sensitization, augmentation of responses, and neurotoxicity during or after chronic treatment. Tissue plasminogen activator (tPA), a serine protease that converts plasminogen to plasmin, is known to play several important roles in the central nervous system. The objectives of this study were to establish whether the tPA-plasmin system contributes to METH-induced sensitization and neurotoxicity in the dopaminergic system.

To establish its role in sensitization, I investigated the involvement of the tPA-plasmin system in tPA-deficient (tPA-/-) mice using microdialysis. In addition, I examined whether protease activated receptor-1 (PAR-1) or laminin could be involved in tPA regulation. My results showed that the tPA-plasmin system contributes substantially to the development of METH-induced sensitization in dopaminergic neurons through laminin degradation by plasmin.

To test the involvement of the tPA-plasmin system in neurotoxicity, I evaluated the changes in known markers of dopaminergic neurons in tPA-/- and PAR-1-deficient (PAR-1-/-) mice. Additionally, rectal temperature was assessed in both tPA-/- and PAR-1-/- mice. My results suggest that neither tPA nor PAR-1 is involved in METH-induced neurotoxicity in the dopaminergic neurons.

In conclusion, the tPA-plasmin system contributes to METH-induced sensitization but not METH-induced neurotoxicity in dopaminergic neurons. These findings would be helpful to develop novel medicines to treat METH addiction.

General Introduction

It is well-known that one in eight deaths occur due to the abuse of addictive substances such as tobacco, alcohol and other drugs, and chronic drug use can cause long-term changes in the brain (Humphreys et al., 2017).

Methamphetamine (METH), one of the most widely used addictive drugs worldwide, is a cause of serious public health concern in many countries. It is well-established that METH acts by inhibiting dopamine re-uptake through the dopamine transporter (DAT), in turn increasing extracellular dopamine levels in the synaptic clefts (Morley et al., 2017). In humans, METH induces diverse behavioral and physiological changes such as wakefulness, hyperactivity, auditory hallucination, psychosis, hyperthermia, and in extreme cases, death (Moszczynska et al., 2017). Although, METH-associated psychological dependence and chronic use is a serious issue, currently, there is no treatment for METH dependence. At best, improvement of symptoms prevents patients from further addiction. In other words, a medication for METH abuse is anticipated to solve an important social issue as well.

It is known that acute treatment with METH increases locomotor activity, a representative METH-associated behavioral response in experimental animals, and augmentation of dopamine release in the nucleus accumbens (NAc) plays a critical role in changes in locomotor activity associated with METH (Kalivas et al., 1988). Chronic treatment with METH leads to a long-lasting promoted behavioral response, a phenomenon defined as sensitization (Steketee et al., 2011). This enhanced locomotor activity is accompanied by increased dopamine release in the NAc (Robinson et al., 1988). In other words, chronic treatment with METH leads to sensitization of both

behavioral response and dopamine release in the NAc. This sensitization phenomenon associated with METH reflects the observed clinical symptoms in METH-dependent patients (Ujike et al., 2004).

Furthermore, high dose of METH induces neurotoxicity in dopaminergic neurons in the striatum of humans (Chang et al. 2007; Volkow et al., 2001) and experimental animal models (Cadet et al., 2005). In humans, positron emission tomography and single-photon emission computed tomography studies have shown that chronic use of high-dose METH can induce downregulation of DAT (London et al., 2015). In experimental animal models, the decrease in dopaminergic neuron-specific markers tyrosine hydroxylase (TH) or DAT were observed with high doses of METH administration in the striatum (O'Callaghan et al., 1994; Deng et al., 2007).

Although METH-induced sensitization and/or neurotoxicity undermines the normal function of the dopaminergic system, the underlying mechanisms of action, downstream to dopamine, is not yet established. Consequently, there are no approved therapies and/or medications available for the treatment of METH-dependent patients. Considering that METH abuse has a huge impact on the social costs and public health in many countries, detailed analyses of the neurobiology behind METH-dependency and drug discovery for METH-dependent patients are matters of urgency. Fortunately, the development of therapeutic medications can be pursued through the investigation of the effects of METH on rodents because common phenotypes (i.e. sensitization and neurotoxicity) exist between humans and these experimental animals.

Tissue plasminogen activator (tPA) is a known serine protease with a crucial role in the fibrinogenolysis system. tPA converts plasminogen (proenzyme) to plasmin (broad-specificity proteinase), which in turn degrades fibrin and dissolves the blood

clots (Collen, 1999). tPA-plasmin system is known to have a critical role in blood fibrinogenolysis and in the central nervous system (CNS) as signaling molecules.

In the present study, I investigated the relationship between METH-induced changes in dopaminergic neurons and the tPA-plasmin system in the brain. In chapter 1, sensitization, as an important aspect of METH-induced changes in dopaminergic neurons, is examined by using *in vivo* microdialysis technique. In chapter 2, neurotoxicity is assessed. These studies demonstrate that the tPA-plasmin system contributes to development of sensitization through dopamine release induced by chronic METH treatment, but not METH-induced neurotoxicity in dopaminergic neurons.

Chapter I: Methamphetamine-induced sensitization of dopamine release

1. Introduction

The mesolimbic dopaminergic system from the ventral tegmental area (VTA) to the NAc plays a crucial role in drug dependence (Wise, 1996; Koob et al., 1998; Mizoguchi et al., 2004; Nestler, 2005). METH, one of the most abused drugs in the world, increases extracellular dopamine levels in the NAc by reversing the DAT, which is associated with the reinforcing effect of METH (Seiden et al., 1993; Giros et al., 1996; Nakajima et al., 2004). In rodents, augmentation of behavioral responses to METH is observed during and after repeated administration. This form of behavioral plasticity, called behavioral sensitization, is regarded as an animal model for the intensification of drug craving in human addicts (Robinson and Berridge, 2003) and for METH-induced psychosis (Sato et al., 1983, 1992). In parallel with behavioral sensitization, repeated METH administration enhances the dopamine release-stimulating effect of the drug in the NAc (O'Dell et al., 1991; Suzuki et al., 1997; Narita et al., 2004).

tPA, a serine protease which catalyzes the conversion of plasminogen to plasmin, plays an important role in the central nervous system. Accumulating evidence has demonstrated that tPA is involved in synaptic plasticity and remodeling, directly by itself or indirectly through plasmin. For instance, tPA is directly involved in long-term potentiation (LTP) by acting on low-density lipoprotein receptor-related proteins (Zhuo et al., 2000) and NMDA receptors (Nicole et al., 2001). On the other hand, neurite outgrowth (Krystosek and Seeds, 1981), cell migration (Moonen et al., 1982; Seeds et al., 1999), and amyloid-β degradation induced by tPA (Melchor et al., 2003; Tucker et

al., 2000) are mediated by plasmin. In addition, recent studies have demonstrated the role of tPA in the regulation of neurotransmitter release. Thus, depolarization-evoked dopamine release in the NAc (Ito et al., 2006) as well as norepinephrine release from hearts (Schaefer et al., 2006) are diminished in tPA-deficient (tPA-/-) mice compared with wild-type mice. Nagai et al. (2004, 2005 and 2006) have demonstrated that tPA plays an important role in the rewarding effects of abused drugs, including METH, morphine and nicotine, through the activation of plasminogen to plasmin, and that plasmin modulates morphine- and nicotine-induced dopamine release in the NAc (Nagai et al., 2004, 2005a, 2005b, 2006).

Regarding the molecular targets of the tPA-plasmin system, protease-activated receptor-1 (PAR-1) is activated by plasmin (Kuliopulos et al., 1999) and plasmin-induced migration requires signaling through PAR-1 (Majumdar et al., 2004). Nagai et al. (2006) and Ito et al. (2007) have demonstrated that PAR-1 is involved in the enhancement of nicotine-induced dopamine release by the tPA-plasmin system (Nagai et al., 2006) as well as morphine-induced dopamine release and hyperlocomotion (Ito et al., 2007). Alternatively, the degradation of laminin, one of the major components of extracellular matrix (ECM), by plasmin is important in the maintenance of LTP in organotypic hippocampal cultures (Nakagami et al., 2000) and excitotoxin-induced neuronal cell death in the hippocampus (Chen and Strickland, 1997).

In a previous study, Nagai et al. (2005) and Yamada et al. (2005) have demonstrated that repeated METH treatment dose-dependently induced tPA mRNA expression as well as enzyme activity in the NAc, whereas acute METH treatment had no effect. Although there was no difference in acute METH-induced hyperlocomotion between wild-type and tPA-/- mice, METH-induced conditioned place preference and behavioral

sensitization after repeated METH treatment were significantly reduced in tPA-/- mice compared with wild-type mice. The defect of behavioral sensitization in tPA-/- mice was reversed by microinjection of exogenous tPA into the NAc (Nagai et al., 2005b; Yamada et al., 2005). These results suggest that tPA plays a role in the development of behavioral sensitization induced by repeated METH treatment, but the underlying mechanism remains to be determined.

In the present study, I investigated whether the tPA-plasmin system participates in the sensitization of METH-induced dopamine release in the NAc of mice after repeated treatment. Furthermore, I examined whether PAR-1 activation or laminin degradation is involved in modulation by the tPA-plasmin system of dopamine release in the NAc after repeated METH treatment.

2. Materials and Methods

2.1. Animals

Male ICR mice (7 weeks old) were obtained from Japan SLC Inc. (Shizuoka, Japan). Wild-type (C57BL/6J) , tPA-/- (stock number 002508, Carmeliet et al., 1994) and PAR-1-deficient (PAR-1-/-) (stock number 002862, Connolly et al., 1996) mice were provided by the Jackson Laboratory (Bar Harbor, ME, USA), and the presence or absence of either PAR-1, tPA, or the neomycin cassette was verified according to the manufacturer's instructions. When comparing the wild-type and knock-out forms, only congenic animals were used. The animals were housed in plastic cages and kept in a regulated environment (23 ± 1˚C, 50 ± 5% humidity), with a 12/12 hr light-dark cycle (lights on at 9:00 A.M.). Food (CA1; Clea Japan Inc., Tokyo, Japan) and tap water were available ad libitum.

All animal care and use were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Kanazawa University.

2.2. METH treatment

METH hydrochloride (Dainihon Pharmaceutical Co. Ltd., Osaka, Japan) was dissolved in physiological saline. For acute METH treatment, mice were given a subcutaneous injection of METH (1 mg/kg). For repeated METH treatment, animals were subjected to a 5-day regimen in which METH (1 mg/kg) was injected subcutaneously once a day for 5 days. Control animals were given the same volume of saline under the same injection schedule as used for acute and repeated administrations of METH.

2.3. In vivo microdialysis

Animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and a guide cannula (MI-AG-6, Eicom Corp., Kyoto, Japan) was implanted in the NAc (+1.5 mm anteroposterior, +0.8 mm mediolateral from the bregma, -4.0 mm dorsoventral from the skull) according to the mouse brain atlas (Franklin and Paxinos, 1997). For repeated METH treatment (Fig. 1-1), mice were given a subcutaneous injection of METH (1 mg/kg) or saline for 4 days one or two days after the operation. One day after the last injection of repeated METH or saline, microdialysis was performed. For acute METH treatment (Fig. 1-2 and 1-3), microdialysis was performed one or two days after the operation. A dialysis probe equipped with a microinjection tube (MIA-6-1; 1 mm membrane length, Eicom Corp.) was inserted through the guide cannula, and perfused with artificial cerebrospinal fluid (aCSF; 147 mM NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$) at a flow rate of 1.0 μl/min (Nagai et al., 2004). The microdialysis probes were constructed of three stainless tubes, two silicatubes (inlet and outlet) for microdialysis with a 75 μm outer diameter and a microinjection silica tube with a 75 μm outer diameter. The microinjection tube was place in parallel with the tubes for microdialysis. The microinjection tube was half the length of the dialysis membrane. These three silica tubes were sealed together with epoxy resin, and each was secured with stainless steel tubing at the top of the probe.

Outflow fractions were collected every 20 min. Following the collection of three baseline fractions, human recombinant tPA (100 ng, provided by Eisai Co. Ltd., Tokyo, Japan) or human plasmin (100 ng, Chromogenix, Molndal, Sweden) dissolved in 1 μl of aCSF solution was injected during a 10-min period through the microinjection tube into the NAc (Nagai et al., 2005a). Ten min after the microinjection, METH (1 mg/kg, s.c.)

was administrated. Dopamine levels in the dialysates were analyzed using an HPLC system equipped with an electrochemical detector (Nagai et al., 2004; 2006).

2.4. Measurement of locomotor activity

Animals were treated with saline on day 1 and METH (1 mg/kg, s.c.) once daily for 7 days (from day 2 to day 8). Mice were habituated to a transparent acrylic cage (25 × 25 × 20 cm) for 120 min before the measurement of locomotor activity at 10-min intervals for 60 min using digital counters with an infrared sensor on days 1, 2, and 8 (Brainscience Idea Inc., Osaka, Japan) (Nagai et al., 2005b).

2.5. Western blotting

Striatal tissues containing NAc were homogenized in 0.1 M Tris-HCl (pH 7.2) containing 0.1% Triton X-100 and centrifuged at 10,000 × g at 4ºC for 20 min. Supernatants was incubated with either 0.3 μM tPA and 0.5 μM plasminogen (Chromogenix) or 1 μM plasmin at 37ºC for 30 min. Samples were subjected to 6% SDS-PAGE under reducing conditions, followed by transfer onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was blocked with 5% skim milk in Tris-buffered saline-Tween 20 (10 mM Tris-HCl (pH. 7.5), 100 mM NaCl, and 1% Tween-20), and incubated with rabbit anti-laminin antibody (1:2,000, Sigma, St. Louis, MO, USA) at 4ºC overnight. After incubation with a horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (1:5,000, GE Healthcare Bio-Science Corp., Piscataway, NJ, USA) at room temperature for 1 h, the immune complex was detected using ECL plus Western blotting detection reagents (GE Healthcare Bio-Science Corp.).

2.6. Immunohistochemistry

Thirty min after the injection of plasmin into the NAc, the animals were anesthetized with ether, and transcardially perfused with isotonic 0.1 M phosphate buffer (pH 7.4) followed by isotonic 4% paraformaldehyde. The brains were removed, post-fixed in 4% paraformaldehyde for 2 h and then cryoprotected in 30% sucrose in 0.1 M phosphate buffer. The brains were embedded in Tissue-Tek O.C.T. compound (Sakura Finetech) and stored at -80°C. Briefly, sections (14 μm) were fixed with 4% paraformaldehyde, and washed with 0.3% Triton X-100 in phosphate-buffered saline. They were blocked with 10% normal goat serum and 1% bovine serum albumin in PBS for 30 min, and incubated in rabbit anti-laminin IgG (1:30) at 4°C overnight. Sections were then incubated in Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200, Invitrogen Corp.) for 1 h. Samples were observed with a confocal microscope (model LSM510, Carl Zeiss).

2.7. Nissl stain

Mice were anesthetized with ether, and transcardially perfused with isotonic 0.1 M phosphate buffer (pH 7.4) followed by isotonic 4% paraformaldehyde. The brains were removed, post-fixed in 4% paraformaldehyde for 2 h and then cryoprotected in 30% sucrose in 0.1 M phosphate buffer. Sections (14 μm) were stained with 1% Cresyl violet. Samples were observed with a microscope (model Axioskop, Carl Zeiss).

2.8. Statistical analysis

All data are expressed as the mean ± SEM. In analysis of the time course of microdialysis, ANOVA with repeated measures was used and followed by the

Student-Newman-Keuls test when F ratios were significant ($p < 0.05$). Statistical differences in the analysis of laminin protein levels were determined using one-way ANOVA, followed by the Student-Newman-Keuls test when F ratios were significant ($p < 0.05$).

3. Results

3.1. Changes in METH-induced dopamine release in tPA-/- mice

First, to investigate whether the tPA-plasmin system is involved in the development of sensitization in METH-induced dopamine release, I measured the levels of extracellular dopamine in the NAc of wild-type and tPA-/- mice after acute or repeated METH treatment, using in vivo microdialysis (Fig. 1-1). Acute METH treatment at 1 mg/kg increased extracellular dopamine levels to 253 ± 32% and 235 ± 44% of the basal levels 40 min after treatment in wild-type and tPA-/- mice, respectively. There was no difference in acute METH-induced dopamine release between the two genotypes. Repeated METH treatment potentiated METH-induced dopamine release in wild-type mice (412 ± 39% of the basal levels), but not in tPA-/- mice (280 ± 45% of the basal levels). One-way ANOVA with repeated measures indicated significant effects of group [$F(3, 25) = 4.841$, $p < 0.01$] and time [$F(5, 125) = 34.961$, $p < 0.0001$], but not their interaction [$F(15, 125) = 1.621$, $p > 0.05$]. Post-hoc test indicated a significant difference between acute and repeated METH treatment in wild-type mice ($p < 0.01$) whereas no difference was seen in tPA-/- mice. Furthermore, dopamine responses in tPA-/- mice were significantly diminished compared with those in wild-type mice after repeated METH treatment ($p < 0.05$). These results suggest that tPA is involved in the development of sensitization on METH-induced dopamine release after repeated treatment.

3.2. Effect of tPA and plasmin on acute METH-induced dopamine release in the NAc of ICR mice

Nagai et al. (2005) have previously demonstrated that repeated, but not acute, treatment with METH increased the enzyme activity of tPA in the NAc (Nagai et al.,

2005b). Accordingly, to clarify the tPA-dependent mechanism underlying the development of sensitization in METH-induced dopamine release, I studied the effects of microinjections of exogenous tPA or plasmin into the NAc on acute METH-induced dopamine release in the NAc of ICR mice (Fig. 1-2). Acute METH-induced dopamine release (226 ± 29% of the basal levels at 40 min in the vehicle-treated group) was significantly potentiated by a prior microinjection of recombinant tPA into the NAc (344 ± 55% of the basal levels at 40 min in the tPA-treated group) as the response observed after repeated METH treatment (Fig. 1-2a). One-way ANOVA with repeated measures revealed significant effects of group [$F(1, 16) = 5.382$, $p < 0.05$] and time [$F(5, 80) = 6.181$, $p < 0.0001$], but not their interaction [$F(5, 80) = 0.914$, $p > 0.05$]. Thus, it is possible that an increase in tPA expression in the NAc after repeated METH treatment may contribute to the development of sensitization in METH-induced dopamine release. Similarly, microinjection of plasmin (100 ng) into the NAc enhanced acute METH-induced dopamine release (394 ± 60% of the basal levels) (Fig. 1-2b). One-way ANOVA with repeated measures revealed significant effects of group [$F(1, 13) = 4.891$, $p < 0.05$] and time[$F(5, 65) = 7.463$, $p < 0.0001$], but not the interaction of group with time [$F(5, 65) = 1.331$, $p > 0.05$]. These results suggest that tPA potentiates METH-induced dopamine release in the NAc, through the conversion of plasminogen to plasmin.

3.3. PAR-1 is not involved in METH-induced dopamine release

In previous studies, Nagai et al and Ito et al demonstrated that the tPA-plasmin system potentiates nicotine- and morphine-induced dopamine release in the NAc by activating PAR-1 expressed on dopaminergic nerve terminals (Nagai et al., 2006; Ito et

al., 2007). Therefore, I examined whether PAR-1 is involved in the potentiation of METH-induced dopamine release by plasmin, using PAR-1-/- mice. Microinjection of plasmin into the NAc significantly potentiated acute METH-induced dopamine release both in wild-type and PAR-1-/- mice (Fig. 1-3). There was no difference between wild-type and PAR-1-/- mice in METH-induced dopamine release in the presence or absence of pretreatment with plasmin. One-way ANOVA with repeated measures indicated significant effects of group [$F(3, 19) = 3.392$, $p < 0.05$] and time [$F(5, 95) = 9.440$, $p < 0.01$], but not their interaction [$F(15, 95) = 1.033$, $p > 0.05$].

I also compared repeated METH-induced behavioral sensitization in PAR-1-/- mice with that in wild-type mice. As shown in Table 1, there was no difference between wild-type and PAR-1-/- mice in either acute METH-induced hyperlocomotion or repeated METH-induced behavioral sensitization. Collectively, it is unlikely that PAR-1 plays a significant role in the development of sensitization in dopamine release and hyperlocomotion induced by repeated METH treatment.

3.4. Effect of plasmin on laminin degradation *in vitro* and *in vivo*

To explore the possible mechanism underlying the plasmin-induced potentiation of acute METH-induced dopamine release in the NAc, I examined the changes in laminin contents in brain tissue after treatment with plasmin in vitro. Treatment of striatal tissues with either tPA or plasminogen alone had no effect, but the combination significantly reduced laminin levels [laminin α1 subunit; $F(3, 12) = 27.199$, $p < 0.01$ (Fig. 1-4a, b), laminin β1γ1 subunits; $F(3, 12) = 34.719$, $p < 0.01$ (Fig. 1-4a, c)]. Similarly, incubation of brain tissues with plasmin decreased laminin levels in a concentration-dependent manner [laminin α1 subunit; $F(2, 9) = 68.48$, $p < 0.01$ (Fig.

1-4d, e), laminin β1γ1 subunits; $F(2, 9) = 54.706$, $p < 0.01$ (Fig. 1-4d, f)]. These results suggest that plasmin produced by tPA from plasminogen can degrade laminin in brain tissue in vitro.

Lastly, I examined the degradation of laminin in the NAc after plasmin treatment in vivo. Immunohistochemical analysis indicated that laminin-like immunoreactivity in the NAc was markedly reduced 30 min after the microinjection of plasmin into the NAc (Fig. 1-5a) compared with the vehicle-injected control group (Fig. 1-5b). Nissl staining indicated no apparent cell damage after plasmin treatment (Fig. 1-5c and d).

4. Discussion

In the present study I demonstrated that repeated METH treatment in wild-type mice resulted in the development of sensitization in METH-induced dopamine release in the NAc whereas such sensitization of dopamine release was not evident in tPA-/- mice. Since there was no difference in acute METH-induced dopamine release between wild-type and tPA-/- mice, it is unlikely that there is a mechanism defect of METH-induced DAT-mediated dopamine release in the NAc (Seiden et al., 1993; Giros et al., 1996; Nakajima et al., 2004). Rather, it is likely that tPA plays a role in the development of sensitization in METH-induced dopamine release. The present findings are consistent with the previous findings that behavioral sensitization after repeated METH treatment was attenuated in tPA-/- mice, although there was no difference in acute METH-induced hyperlocomotion between wild-type and tPA-/- mice (Nagai et al., 2005b). It should be noted that the levels of tPA mRNA and its enzyme activity in the NAc were markedly increased after repeated METH treatment, although acute METH treatment had no effect (Nagai et al., 2005b). Taken together, it is suggested that the induction of tPA in the NAc after repeated METH treatment plays a role in the development of sensitization to METH.

In contrast to acute METH-induced dopamine release, Nagai et al. and Ito et al. have demonstrated that acute morphine- (Nagai et al., 2004) and nicotine-induced dopamine release (Nagai et al., 2006) as well as potassium depolarization-evoked dopamine release (Ito et al., 2006) are all diminished in tPA-/- mice compared with wild-type mice. One possible explanation for this discrepancy is that METH-induced dopamine release is a DAT-mediated Ca^{2+}-independent process (Nakajima et al., 2004), whereas dopamine release induced by morphine and nicotine is Ca^{2+}-dependent (Keren

et al., 1997; Harsing et al., 1992). However, the fact that microinjection of exogenous tPA and plasmin into the NAc can enhance METH-induced dopamine release does not support the aforementioned hypothesis. Rather, it is likely that the tPA-plasmin system can potentiate METH-induced, DAT-mediated and Ca^{2+}-independent dopamine release as it does in morphine-, nicotine and depolarization-induced dopamine release. An alternative explanation is that because acute METH treatment has no effect on tPA release and expression in the brain (Nagai et al., 2005b), acute METH-induced dopamine release is not affected by the deficiency of tPA. On the other hand, since acute treatment with morphine (Ito et al., 2007), nicotine (Nagai et al., 2006) and potassium depolarization (Gualandris et al., 1996) induces tPA expression and increases extracellular tPA activity in the brain, dopamine release is diminished in tPA-/- mice compared with wild-type mice.

Nagai et al. (2005) have reported that METH-induced locomotor sensitization is significantly attenuated in tPA-/- mice although there is no difference in acute METH-induced hyperlocomotion between wild-type and tPA-/- mice (Nagai et al., 2005b). Furthermore, dopamine release in the NAc is thought to play a crucial role in the locomotor-stimulating effects of drugs of abuse (Koob et al., 1998; Ito et al., 2007). Thus, it is suggested that tPA-plasmin system plays a role in the development of behavioral sensitization induced by repeated METH treatment through the regulation of processes underlying the sensitization of dopamine release in the NAc. I propose that the induction of tPA in the NAc following repeated METH treatment is a critical step for the development of sensitization in METH-induced dopamine release, leading to behavioral sensitization.

There are several potential targets of the tPA-plasmin system in the brain. For

example, tPA enhances NMDA receptor signaling by cleaving the NR1 subunit at the arginine 260 of the amino-terminal domain (Nicole et al., 2001; Fernández-Monreal et al., 2004). Thus, the NR1 subunit of NMDA receptors may be a possible target for the tPA-plasmin system to potentiate METH-induced dopamine release in the NAc. This assumption is consistent with observations that NMDA receptor antagonists prevent the development of locomotor sensitization to amphetamines (Karler et al., 1991; Stewart and Druhan, 1993; Wolf and Jeziorski, 1993). However, since plasmin leads to complete degradation of the amino-terminal domain of NR1, the proteolytic pattern being different from that of tPA (Fernández-Monreal et al., 2004), it is unlikely that NMDA receptor activation caused by cleavage of the NR1 subunit is involved in the sensitization of METH-induced dopamine release by the tPA-plasmin system.

Alternatively, it has been demonstrated that tPA, by activating plasmin, converts the precursor pro-brain-derived neurotrophic factor (BDNF) to mature BDNF, and that this conversion is critical for the expression of late-phase LTP (Pang et al., 2004). Furthermore, BDNF is implicated in METH-induced dopamine release and behavior effects (Narita et al., 2003). Thus, it is possible that BDNF is involved in the sensitization of METH-induced dopamine release by the tPA-plasmin system.

Regarding a target molecule for the tPA-plasmin system, I have demonstrated that plasmin activates PAR-1 expressed on the nerve terminals of dopaminergic neurons in the NAc. In addition, Nagai et al indicated that nicotine-induced dopamine release and reward was diminished in PAR-1-/- mice and that tyrTRAP7, a PAR-1 antagonist peptide, blocked the ameliorating effect of plasmin on the defect of nicotine-induced dopamine release in tPA-/- mice (Nagai et al., 2006). Moreover, the PAR-1 antagonist blocked the ameliorating effect of plasmin on the defect of morphine-induced dopamine

release in tPA-/- mice (Ito et al., 2007). Accordingly, I examined METH-induced dopamine release and locomotor sensitization in PAR-1-/- mice. As there was no difference in METH-induced dopamine release and locomotor sensitization, it is unlikely that PAR-1 is a target molecular for the tPA-plasmin system to potentiate METH-induced dopamine release.

I therefore focused on the role of laminin, an ECM protein susceptible to plasmin since previous studies indicated that laminin degradation by plasmin is involved in the maintenance of LTP in the hippocampus (Nakagami et al., 2000) and excitotoxin-induced cell death in the hippocampus (Chen and Strickland, 1997). Laminin levels in brain tissue containing the NAc were reduced by treatment with tPA plus plasminogen or plasmin *in vitro*. Furthermore, microinjection of plasmin into the NAc resulted in a marked decrease in laminin immunoreactivity without any apparent cell damage. These findings suggest that degradation of laminin may be involved in the potentiation of METH-induced dopamine release by the tPA-plasmin system. Although further studies are necessary to substantiate this hypothesis, the previous study that the microinjection of purified matrix metalloprotease-2, an enzyme known to cleave ECM such as laminin (Yong et al., 2001), into the NAc enhances METH-induced dopamine release without affecting basal dopamine levels (Mizoguchi et al., 2007) may support the hypothesis.

On the other hand, repeated treatment with drugs of abuse produces structural adaptations. Exposure to amphetamine produces a long-lasting increase in the length of dendrites and the number of branched spines on medium spiny neurons (Robinson and Kolb 1997). Development of sensitization in METH-induced dopamine release in the NAc and locomotor activity was significantly attenuated in tPA-/- mice compared with

wild-type mice. Thus, it is possible that tPA may play a role in repeated METH-induced structural changes through the degradation of laminin, which may underlie the behavioral and neurochemical sensitization to the methamphetamine.

In conclusion, I have demonstrated that the tPA-plasmin system participates in the development of behavioral sensitization induced by repeated METH treatment by regulating the processes underlying the sensitization of METH-induced dopamine release in the NAc, in which degradation of laminin by plasmin may play a role.

5. Table

Table 1. Behavioral sensitization induced by repeated METH treatment in wild-type and PAR-1-/- mice.

Genotypes	Treatment		
	Saline (day 1)	METH (day 2)	METH (day 8)
Wild-type mice	1120 ± 208	1844 ± 249*	2705 ± 235$^{*,\#}$
PAR-1-/- mice	1223 ± 152	1965 ± 166*	2620 ± 101$^{*,\#}$

Animals were treated with saline on day 1 and METH (1 mg/kg, s.c.) once daily for 7 days (day 2 to 8). Locomotor activity was measured for 1 h after treatment on days 1, 2 and 8. Values indicate the means ± SEM (n = 9-10). One-way ANOVA revealed significant effects of METH treatment [F(5, 51) = 12.66, $p < 0.0001$]. * $p < 0.05$ vs. respective saline treatment (day 1). #$p < 0.05$ vs. respective acute METH treatment (day 2).

6. Figures

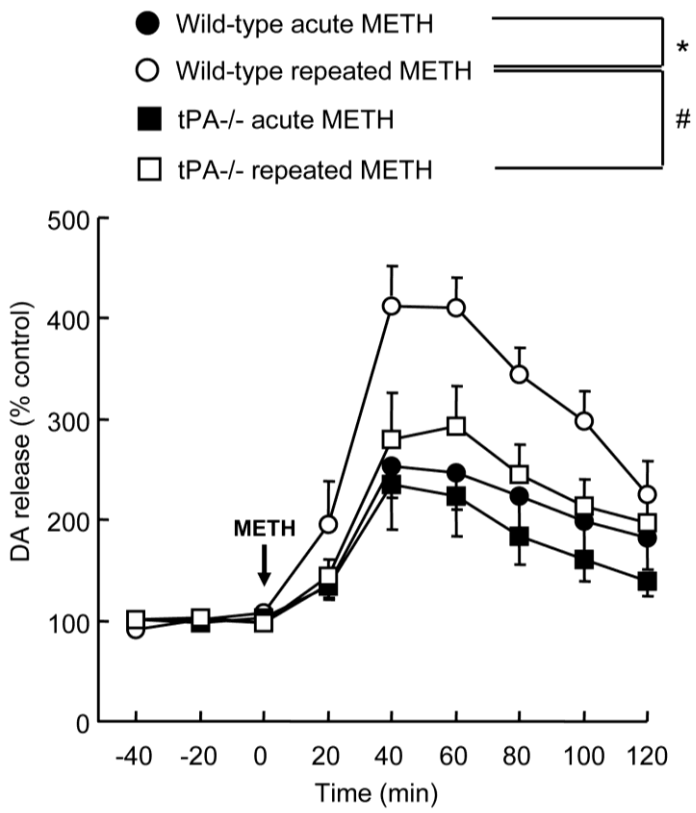

Fig. 1-1. Acute and repeated METH-induced changes in extracellular dopamine levels in the NAc of wild-type and tPA-/- mice. METH was administered at a dose of 1 mg/kg for 5 days in the repeated METH groups. Values indicate the means ± SEM (n = 7 for acute METH-treated wild-type mice; n = 7 for repeated METH-treated wild-type mice; n = 8 for acute METH-treated tPA-/- mice; n = 7 for repeated METH-treated tPA-/- mice). Basal levels of dopamine (pg/20 min/20 μl) were 1.2 ± 0.2 in acute METH-treated wild-type mice, 1.2 ± 0.2 in repeated METH-treated wild-type mice, 1.7 ± 0.4 in acute METH-treated tPA-/- mice and 1.3 ± 0.4 in repeated METH-treated tPA-/- mice. One-way ANOVA with repeated measures indicated significant effects of group [$F(3, 25) = 4.841$, $p < 0.01$] and time [$F(5, 125) = 34.961$, $p < 0.0001$], but not the interaction of group with time [$F(15, 125) = 1.621$, $p > 0.05$]. *$p < 0.05$ vs. acute METH-treated wild-type mice. #$p < 0.05$ vs. repeated METH-treated wild-type mice. DA: dopamine.

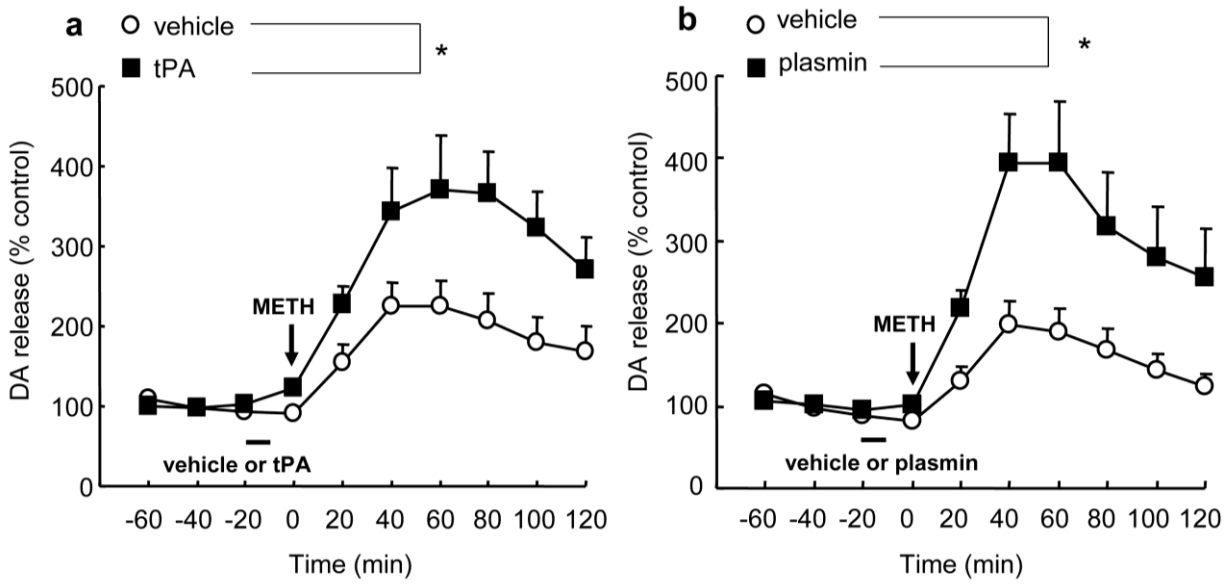

Fig. 1-2. Effect of microinjections of either tPA (a) or plasmin (b) into the NAc on acute METH-induced dopamine release in ICR mice. (a) tPA (100 ng) was microinjected during a 10-min period into the NAc at a volume of 1 μl. Ten min after the microinjection, METH (1 mg/kg) was administrated s.c.. Values indicate the means ± SEM (n = 8 for vehicle-treated mice, n = 10 for tPA-treated mice). One-way ANOVA with repeated measures revealed significant effects of group [$F(1, 16) = 5.382$, $p < 0.05$] and time [$F(5, 80) = 6.181$, $p < 0.0001$], but not the interaction of group with time [$F(5, 80) = 0.914$, $p > 0.05$]. *$p < 0.05$ vs. vehicle-treated group. (b) Plasmin (100 ng) was microinjected during a 10-min period into the NAc in a volume of 1 μl. Ten min after the microinjection, METH (1 mg/kg) was administrated s.c. Values indicate the means ± SEM (n = 6 for vehicle-treated mice; n = 9 for plasmin-treated mice). One-way ANOVA with repeated measures revealed significant effects of group [$F(1, 13) = 4.891$, $p < 0.05$] and time [$F(5, 65) = 7.463$, $p < 0.0001$], but not the interaction of group with time [$F(5, 65) = 1.331$, $p > 0.05$]. *$p < 0.05$ vs. vehicle-treated group. DA: dopamine.

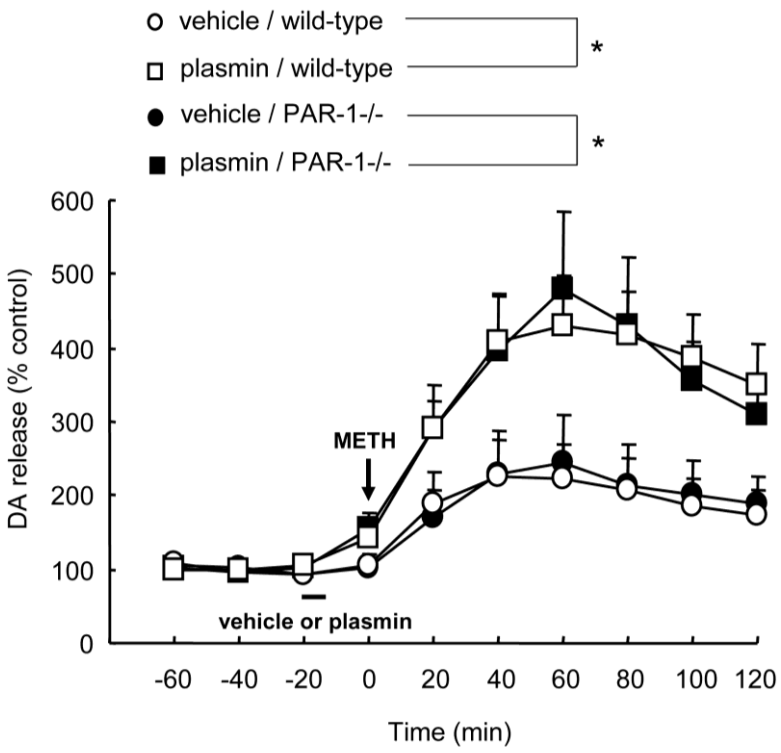

Fig. 1-3. Effect of microinjection of plasmin into the NAc on METH-induced dopamine release in PAR-1-/- mice. Plasmin (100 ng) was microinjected during a 10-min period into the NAc in a volume of 1 μl. Ten min after the microinjection, METH (1 mg/kg) was administrated s.c.. Values indicate the means ± SEM (n = 5 for vehicle-treated wild-type mice, n = 8 for plasmin-treated wild-type mice, n = 5 for vehicle-treated PAR-1-/- mice, n = 5 for plasmin-treated PAR-1-/- mice). One-way ANOVA with repeated measures indicated significant effects of group [$F(3, 19) = 3.392$, $p < 0.05$] and time [$F(5, 95) = 9.440$, $p < 0.01$], but not the interaction of group with time [$F(15, 95) = 1.033$, $p > 0.05$] There was no difference in METH-induced dopamine release between wild-type and PAR-1-/- mice in the presence or absence of pretreatment with plasmin. DA: dopamine

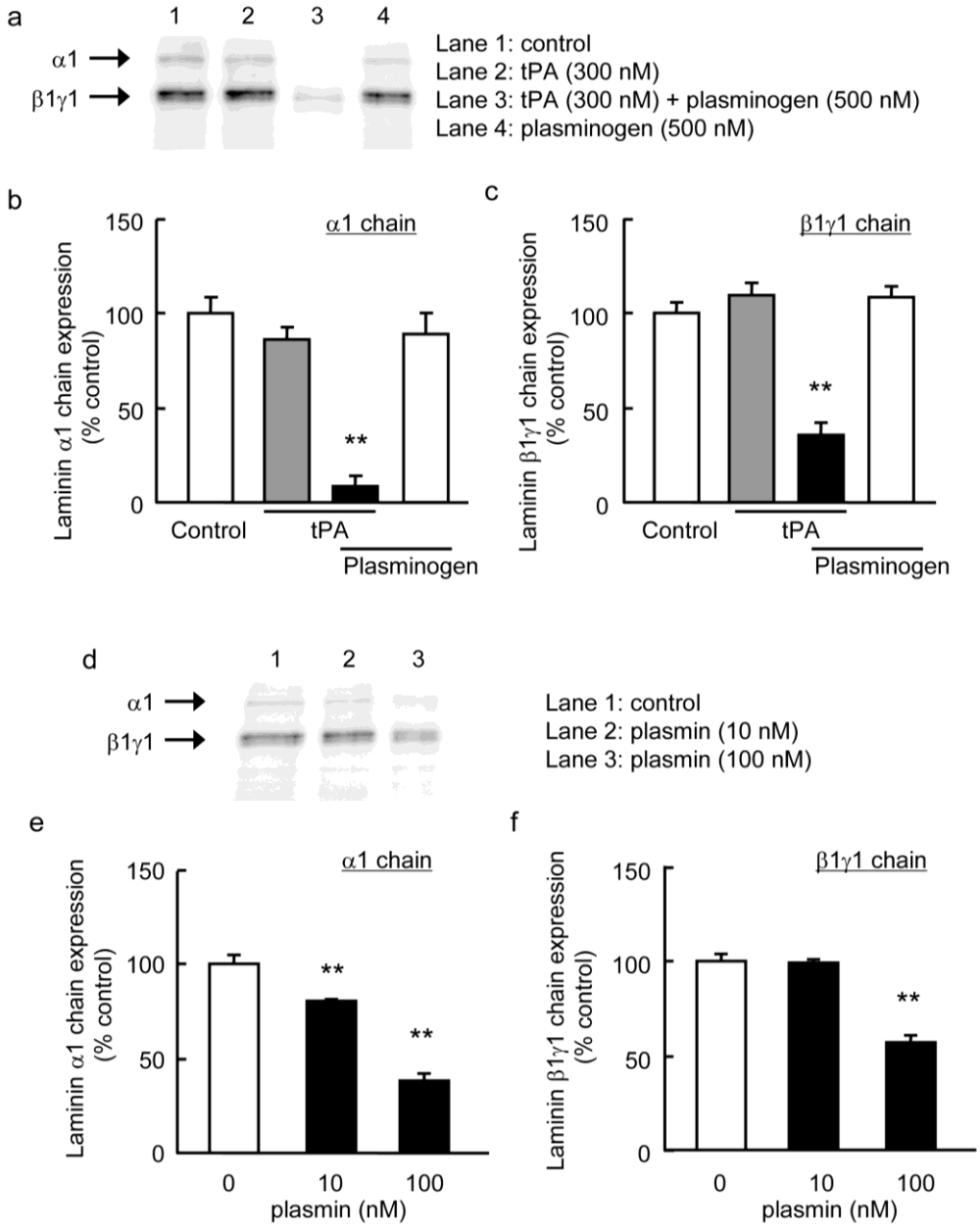

Fig. 1-4. Effects of tPA, plasminogen and plasmin on laminin degradation in vitro. (a, d) Representative photograph of Western blotting for laminin after treatment with tPA, plasminogen (a) and plasmin (d). (b, c, d, f) Densitometric analysis of laminin α1 (b, e) and β1γ1 (c, f) chain. (a-c) The homogenate of striatum including the NAc was incubated with recombinant human tPA (300 nM) with or without human plasminogen (500 nM) for 30 min at 37ºC. (b) ANOVA [$F(3, 12) = 27.199$, $p < 0.01$]. (c) ANOVA [$F(3, 12) = 34.719$, $p < 0.01$]. (d-f) The homogenate of striatum was incubated with

recombinant human plasmin (10 or 100 nM) for 30 min at 37ºC. (e) ANOVA [$F(2, 9) = 68.48$, $p < 0.01$]. (f) ANOVA [$F(2, 9) = 54.706$, $p < 0.01$]. **$p < 0.01$ vs. control.

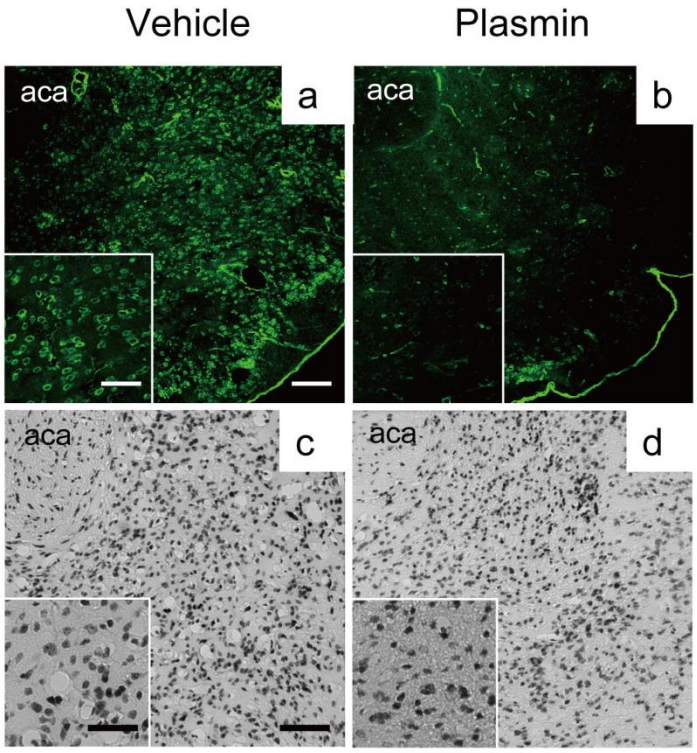

Fig. 1-5.

Immunohistochemical detection of laminin in the NAc after microinjection of plasmin. (a, b) Representative photographs of laminin immunoreactivity in the NAc of vehicle- (a) or plasmin-microinjected mice (b). (c, d) Representative photographs of Nissl-stained sections in the NAc of vehicle- (c) or plasmin-microinjected mice (d). Scale bar indicates 100 μm in Fig. 6a and c and 50 μm in Fig. 5a and c insert. aca: anterior commissure anterior part.

Chapter II: Methamphetamine-induced neurotoxicity

1. Introduction

METH is an abused drug. It has a strong rewarding effect that is related to the stimulating effect of dopamine release in the brain (Yamada et al., 2004; Nakajima et al., 2004). Repeated treatment with METH in rodents leads to the development of behavioral sensitization, which may be associated with METH psychosis or a strong craving in METH abusers. METH dependence and craving can be modeled by a self-administration paradigm in mice. A recent study demonstrated that glial-derived neurotrophic factor (GDNF) plays an important role in the craving for METH after long-term abstinence using GDNF-deficient mice (Yan et al., 2007).

Yamada et al. and Nakajima et al. (2004 and 2005) have previously demonstrated that endogenous tumor necrosis factor-α (TNF-α) is induced in the brain after repeated administration of METH, and this cytokine acts to suppress METH dependence and its dopaminergic neurotoxicity (Yamada et al., 2004; Nakajima et al., 2004; Yamada et al., 2005). Thus, METH-induced conditioned place preference (CPP), behavioral sensitization and loss of TH in the striatum are potentiated in TNF-α-deficient (TNF-α-/-) mice compared with wild-type mice, whereas systemic administration of TNF-α in mice suppresses METH-induced hyperlocomotion, behavioral sensitization, conditioned place preference and the loss of TH in the striatum (Nakajima et al., 2004).

On the other hand, the recent studies demonstrated that tPA acts as a pro-addictive substance to enhance the reward of and behavioral sensitization to METH (Yamada et al., 2004; Nagai et al., 2005); thus, METH-induced CPP and behavioral sensitization are reduced in tPA-deficient (tPA-/-) mice compared to wild-type mice. It remains to be

determined, however, whether tPA is also involved in the dopaminergic neurotoxicity of METH.

In the present study, to investigate the role of tPA in METH-induced dopaminergic neurotoxicity, I compared TH and DAT levels in the striatum between wild-type and tPA-/- mice after repetitive treatment of METH at 4 mg/kg. As Nagai et al. (2006) have recently demonstrated that PAR-1 is involved in regulation by the tPA-plasmin system of dopamine release induced by nicotine (Nagai et al., 2006), I also examined METH neurotoxicity in PAR-1-/- mice.

2. Materials and Methods

2.1. Animals

Male ICR mice (7 weeks old) were obtained from Japan SLC Inc., and were used in gel zymography experiment. Male wild-type (C57BL/6J), tPA-/- (Nagai et al., 2006) and PAR-1-/- mice (Nagai et al., 2006) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The animals were housed in plastic cages and kept in a regulated environment (23 ± 1˚C, 50 ± 5% humidity), with a 12/12 hr light-dark cycle (lights on at 9:00 A.M.). Food (CA1; Clea Japan Inc., Tokyo, Japan) and tap water were available ad libitum.

All animal care and use were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the study was approved by the Institutional Animal Care and Use Committee of Kanazawa University.

2.2. Measurement of locomotor activity and rectal temperature

METH hydrochloride (Dainihon Pharmaceutical Co. Ltd., Osaka, Japan) was dissolved in physiological saline. Mice were given subcutaneous injections of METH at 4 mg/kg for 4 times at 2-hr intervals. Control animals were given the same volume of saline under the same schedule as used for administrations of METH. Rectal temperature was recorded with a Physitemp BRC BDT-100 digital thermometer (Physitemp Instrument, Clifton, NJ) after 30 min administration of METH.

2.3. Western blotting

Mice were sacrificed 10 days after METH treatment. Brains were removed rapidly, and striatal tissues containing NAc were dissected out on an ice-cold plate. Each tissue

was frozen quickly and stored in a freezer at -80ºC until assayed. Samples were homogenized in a lysis buffer composed of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 1mM sodium orthovanadate, 0.1% SDS, 1% sodium deoxycholate, 0.5mM dithiothreitol, 10 mM sodium pyrophosphate decahydrate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL of aprotinin, 10 μg/mL of leupeptin, and 10 μg/mL of pepstatin for the western blotting of TH. Alternatively, the tissues were homogenized in ice-cold 10 mM Tris–HCl (pH 7.4)/5 mM EDTA buffer containing 320 mM sucrose, protease inhibitors (0.1 mM PMSF, 1 mM EGTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin) and phosphatase inhibitors (0.1 mM Na_3VO_4, 1 mM NaF) and centrifuged at 700 g for 10 min. The supernatant was centrifuged again at 37,000 g for 40 min at 4 ºC and the pellet was resuspended in 10 mM Tris–HCl in the presence of protease inhibitors and phosphatase inhibitors for the measurement of DAT. Ten μg of protein was boiled in a sample buffer (0.125 M Tris-HCl at pH. 6.8, 2% SDS, 5% glycerol, 0.002% bromophenol blue, and 5% 2-mercaptoethanol), applied onto a 8-10% SDS-polyacrylamide gel, and subsequently transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was blocked with 3% BSA in Tris-buffered saline-Tween 20 (TBS-T: 10 mM Tris-HCl at pH. 7.5, 100 mM NaCl, and 1% Tween-20), and incubated with mouse anti-TH monoclonal antibody (1:2000; MAB318, Chemicon, Temecula, California) or rat anti-DAT monoclonal antibody (1:1000; MAB369, Chemicon, Temecula, California) at 4ºC overnight. After incubation with horseradish peroxidase-conjugated anti-mouse or rat IgG at room temperature for 1 h, the immune complex was detected using ECL Western blotting detection reagents (GE Healthcare Bio-Science Corp.).

2.4. Gel zymography

To measure tPA activity in the brain, mice were decapitated 30 min or 2 h after the injection of METH, and the brains were quickly removed. Striatal tissues containing NAc were dissected out on an ice-cold plate. Each tissue was frozen quickly and stored in a freezer at -80 ºC until assayed. Gel zymography was performed as described previously (Nagai et al., 2004). Destaining with the same solvent revealed a transparent lysis zone against the dark protein background at 65 kDa corresponding to tPA.

2.5. Statistical analysis

All data were expressed as the mean ± SEM. In the analysis of the time course of the rectal temperature, analysis of variance (ANOVA) with repeated measures was used, followed by the Bonferroni test when F ratios were significant ($p < 0.05$). Statistical differences in the analysis of protein levels were determined using one-way ANOVA, followed by the Bonferroni test when F ratios were significant ($p < 0.05$).

3. Results

3.1. High dose of METH-induced changes of DAT and TH in tPA-/- mice and PAR-1-/- mice

The administration of high doses of METH (4 mg/kg) has been shown to result in damage to dopamine terminals, and perhaps in cell loss, in the brains of rodents and nonhuman primates (Ricaurte et al., 1980). Evidence for dopamine axon terminal damage includes a long-term decrease in dopamine content, the depletion of DAT, and a decrease in TH levels (Nakajima et al., 2004). I examined TH and DAT protein levels in mice killed 10 days after METH treatment. METH markedly decreased both TH and DAT proteins in the striatum (Fig. 2-1), and there was no difference in the magnitude of reduction of both proteins among wild-type, tPA-/- and PAR-1-/- mice.

3.2. High dose of METH-induced hyperthermia in tPA-/- mice and PAR-1-/- mice

It is known that hyperthermia consistently exacerbates METH-induced dopaminergic neurotoxicity, whereas hypothermia is neuroprotective (Nakajima et al., 2004). As shown in Fig. 2-2, METH significantly increased rectal temperature in wild-type, tPA-/- and PAR-1-/- mice by a similar magnitude. There was also no difference in the magnitude of METH-induced abnormal behavior including biting and gnawing during hyperthermia produced by high dose of METH treatment among those mice (data not shown). Finally, I measured the effect of METH at 4 mg/kg on the enzymatic activity of tPA in the striatum by using gel zymography. METH treatment had no effect on tPA activity in the striatum 30 min (109±3% of control) or 2 h (111±5%) after METH treatment (data not shown).

4. Discussion

The administration of high doses of METH (4 mg/kg) has been shown to result in damage to dopamine terminals, and perhaps in cell loss, in the brains of rodents and nonhuman primates (Ricaurte et al., 1980). Evidence for dopamine axon terminal damage includes a long-term decrease in dopamine content, the depletion of DAT, and a decrease in TH levels (Nakajima et al., 2004). I examined TH and DAT protein levels in mice killed 10 days after METH treatment. METH markedly decreased both TH and DAT proteins in the striatum (Fig. 2-1), and there was no difference in the magnitude of reduction of both proteins among wild-type, tPA-/- and PAR-1-/- mice. It is known that hyperthermia consistently exacerbates METH-induced dopaminergic neurotoxicity, whereas hypothermia is neuroprotective (Nakajima et al., 2004). As shown in Fig. 2-2, METH significantly increased rectal temperature in wild-type, tPA-/- and PAR-1-/- mice by a similar magnitude. There was also no difference in the magnitude of METH-induced abnormal behavior including biting and gnawing during hyperthermia produced by high dose of METH treatment among those mice (data not shown). Finally, I measured the effect of METH at 4 mg/kg on the enzymatic activity of tPA in the striatum by using gel zymography. METH treatment had no effect on tPA activity in the striatum 30 min (109±3% of control) or 2 h (111±5%) after METH treatment (data not shown).

I found that repetitive treatment with METH at 4 mg/kg decreased TH and DAT levels equivalently in the striatum in wild-type, tPA-/- and PAR-1-/- mice. Furthermore, there was no difference in METH-induced abnormal behavior and hyperthermia after METH treatment among wild-type, tPA-/- and PAR-1-/- mice. These results suggest that neither tPA nor PAR-1 is involved in METH-induced dopaminergic neurotoxicity.

It has been reported that tPA-/- mice exhibited an approximately 50% reduction of brain damage after focal cerebral ischemia compared to wild-type mice (Wang et al., 1998) and that tPA-/- mice are less susceptible to excitotoxin-induced seizures than wild-type mice (Tsirka et al., 1995), indicating that tPA play a crucial role in neuronal damage mediated by excitotoxins. Furthermore, the deficiency of PAR-1, a receptor for thrombin and plasmin (Kuliopulos et al., 1999), protects against neuronal damage caused by transient focal cerebral ischemia (Junge et al., 2003) or unilateral cerebral hypoxia/ischemia (Olson et al., 2004). Taken together with my results, it is suggested that the mechanisms underlying METH-induced dopaminergic neurotoxicity are distinct from those in excitatory amino acid (glutamate)-mediated neuronal and vascular damage, and that endogenous tPA and PAR-1 are not important in METH-induced dopaminergic neurotoxicity.

In agreement with the evidence that release and subsequent actions of dopamine are important in mediating METH-induced dopaminergic neurotoxicity (Nakajima et al., 2004), I found no difference in single METH (1 mg/kg)-induced dopamine release in NAc between wild-type and tPA-/- mice (Chapter 1 in this manuscript), whereas tPA/plasmin system participates in the development of behavioral sensitization induced by repeated METH treatment, by regulating the processes underlying the sensitization of METH-induced dopamine release (Chapter 1 in this manuscript). Therefore, it may be unlikely that tPA plays a significant role in the dopamine release induced by repetitive administration of high dose of METH (4 mg/kg), which results in dopaminergic neurotoxicity in the striatum.

In conclusion, I have demonstrated that neither tPA nor PAR-1 is involved in METH-induced dopaminergic neurotoxicity in vivo.

5. Figures

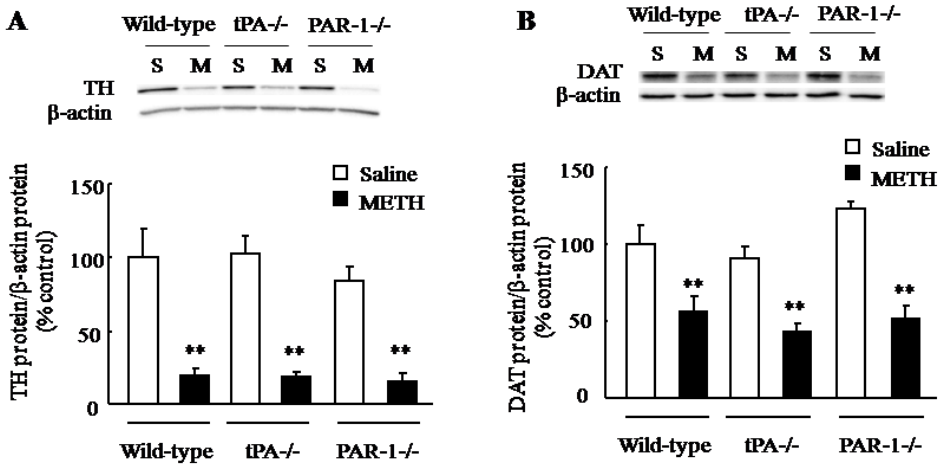

Fig. 2-1. METH-induced decrease in TH (A) and DAT (B) protein in the striatum of wild-type, tPA-/- and PAR-1-/- mice. METH was administered at a dose of 4 mg/kg for 4 times at 2-hr intervals. Ten days after METH treatment, mice were killed to measure TH levels. Control was saline-treated wild-type mice. Values indicate the means ± SE (A, n=4 for saline-treated wild-type mice; n=4 for METH-treated wild-type mice; n=4 for saline-treated tPA-/- mice; n=4 for METH-treated tPA-/- mice; n=4 for saline-treated PAR-1-/- mice; n=4 for METH-treated PAR-1-/- mice, and B, n=4 for saline-treated wild-type mice; n=4 for METH-treated wild-type mice; n=3 for saline-treated tPA-/- mice; n=4 for METH-treated tPA-/- mice; n=4 for saline-treated PAR-1-/- mice; n=4 for METH-treated PAR-1-/- mice). One-way ANOVA revealed significant effects [F(5, 18)=15.8, $p < 0.05$ for A; F(5, 17)=13.8, $p < 0.05$ for B]. **$p < 0.05$ vs. saline-treated respective genotype group. S, saline; M, METH.

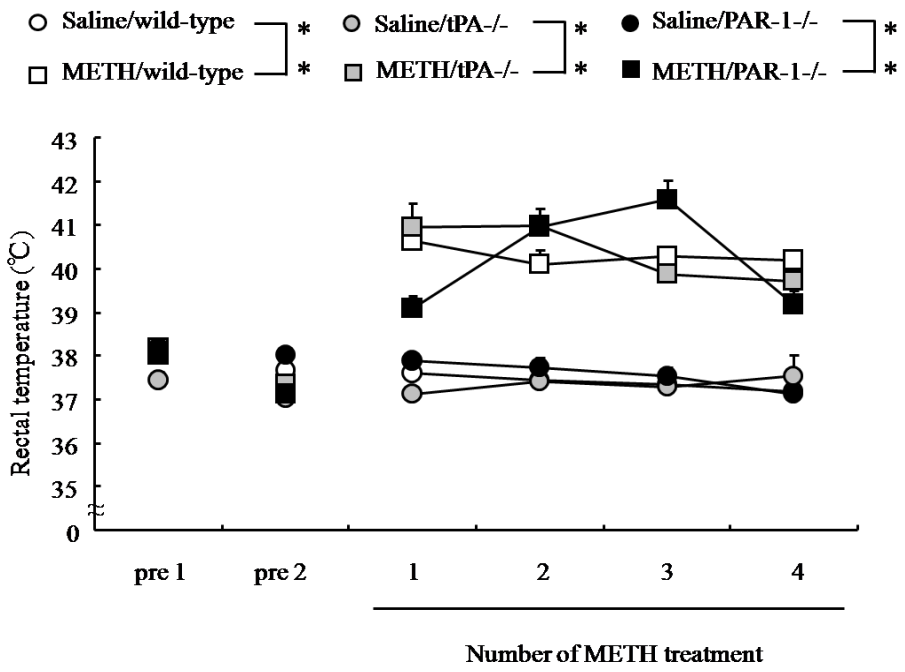

Fig. 2-2. METH-induced hyperthermia in wild-type, tPA-/- and PAR-1-/- mice. METH was administered at a dose of 4 mg/kg for 4 times at 2-hr intervals. Rectal temperature was measured before (pre) and 30 min after each METH treatment. Values indicate the means±SE (*n*=9 for saline-treated wild-type mice; *n*=9 for METH-treated wild-type mice; *n*=5 for saline-treated tPA-/- mice; n=5 for METH-treated tPA-/- mice; *n*=5 for saline-treated PAR-1-/- mice; *n*=5 for METH-treated PAR-1-/- mice). One-way ANOVA with repeated measures revealed significant effects of group [$F(5, 32)=90.8$, $p < 0.05$]. **$p < 0.05$ vs. saline-treated respective genotype group.

General Conclusion

In this study, I investigated the role of the tPA-plasmin system on the two important aspects of METH-induced changes in dopaminergic neurons: sensitization and neurotoxicity.

Earlier, it has been reported that dopaminergic neurons are neurobiologically and functionally altered by METH abuse; however, the downstream mechanism was not yet established. My report would contribute to the development of novel therapeutics for METH addicted patients given that the detailed mechanism of action is also elucidated.

In chapter 1, I described that there is a relationship between the tPA-plasmin system and METH-induced sensitization in dopaminergic neurons. By using tPA-/- mice, it was confirmed that the tPA-plasmin system has an important role in sensitization generation. I assessed whether PAR-1 and laminin, known downstream signals of tPA-plasmin system in the brain, modulate the sensitization downstream to dopamine. My results suggest that the degradation of laminin contributed to sensitization but PAR-1 did not. As laminin is an ECM, it was interesting that it would contribute to METH-induced changes. During the last decade, growing evidence has suggested crucial roles for ECMs in neuronal plasticity (Gundelfinger et al., 2010; Lubbers et al., 2014). Gundelfinger et al. (2010) reported that ECMs are important regulators of neurotransmission or local environment plasticity through controlling diffusion rates of receptors and neurotransmitters (Gundelfinger et al., 2010). Although my data suggest a role for laminin in METH-induced changes, no evidence is currently available as to which downstream pathways or processes could be involved. Recently, Conant et al. (2011) showed that intracellular adhesion molecule-5, a substrate of matrix metalloprotease and cleaved by METH administration, activates integrin and cofilin as

downstream signaling molecules after METH treatment (Conant et al., 2011). Therefore, integrin/cofilin signals may be the possible downstream signals following laminin degradation; however, further investigation is needed to understand the relationship between METH and ECM dynamics.

In chapter 2, I described the relationship between the tPA-plasmin system and METH-induced neurotoxicity in dopaminergic neurons. I evaluated the effect of METH on protein analysis of TH and DAT as indicators of dopaminergic neurotoxicity using tPA-/- mice and PAR-1-/- mice. I also assessed changes in rectal temperature as a measure of hyperthermia, a representative marker induced by toxic METH doses. No difference was observed in protein levels as well as hyperthermia among wild type tPA-/- mice and PAR-1-/- mice. Therefore, it was concluded that tPA and PAR-1 are dispensable in dopaminergic neurotoxicity caused by METH.

Although TNF-α, an immunological molecule, has a role both in sensitization and neurotoxicity (Nakajima et al., 2004), interestingly, the tPA-plasmin system seemed to be involved only in sensitization, even though the similar experimental model (i.e. dopaminergic neuron after METH treatment) was used. More recently, Gou et al. (2015) demonstrated that cholecystokinin-8, a neuropeptide involved in feeding, pain, and learning, contributes both to behavioral sensitization and to neurotoxicity after chronic METH treatment (Gou et al., 2015). It is possible that the tPA-plasmin system is regulated by METH in a strict manner in sensitization unlike other molecules. However, these complicated regulations associated with METH-induced changes in dopaminergic neurons should be investigated in the future.

Even though almost all drugs that are abused, including METH, nicotine, and morphine, increase dopamine release in the NAc, PAR-1 showed no correlation to

METH-induced changes in this study when compared to other abuse drugs such as nicotine and morphine (Ito et al., 2006 and 2007). It is possible that differences in downstream signals from dopamine exist for the symptoms associated with each abuse drugs. Therefore, each addictive drug needs specific medicines based on their specific mechanism of action, which causes difficulty in developing new drugs to treat addiction.

Over the last decade, many agents that showed a decrease in markers of METH dependence in animals have been investigated for use in humans. In these clinical trials, some signals of efficacy has been observed; however, no medication has showed a broad and robust effect against METH-dependence (Morley et al., 2017). Consequently, novel treatments are in urgent need for METH abusers. Finally, the findings indicated in this publication may provide a basis for the development of novel medications for the treatment of METH-addicted patients as well as solve drug addiction-related issues worldwide.

Acknowledgments

The author is greatly indebted to all the people who made this thesis possible.

The author expresses her deepest appreciation to Dr. Kiyofumi Yamada, Dr. Taku Nagai and Dr. Hiroyuki Mizoguchi for providing the opportunity to carry out this study.

Finally, the author expresses her deepest gratitude to Associate Professor Hidekazu Kuwayama, the University of Tsukuba, for his peer-review and invaluable advice.

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