サブトラクション法を用いて覚せい剤依存の形成に関連する新規遺伝子の同定を試み、昨年度までに shati と piccolo の 2 つの遺伝子を見出した。両遺伝子とも覚せい剤による薬物依存を軽減する可能性を示唆する報告をしている[1,6,7]。両遺伝子とも、覚せい剤を培養細胞に投与した場合に見出されるドパミントランスポーターの内在化による不活化を阻止することから、新たな遺伝子として非常に興味深い。本年度は、両遺伝子の遺伝子過剰発現マウスと shati の遺伝子欠損マウスの作成に着手し、その中から得られた、若干の知見を紹介する。また、shati については測定系の作成に成功し、この測定系による精神神経変性疾患の判定の可能性について考えている。

B. 研究方法

1．実験動物

実験には、10 週齢の c57BL / 6J 雄性マウス（日本 SLC、静岡）および遺伝子組み換えマウスの作成には同一バックグランドの系統を使用した。本実験における動物実験は名古屋大学医学部動物実験指針、文部科学省動物実験指針および the *Guidelines for Proper Conduct of Animal Experiments* Science Council of Japan, 2006 に準じ動物実験委員会で承認された上で行った。行動および生化学実験に用いるマウスについては、午前 9 時から午後 9 時を明期とする部屋で飼育し、行動実験中以外は、餌および水は自由摂取とした。

2．遺伝子組み換え動物の作成

*Piccolo* 遺伝子の一部である C2A ドメインまたは *Shati* 遺伝子の翻訳部分を pDEST26 ベクターに組み込んだ。本ベクターはサイトメガウィルス (CMV) プロモーターを含んでおり、その下流に、*Piccolo* C2A ドメインまたは *shati* を組み込んだ。C2A ドメイン遺伝子の前には myc を、*shati* 遺伝子の前には GFP をそれぞれ組み込み、共発現するようにした。図 1 に示すベクターを線形にし、受精卵に注入した。今回用いた CMV プロモーターは、図 2 に示すように、発達または増殖過程にある細胞のみで過剰発現することがわかっている。そのため、本マウスでも、胎生期および生後 1 週間程度の間のみ該当蛋白質の増加がおこっていると考えられる。また、このベクターが遺伝子にランダムに組み込まれることで染色体上のどのような位置に配置されるか不明であり、元来存在していた機能遺伝子の転写が阻害される可能性があるという不確定な部分がある。

3．Social interaction 試験

実験を行う前 2 日間、観察箱にマウスを入れ 10 分間慣れさせた。観察の際には、マウスを普段同じケージで飼育していない未知のマウスと共に実験箱に入れ、10 分間にお互いが接触する時間を計測した。

4．強制水泳試験法

25℃の水を 15cm の水深で張った円筒形の水槽にマウスを投入し 6 分間の強制水泳を行い、後半 5 分間における無動時間を赤外線装置により測定した。無動時間が長いほどうつ様症状を示していると考えられる。

5．条件性場所嗜好 (Conditioned Place Preference; CPP) 試験

CPP 試験には 2-compartment box を用いて、既報に従って行った[12]。メタンフェタミン (0.3 mg/kg) の投与は実験の直前に行った。

6．図 3 に示すように、サンドイッチ型の酵素免疫測定法を用いて、マウスの血液および尿中成分の測定を行った。

7．統計処理

実験結果は平均±標準誤差で示した。有意差検定は一元配置分散分析の後、Bonferroni's テストを用いて行った。2 群間比較には、Student's *t*-test を用いて検定した。

C. 研究結果

１．遺伝子過剰発現マウスの選別

受精卵にそれぞれの遺伝子を含むベクターを注入した後、メスマウスの子宮に入れ、着床および妊娠させた。その結果、得られた子供を F1 として、便宜上、由来受精卵ごとに系統に番号をつけ、いずれの系統が過剰にそれぞれの蛋白質を発現しているかを比較し、実験に用いるマウスの系統を決定した。そのために、同じ番号の系統の F1 のオスとメスを交配させ、産まれてきたマウスの尾から DNA を抽出し、その中で PiccoloC2A ドメイン過剰発現マウスの場合は C2A ドメイン、Shati 過剰発現マウスの場合は GFP について、それぞれ発現量のチェックを行い、発現が観察されたものを選択した。この操作は、遺伝子過剰発現マウスの場合、挿入された外来遺伝子のコピー数が系統毎に異なること、また染色体の位置によっては、交配を続けている間に外来遺伝子が失われる可能性があるためである。図 4A に示すように、Piccolo C2A ドメインでは 111 と番号をつけた系統が当該遺伝子の発現量が多かったため、その生後１日目のマウス脳を用いて Piccolo C2A ドメインの発現量をリアルタイム RT-PCR 法で測定したところ、mRNA が 1.5 倍に増加していた。以降の実験は、本マウスを用い、またコントロールには、Piccolo C2A 過剰発現マウスと同腹の野生型マウスを用いた。同様に、Shati 過剰発現マウスについても選別を行い、コントロールと比較して生後１日目に 1.3 倍の Shati mRNA を発現していた 011 の系統を実験に用いた（図 4B)。本年度は、生育が早く、行動実験可能な数のマウスが確保できた Piccolo C2A ドメイン過剰発現マウスの結果を示す。

２．Social interaction 試験

図 5 に示すようにコントロールマウスと比較して Piccolo C2A ドメイン過剰発現マウスは、未知のマウスとの接触時間が有意に減少し、他者との社会性が減少していた。

３．強制水泳試験法

図 6 に示すように 10 分間の強制水泳をコントロールマウスと Piccolo C2A ドメイン過剰発現マウスに課したところ、コントロールマウスと比較して Piccolo C2A ドメイン過剰発現マウスに無動時間の有意な延長が観察された。このことから、Piccolo C2A ドメイン過剰発現マウスは、うつ様症状を示すことが考えられる。

４．条件付場所嗜好 (Conditioned Place Preference; CPP) 試験

0.3mg/kg のメタンフェタミンは、コントロールマウスにおいて薬物依存を誘導しない程度の低濃度である。図 7 に示すように、Piccolo C2A ドメイン過剰発現マウスは、メタンフェタミン投与を行った部屋への嗜好性がコントロールマウスより高まっていた。このことは、Piccolo C2A ドメインの過剰発現によって依存性薬物への感受性が高まったことを示すと考えられる。

５．覚せい剤精神病モデルマウスの血中および尿中の shati の濃度

覚せい剤精神病モデルマウスの血中および尿中では、酵素免疫測定法の結果、有意に濃度の増加が観察された。また血中の白血球内の shati mRNA は覚せい剤精神病モデルマウス内で変化は無く、酵素免疫測定法により増加が検出された Shati は白血球由来では無いことが示唆された。

D. 考察

昨年度までの研究において、shati と Piccolo という２つの薬物依存形成に関与する可能性の高い蛋白質を同定および見出した。shati は、N-アセチルトランスフェラーゼ 8 と類似の構造を持つことから N-acetyltransferase 8-like (NAT8l) としても NCBI に登録されている(http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=134288911)。しかし、生理活性についての研究はほとんどなされていない。N-アセチルトランスフェラーゼは蛋

白質のN末端のアセチル化を行う酵素であり、多くの蛋白質においてN-アセチル化は機能発現に重要である。NAT8は腎臓と肝臓の組織形成と機能維持に重要であることが報告されていることから、脳で発現するShati/NAT8lも神経やグリアの分化や機能維持に重要な役割を担っている可能性が考えられる。薬物依存の形成にはドパミンだけでなく記憶・学習も重要なファクターの1つであることから、shatiの神経分化やシナプス可塑性などへの影響についての検討が今後の課題である。また、本研究でPiccoloは、インシュリン分泌を調節する蛋白質として報告されているが、ドパミンの遊離量も調節している可能性が本研究から示された。Piccoloのドパミン遊離量調節には、生合成よりも再取り込みに関わっている、即ち、C2Aドメインの過剰発現細胞においてドパミントランスポーターの内在化が阻止されている事を示唆する研究成果も得ており、今後は、培養細胞を用いてこれらの仮説を証明したいと考えている。これらのことを *in vivo* で一挙に解決するために、両遺伝子の過剰発現マウスおよびshatiマウスの遺伝子欠損マウスの作成に着手した。繁殖ならびに実験に使用するために、多くの時間とマンパワーを費やしたため、一般行動に実験結果しか本年は示すことは出来なかったが、社交性に欠けることや、うつ様の傾向があること、また、覚せい剤への場所嗜好性が強いことを示すことが出来た。

## E. 結論

今までにも薬物依存形成に関連することが報告されている蛋白質はいくつかあるが、Key蛋白質となるものについての統一した見解は得られていない。そこで、本研究で見出した2つの蛋白質が薬物依存形成にどのように寄与しているかについて今後検討を重ねる予定である。本年度はCMVプロモーターを用いた遺伝子過剰発現マウスの作成を試みた。またshatiの定量方法を確立した。次年度は、shatiの遺伝子過剰発現マウスおよび遺伝子欠損マウスを用いて、詳細な生理機能を明らかにする。また、今回報告した酵素免疫測定法本測定系による血中および尿中のShati蛋白質の検出系を用い、精神疾患の早期発見ができるようになることを信じている。

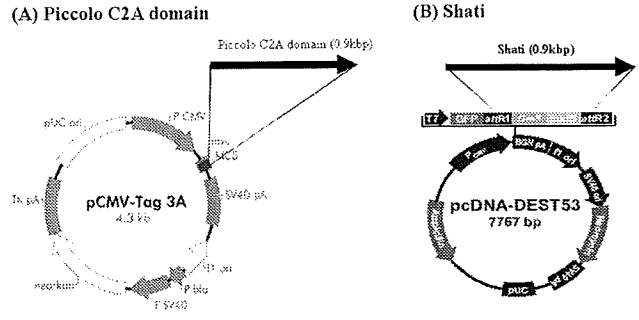



**図1 Expression vector of Piccolo C2A domain and Shati**

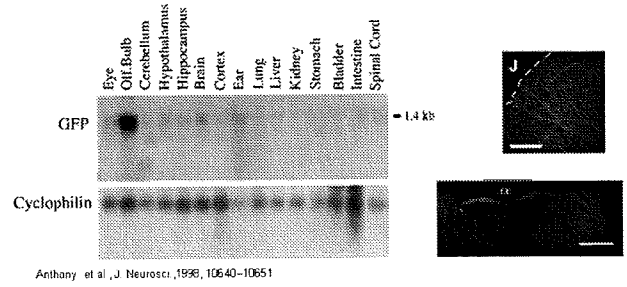



**図2 CMV promoter の下流遺伝子は神経発達期に発現する**

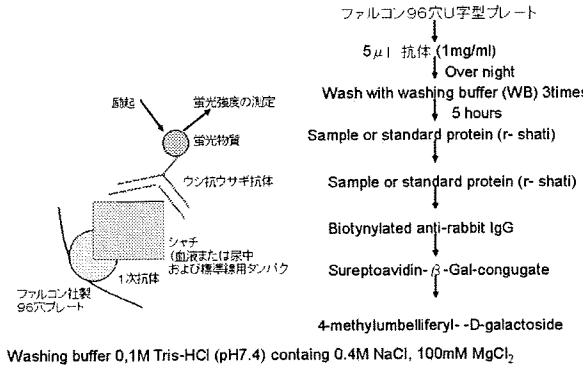
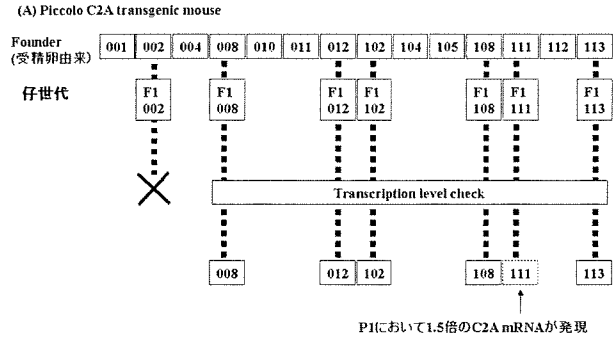



**図3 サンドイッチ型酵素免疫測定法の原理**



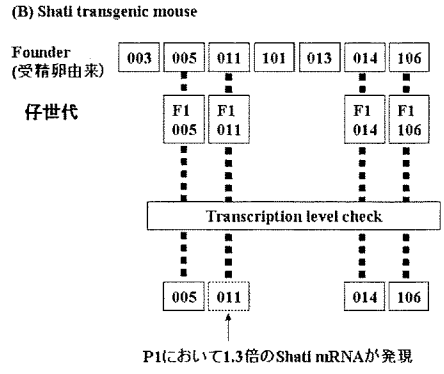
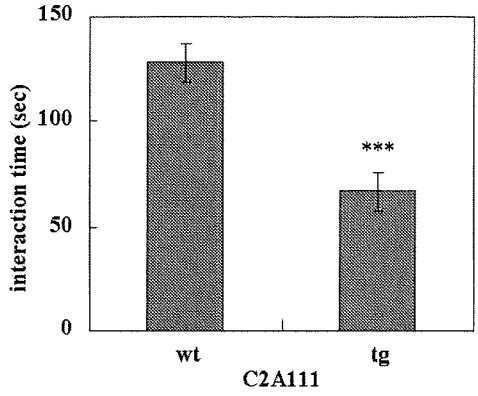
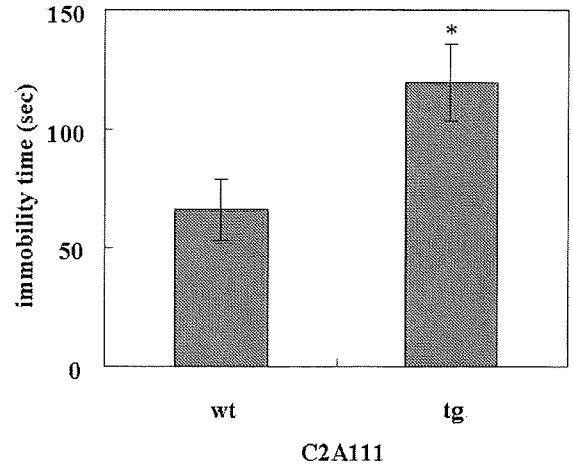




**図4　Transgenic mouse 系統図**




*** P<0.005　　N=12

**図5　Social interaction data of Piccolo C2A domain transgenic mouse (C2A111)**




*P<0.05　N=12

**図6　Immobility time of Piccolo C2A domain transgenic mouse (C2A111) in forced swim test**

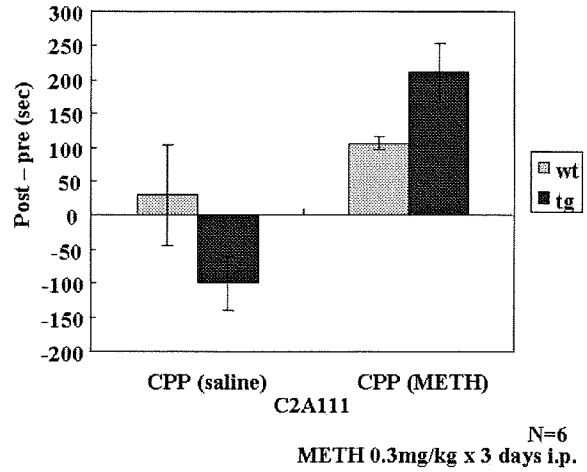



**図7　Conditioning place preference of Piccolo C2A transgenic mouse (C2A111)**

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G. 知的財産権の出願・登録状況

1．特許取得

1) 発明の名称：脳内酸化抑制剤およびその使用
2008年5月2日国際公開（WO2008/050754A1）
出願人：国立大学法人名古屋大学，協和発酵バイオ株式会社（米国を除くすべての指定国），新田淳美，鍋島俊隆（米国のみ）
発明者：新田淳美，鍋島俊隆

2) 発明の名称：抗うつ・抗不安剤
2009年1月23日国際出願(PCT/JP2009/051027)
出願人：協和発酵バイオ株式会社，国立大学法人名古屋大学（米国を除くすべての指定国），新田淳美，日比陽子，鍋島俊隆，森下幸治，池田武史（米国のみ）
発明者：新田淳美，日比陽子，鍋島俊隆，森下幸治，池田武史

3) 発明の名称：眼科用薬剤
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出願人：株式会社ニデック，国立大学法人名古屋大学
発明者：中谷正義，篠原結子，平林美紀，鈴森千智，西村茂，新田淳美，日比陽子

２．実用新案登録
なし

３．その他
なし

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# A novel molecule 'shati' increases dopamine uptake via the induction of tumor necrosis factor-α in pheochromocytoma-12 cells

Minae Niwa,*,†,‡ Atsumi Nitta,† Xiaobo Cen,† Kiyoyuki Kitaichi,§ Norio Ozaki,* Kiyofumi Yamada† and Toshitaka Nabeshima†,‡

*Department of Psychiatry, Nagoya University Graduate School of Medicine, Nagoya, Japan

†Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Japan

‡Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Meijo University, Nagoya, Japan

§Department of Pharmacology, Faculty of Pharmaceutical Sciences, Nagasaki International University, Nagasaki, Japan

**Abstract**

The psychostimulant properties of methamphetamine (METH) are associated with an increase in extracellular dopamine (DA) levels in the brain, via facilitation of DA's release from pre-synaptic nerve terminals and inhibition of its reuptake through DA transporter. Recently, we have demonstrated that tumor necrosis factor-α (TNF-α) increases DA uptake and inhibits METH dependence. Moreover, we have clarified 'shati' identified in the nucleus accumbens of mice treated with METH is involved in METH dependence. In the present study, we investigated the effects of TNF-α on DA uptake in PC12 cells and established a PC12 cell line transfected with a vector containing shati cDNA to examine the precise mechanism behind the role of shati in DA uptake. Moreover, we examined the relationship between shati and TNF-α. TNF-α increased DA uptake via the mitogen-activated protein kinase kinase pathway and inhibited the METH-induced decrease in DA uptake in PC12 cells. Transfection of the vector containing shati cDNA into PC12 cells, induced the expression of shati and TNF-α mRNA, accelerated DA uptake, and inhibited the METH-induced decrease in DA uptake. These results suggest that the functional roles of shati in METH-regulated behavioral changes are mediated through inhibition of the METH-induced decrease in DA uptake via TNF-α.

**Keywords:** addiction, dopamine (DA) uptake, methamphetamine, shati, tumor necrosis factor-α (TNF-α).

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The abuse of methamphetamine (METH) has significant psychiatric and medical consequences, including dependence, psychosis, overdose, and even death (Rawson *et al.* 2002). Drugs of abuse, including METH, modulate the activity of mesolimbic dopaminergic neurons, projecting from the ventral tegmental area to the nucleus accumbens

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Address correspondence and reprint requests to Toshitaka Nabeshima, PhD, Department of Chemical Pharmacology, Meijo University Graduate School of Pharmaceutical Sciences, 150 Yagotoyama, Tenpaku-ku, Nagoya 468-8503, Japan. E-mail: tnabeshi@ccmfs.meijo-u.ac.jp

*Abbreviations used*: AMPH, amphetamine; ANOVA, analysis of variance; AP, anteroposterior; cDNA, complementary DNA; CNS, central nervous system; CSF, cerebrospinal fluid; D1-R, dopamine D1 receptor; D2-R, dopamine D2 receptor; DA, dopamine; DAT, dopamine transporter; DV, dorsoventral; ERK1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum; GABA, γ-aminobutyric acid; GBR 12909, [1-(2[bis(4-fluorophenyl-) methoxy]ethyl)-4-(3-phenylpropyl)piperazine] bimesylate hydrate; GFP, green fluorescent protein; GNAT, GCN5-related N-acetyltransferase; JNK, c-Jun N-termial kinase; MAO, monoamine oxidase; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase kinase; METH, methamphetamine; ML, mediolateral; NAc, nucleus accumbens; NF-κB, nuclear factor-κB; p38, p38 mitogen-activated protein kinase; PC12, pheochromocytoma-12; PCR, polymerase chain reaction; PD98059, 2-(2-amino-3-methyoxyphenyl)-4H-1-benzopyran-4-one; RIP, serine and threonine protein kinase receptor-interacting protein; RT-PCR, reverse transcription-polymerase chain reaction; shati-AS, shati antisense oligonucleotide; shati-SC, shati-scrambled oligonucleotide; SLC6, solute carrier 6; TH, tyrosine hydroxylase; TNFR I, tumor necrosis factor type I receptor; TNF-α, tumor necrosis factor-α; TRAF2, TNF receptor-associated factor 2; VMAT-2, vesicular monoamine transporter; VTA, ventral tegmental area.

(NAc) (Koob 1992; Wise 1996; Koob *et al.* 1998). The principal target for the action of METH is believed to be the dopamine transporter (DAT), which is a member of the solute carrier 6 (SLC6) gene family of $Na^+/Cl^-$ coupled transporters that also includes the neurotransmitter transporters of norepinephrine, serotonin, glycine, and γ-aminobutyric acid (GABA) (Amara and Kuhar 1993; Torres *et al.* 2003; Chen *et al.* 2004). The DAT controls dopaminergic signaling by the rapid reuptake of dopamine (DA) from synaptic clefts. As a substrate, METH not only competitively inhibits DA uptake and thereby increases synaptic DA but also promotes the reverse transport of nonvesicular DA, resulting in an efflux of DA via the DAT (Sulzer *et al.* 2005; Fog *et al.* 2006). This efflux results in a dramatic increase in extracellular DA and is believed to be of major importance for the psychostimulant properties of METH (Sulzer *et al.* 2005; Fog *et al.* 2006). However, the exact neuronal circuits and molecular cascade essential for drug dependence are still poorly understood. Moreover, the molecules related to the METH-induced increase in DA efflux are unclear.

Tumor necrosis factor-α (TNF-α) plays an important role in a variety of infectious, inflammatory, and autoimmune conditions (Vassalli 1992). TNF-α also affects the CNS directly or indirectly through the stimulation of vagal afferents (Maier and Watkins 1998). Thus, this proinflammatory cytokine is emerging as a modulator of CNS function. Recently, we have demonstrated that TNF-α activates synaptosomal and vesicular DA uptake (Nakajima *et al.* 2004). Moreover, we have reported that TNF-α and its inducer diminish METH and morphine-induced behavioral sensitization and rewarding effects by promoting plasmalemmal and vesicular DA uptake as well as attenuating the METH and morphine-induced increase in overflow of DA in the NAc (Nakajima *et al.* 2004; Niwa *et al.* 2007b,d; Niwa *et al.* 2008). TNF-α modulates cellular responses through the extracellular signal-regulated kinase 1/2 (ERK1/2) and nuclear factor-κB (NF-κB) signaling pathways (van Vliet *et al.* 2005). ERK1/2 regulates the surface expression and capacity of DAT (Morón *et al.* 2003). However, the mechanisms by which TNF-α regulates the uptake of DA are poorly understood.

Recently, we have identified a novel molecule 'shati' in the NAc of mice treated with METH repeatedly using the polymerase chain reaction (PCR)-select complementary DNA (cDNA) substraction method, which is a differential and epochal cloning technique. Further, we have demonstrated that shati, which contains the sequence of GCN5-related *N*-acetyltransferase (GNAT), acetyl-CoA-binding sites, and ATP-binding sites, is involved in METH-induced hyperlocomotion, sensitization, and conditioned place preference (Niwa *et al.* 2007a). Blockage of shati expression by shati antisense oligonucleotide (shati-AS) potentiates not only the increase in extracellular DA levels, but also the decrease in synaptosomal and vesicular DA uptake in the NAc induced by repeated METH treatment, resulting in potentiation of the METH-induced dependence (Niwa *et al.* 2007a).

Pheochromocytoma-12 (PC12) cells are useful as a model of the neuronal system and have DATs. In the present study, we investigated the effects of TNF-α on DA uptake in PC12 cells and the involvement of the mitogen-activated protein kinase kinase (MEK) pathway in the effects of TNF-α on DA uptake. Moreover, we succeeded in the transfection of a vector containing shati cDNA into PC12 cells, investigated the involvement of shati in DA uptake and the METH-induced decrease in DA uptake, and examined the relationship between shati and TNF-α by using these PC12 cells.

## Materials and methods

### Cell culture and transfection

PC12 cells purchased from the Riken cell bank (No. RCB0009) were cultured on poly-ornithine-coated coverslips in Dulbecco's modified Eagle's medium (Sigma-Aldrich St Louis, MO, USA) supplemented with 10% heat inactivated horse serum and 5% fetal bovine serum (Loder and Melikian 2003). We made the vector containing shati cDNA with the suggested sequence of NM_001001985 using the plasmid pcDNA-DEST53 (Invitrogen, Carlsbad, CA, USA) as an expression vector with green fluorescent protein (GFP), although N-terminal of seven amino acids of shati was missing (CDS 882-1760) in this vector. For transient expression, the cells were transfected with the plasmid expressing shati using Lipofectamine 2000 (Invitrogen).

### [$^3$H] DA uptake in PC12 cells

The uptake of [$^3$H] DA in PC12 cells was performed as described before (Melikian and Buckley 1999). The cells were washed in Krebs-Ringers- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer twice before the assay. Uptake was initiated by adding 1 μM [$^3$H] DA (Perkin Elmer, Waltham, MA, USA) containing $10^{-5}$ M pargyline and $10^{-5}$ M ascorbic acid. Uptake proceeded for 10 min at 23°C and was terminated with three rapid washes in ice-cold Krebs-Ringers-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. The amount of [$^3$H] DA accumulated was determined by liquid scintillation counting (Beckman Coulter, Inc., Fullerton, CA, USA). Non-specific uptake was defined in the presence of 10 μM [1-(2[bis(4-fluorophenyl-) methoxy]ethyl)-4-(3-phenylpropyl)piperazine] bimesylate hydrate (GBR 12909). The cells were pre-treated with TNF-α (0.1, 1, and 10 ng/mL) for 40 min, and assayed for [$^3$H] DA uptake. To neutralize TNF-α in PC12 cells, the cells were pre-treated with polyclonal goat anti-TNF-α antibody (R&D Systems Ltd., Minneapolis, MN, USA; Ab; 1, 10, 50, and 100 ng/mL) or soluble TNF receptor I (R&D Systems Ltd, sRI; 0.1, 0.5, 1, and 10 ng/mL) 10 min before the treatment with TNF-α (10 ng/mL, 40 min) (Barone *et al.* 1997), and assayed for [$^3$H] DA uptake. The function of TNF-α is mediated through two distinct cell surface receptors, TNF receptor I and TNF receptor II. The majority of TNF functions are mediated primarily through TNF receptor I, whereas TNF receptor II seems to play a role in only a limited number of

TNF responses (Hsu *et al.* 1995). Moreover, it has been reported that immunoreactivity for TNF receptor I is found in cell bodies and process of dopaminergic neurons (Boka *et al.* 1994). Therefore, we have used soluble TNF receptor I for neutralization for TNF-α. To examine the involvement of the MEK pathway in the TNF-α-induced increase in DA uptake in PC12 cells, the cells were pre-treated with a selective MEK inhibitor 2-(2-amino-3-methyoxy-phenyl)-4H-1-benzopyran-4-one (Calbiochem, San Diego, CA, USA; PD98059; 1, 10, 100, and 500 μM) 10 min before their treatment with TNF-α (10 ng/mL, 40 min), and assayed for [$^3$H] DA uptake. PD98059 was dissolved in dimethyl sulfoxide to give a concentration of 50 mM, stored in aliquots at −80°C, and diluted in Dulbecco's modified Eagle's medium to 1–500 μM immediately prior to use. To examine the effects of TNF-α on the METH-induced decrease in DA uptake in PC12 cells, the cells were pre-treated with TNF-α (10 ng/mL) 10 min before being treated with METH (1 μM, 30 min), and assayed for [$^3$H] DA uptake, following previous observations (Nakajima *et al.* 2004). Cen *et al.* (2008) have reported that METH (1 μM) decreases plasmmalemmal DAT expression in time-dependent manner (0, 5, 15, 30, 60 min), which is paralleled with the decrease in [$^3$H] DA uptake. Since treatment of METH (1 μM) for 30 min significantly decreases DA uptake compared with control group (Cen *et al.*, 2008), we have selected this time point for treatment of METH before the uptake assay. To examine the involvement of TNF-α in the shati-induced increase in [$^3$H] DA uptake in the shati-over-expressing PC12 cells, the cells were pre-treated with polyclonal goat anti-TNF-α antibody (R&D Systems Ltd, Ab; 50 ng/mL) or soluble TNF receptor I (R&D Systems Ltd, sR I; 1 ng/mL) 10 min before their treatment with METH (1 μM, 30 min), and assayed for [$^3$H] DA uptake.

### Immunocytochemistry

Two antibodies against the peptide of the hypothetical protein, CNTAFRGLRQHPRTQLL (S-3) and CMSVDSRFRGKGIAKALG (S-4) unique to shati were generated. These peptides were conjugated to keyhole limpet hemocyanin and injected into rabbits six times at 1-week intervals. Serum was taken from the rabbits 1 week after the final injection. The serum was diluted 200 times for immunostaining (Niwa *et al.* 2007a).

Transfected PC12 cells attached to glass coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline for 20 min, and then blocked in 3% normal sera and 0.1% Triton X-100 for 1 h. The coverslips were incubated with primary antibodies at 4°C overnight, washed with phosphate-buffered saline, and then incubated with appropriate secondary antibodies for 2 h. Polyclonal rabbit anti-S-3 or anti-S-4 antibody (1 : 200), monoclonal mouse anti-tyrosine hydroxylase (TH) antibody (1 : 200, Chemicon, Temecula, CA, USA), monoclonal mouse anti-GFP antibody (1 : 500, Chemicon), polyclonal goat anti-rat TNF-α antibody (1 : 100, R&D Systems Ltd), and polyclonal rabbit anti-GFP antibody (1 : 100, Chemicon) served as primary antibodies. Goat anti-mouse Alexa Fluor 546 (1 : 1000, Invitrogen), donkey anti-goat Alexa Fluor 546 (1 : 1000, Invitrogen), rabbit anti-mouse Alexa Fluor 488 (1 : 1000, Invitrogen), and donkey anti-rabbit Alexa Fluor 488 (1 : 1000, Invitrogen) were used as secondary antibodies. After being washed and mounted, stained cells were observed under a fluorescence microscope (Axioskoop 2 plus). Because similar results were obtained using the anti S-3 and anti-S-4 antibodies in the immunohistochemical experiments, only the data obtained with the anti-S-4 antibody is described.

### Real time reverse transcription-polymerase chain reaction

Total RNA was isolated using an RNeasy Kit (Qiagen, Hilden, Germany) and converted into cDNA using a SuperScript™ First-Strand System for RT-PCR Kit (Invitrogen). The levels of shati and TNF-α mRNA were determined by real-time RT-PCR using a TaqMan probe. The 18S ribosomal RNA was used as the internal control (Applied Biosystems, CA, USA). The shati primers used for real-time RT-PCR were as follows: 5′-TGTAAACACCCCTAA AGTGCCCT-3′ (forward; bp 2967–2989) and 5′-TCAATCCTGC ATACAAGGAATCAA-3′ (reverse; bp 3022–3045), and the Taq-Man probe was 5′-CACAGTCTGTGAGGCTCAGGTTGCCC-3′ (probe; bp 2995–3020). The amplification consisted of an initial step (95°C for 5 min) and then 40 cycles of denaturation for 30 sec at 95°C, annealing for 40 s at 59°C, and the extension time for 1 min at 72°C in an iCycle iQ Detection System (Bio-Rad Laboratories, Inc., CA, USA) (Niwa *et al.* 2007a). The expression levels were calculated as described previously (Wada *et al.* 2000).

### Animals

The male C57BL/6J- wild-type mice were obtained from Slc Japan (Hamamatsu, Japan). Animals were housed in plastic cages and kept in a temperature-, humidity-, and light-controlled room (23 ± 1°C; 50 ± 5% humidity; 12 : 12 h light/dark cycle starting at 8:00 AM) and had free access to food and water, except during behavioral experiments. All animal care and use was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine. Animals were treated according to the Guidelines of Experimental Animal Care issued from the Japanese Pharmaceutical Society.

### Shati-antisense oligonucleotide (shati-AS) treatment

Mice were anesthetized with pentobarbital (40 mg/kg, i.p.) and placed in a stereotaxic apparatus. The infusion cannula was connected to a miniosmotic pump (total capacity was 90 μL, Alzet 1002; Alza, Palo Alto, CA, USA) filled with shati-antisense oligonucleotide (shati-AS) or -scrambled oligonucleotide (shati-SC). The pump was implanted into the right ventricle [anteroposterior (AP) −0.5 mm, mediolateral +1.0 mm from the bregma, and dorsoventral −2.0 mm from the skull, according to the atlas of Franklin and Paxinos (1997)]. Phosphorothionate oligonucleotides were custom-synthesized at Nisshinbo Biotechnology (Tokyo, Japan) and dissolved in artificial CSF (147 mM NaCl, 3 mM KCl, 1.2 mM $CaCl_2$, and 1.0 mM $MgCl_2$, pH 7.2). The oligonucleotides were phosphorothioated at the first three bases of both the 5′- and 3′-ends, which results in increased stability and less toxicity. The sequences of shati-AS and -SC were 5′-TCTTCGTCTCGCAGAC CATGTCG-3′ and 5′-GGTCTGCTACACTGCTGCTAGTC-3′, respectively. Shati-AS and -SC were continuously infused into the cerebral ventricle at a dose of 1.8 nmol/6 μL/day (flow rate, 0.25 μL/h). Additionally, shati-SC was used as a control. Three days after the start of oligonucleotide infusion, mice were

administered METH (1 mg/kg, s.c.) for 5 days and decapitated 2 h after the final treatment (Niwa *et al.* 2007a).

### Statistical analysis

All data were expressed as means ± SE. Statistical differences between two groups were determined with Student's *t*-test. Statistical differences among three groups or more were determined using a one-way analysis of variance (ANOVA), two-way ANOVA, or three-way ANOVA, followed by the Bonferroni multiple comparison test. $p < 0.05$ was regarded as statistically significant.

### Nucleotide sequences

The DNA Data Bank of Japan/GenBank/European Molecular Biology Laboratory accession number for the primary nucleotide sequence of shati is DQ174094.

## Results

### Effect of TNF-α on DA uptake in PC12 cells

First, we investigated the effects of TNF on DA uptake in PC12 cells, since we have recently demonstrated that TNF-α activates synaptosomal and vesicular DA uptake in mice (Nakajima *et al.* 2004).

TNF-α (10 ng/mL, 40 min) increased [$^3$H] DA uptake compared with the control group ($F_{3,28} = 4.933$, $p < 0.01$, one-way ANOVA) (Fig. 1a). Moreover, we investigated whether the TNF-α-induced increase was antagonized by the anti-TNF-α antibody and soluble TNF receptor in PC12 cells. Pre-treatment with the antibody (10, 50, and 100 ng/

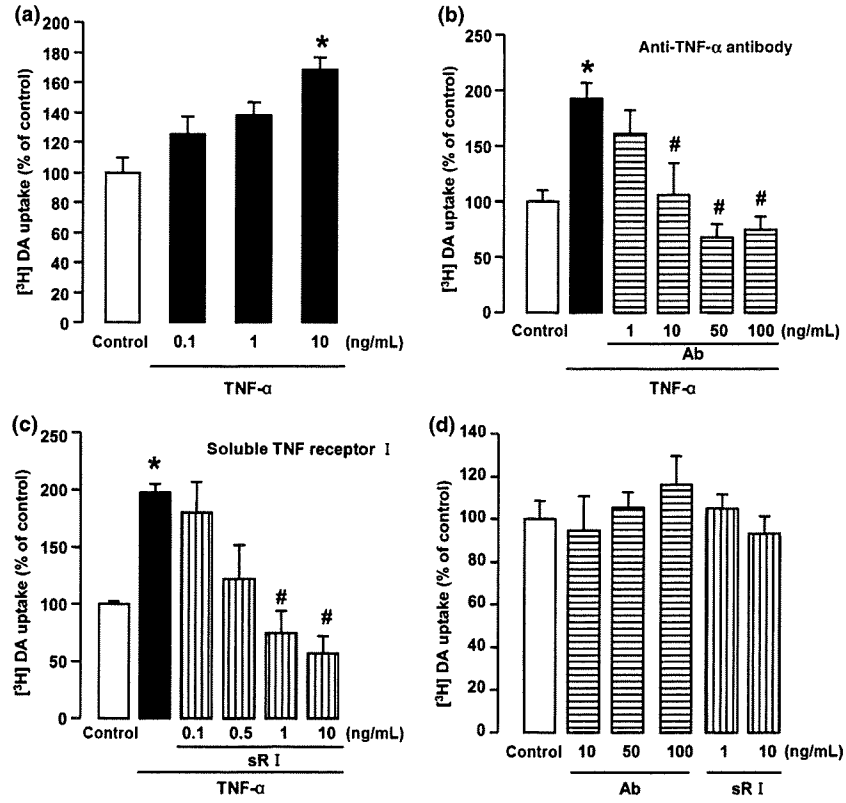



**Fig. 1** Effects of anti-TNF-α antibody (Ab) or soluble TNF receptor I (sR I) on TNF-α-induced increase in [$^3$H] DA uptake in PC12 cells. (a) The cells were pre-treated with TNF-α (0.1, 1, and 10 ng/mL) for 40 min, and assayed for [$^3$H] DA uptake. The [$^3$H] DA uptake was 0.14 ± 0.01 pmol/10 min for control. The final concentration of [$^3$H] DA was 20 nM. Value are means ± SE ($n = 8$). $^*p < 0.05$ versus control. (b) Effects of anti-TNF-α antibody (Ab) on TNF-α-induced increase in [$^3$H] DA uptake in PC12 cells. The cells were pre-treated with anti-TNF-α antibody (1, 10, 50, and 100 ng/mL) 10 min before their treatment with TNF-α (10 ng/mL, 40 min), and assayed for [$^3$H] DA uptake. The [$^3$H] DA uptake was 0.10 ± 0.02 pmol/10 min for the control. The final concentration of [$^3$H] DA was 20 nM. Values are means ± SE ($n = 6$–7). $^*p < 0.05$ versus control. $^\#p < 0.05$ versus TNF-α-treated cells. (c) Effects of soluble TNF receptor I (sR I) on TNF-α-induced increase in [$^3$H] DA uptake in PC12 cells. The cells were pre-treated with soluble TNF receptor I (0.1, 0.5, 1, and 10 ng/mL) 10 min before being treated with TNF-α (10 ng/mL, 40 min), and assayed for [$^3$H] DA uptake. The [$^3$H] DA uptake was 0.06 ± 0.00 pmol/10 min for the control. The final concentration of [$^3$H] DA was 20 nM. Values are means ± SE ($n = 6$–7). $^*p < 0.05$ versus control. $^\#p < 0.05$ versus TNF-α-treated cells. (d) Effects of anti-TNF-α antibody (Ab) or soluble TNF receptor I (sR I) on [$^3$H] DA uptake in PC12 cells. The cells were pre-treated with anti-TNF-α antibody (10, 50, and 100 ng/mL) or soluble TNF receptor I (1 and 10 ng/mL) for 50 min, and assayed for [$^3$H] DA uptake. The [$^3$H] DA uptake was 0.08 ± 0.01 pmol/10 min for the control. The final concentration of [$^3$H] DA was 20 nM. Values are means ± SE ($n = 6$–8).

mL, 50 min) or soluble TNF receptor I (1 and 10 ng/mL, 50 min) significantly inhibited the TNF-α-induced increase in [$^3$H] DA uptake ($F_{5,34}$ = 7.370 for anti-TNF-α antibody; $F_{5,34}$ = 7.526 for soluble TNF receptor I, $p < 0.01$, one-way ANOVA) (Fig. 1b and c), although the anti-TNF-α antibody (10, 50, and 100 ng/mL, 50 min) or soluble TNF receptor I (1 and 10 ng/mL, 50 min) itself had no effect on DA uptake (Fig. 1d). These results suggest that TNF-α activates DA uptake in PC12 cells.

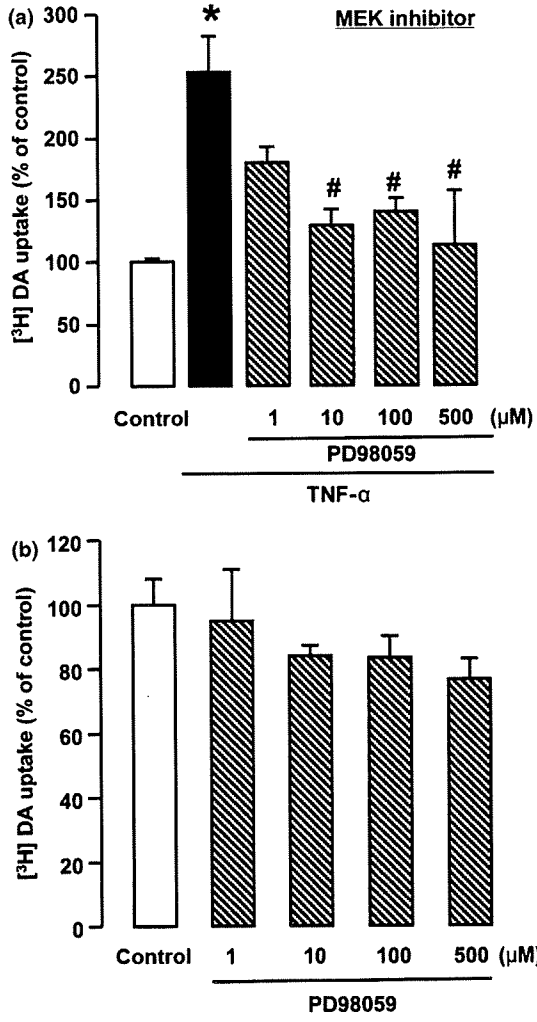



**Fig. 2** Effects of MEK inhibitor on TNF-α-induced increase in [$^3$H] DA uptake in PC12 cells. (a) Effects of the MEK inhibitor PD98059 on TNF-α-induced increase in [$^3$H] DA uptake in PC12 cells. The cells were pre-treated with PD98059 (1, 10, 100, and 500 μM) 10 min before their treatment with TNF-α (10 ng/mL, 40 min), and assayed for [$^3$H] DA uptake. The [$^3$H] DA uptake was 0.10 ± 0.00 pmol/10 min for control. The final concentration of [$^3$H] DA was 20 nM. Values are means ± SE ($n$ = 4). *$p < 0.05$ versus control. #$p < 0.05$ versus TNF-α-treated cells. (b) Effects of PD98059 on [$^3$H] DA uptake in PC12 cells. The cells were pre-treated with PD98059 (1, 10, 100, and 500 μM) for 50 min, and assayed for [$^3$H] DA uptake. The [$^3$H] DA uptake was 0.12 ± 0.00 pmol/10 min for the control. The final concentration of [$^3$H] DA was 20 nM. Values are means ± SE ($n$ = 4).

### Effects of mitogen-activated protein kinase kinase inhibitor on TNF-α-induced increase in DA uptake in PC12 cells

TNF-α modulates cellular responses through the ERK1/2 signaling pathway (van Vliet *et al.* 2005). Therefore, we investigated whether the TNF-α-induced increase in DA

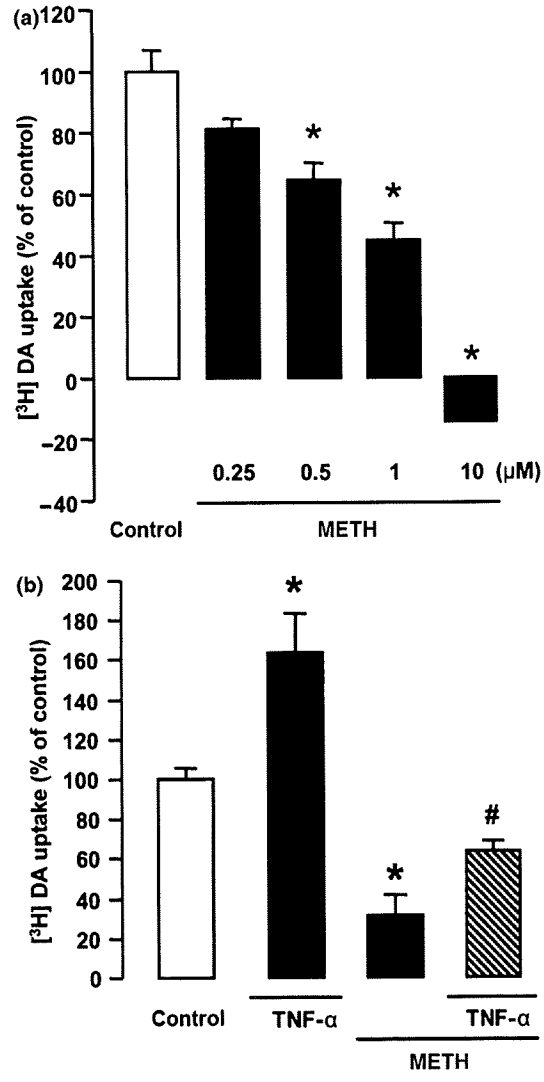



**Fig. 3** Effects of TNF-α on METH-induced decrease in [$^3$H] DA uptake in PC12 cells. (a) Dose-response effects of METH on [$^3$H] DA uptake in PC12 cells. The cells were pre-treated with METH (0.25, 0.5, 1, and 10 μM) for 30 min, and assayed for [$^3$H] DA uptake. The [$^3$H] DA uptake was 0.28 ± 0.02 pmol/10 min for the control. The final concentration of [$^3$H] DA was 20 nM. Values are means ± SE ($n$ = 4). *$p < 0.05$ versus control. (b) Effects of TNF-α on METH-induced decrease in [$^3$H] DA uptake in PC12 cells. The cells were pre-treated with TNF-α (10 ng/mL) 10 min before being treated with METH (1 μM, 30 min), and assayed for [$^3$H] DA uptake. The [$^3$H] DA uptake was 0.19 ± 0.01 pmol/10 min for the control. The final concentration of [$^3$H] DA was 20 nM. Values are means ± SE ($n$ = 5). *$p < 0.05$ versus control. #$p < 0.05$ versus METH-treated cells.

uptake was antagonized by the MEK inhibitor PD98059 in PC12 cells.

Pre-treatment with PD98059 (10, 100, and 500 μM, 50 min) significantly inhibited the TNF-α-induced increase in [$^3$H] DA uptake ($F_{5,18} = 5.961, p < 0.01$, one-way ANOVA) (Fig. 2a), although PD98059 (1, 10, 100, and 500 μM, 50 min) itself had no effect on the uptake (Fig. 2b). These results suggest that TNF-α activates DA uptake via the MEK signaling pathway in PC12 cells.

### Effects of TNF-α on METH-induced decrease in DA uptake in PC12 cells

We have previously demonstrated that TNF-α and its inducer diminish the METH-induced decrease in DA uptake and inhibit the rewarding effects of and sensitization to METH (Nakajima *et al.* 2004; Niwa *et al.* 2007c, e). Therefore, we confirmed the effects of TNF-α on the METH-induced decrease in DA uptake in PC12 cells.

METH (0.5, 1, and 10 μM, 30 min) decreased [$^3$H] DA uptake compared with the control group in a dose-dependent manner ($F_{4,15} = 83.675$, $p < 0.01$, one-way ANOVA) (Fig. 3a). Moreover, TNF-α (10 ng/mL, 40 min) inhibited the METH-induced decrease in [$^3$H] DA uptake (TNF-α, $F_{1,16} = 14.759$, $p < 0.01$; METH, $F_{1,16} = 45.994$, $p < 0.01$; TNF-α·METH $F_{1,16} = 1.573$, $p = 0.228$; two-way ANOVA) (Fig. 3b). These results suggest that TNF-α inhibits the METH-induced decrease in DA uptake in PC12 cells (Fig. 3) as well as promoting plasmalemmal and vesicular DA uptake to diminish METH and morphine-induced behavioral sensitization and rewarding effects (Nakajima *et al.* 2004; Niwa *et al.* 2007b; Niwa *et al.* 2008).

### Transfection of the vector containing shati cDNA into PC12 cells

We established a PC12 cell line transfected with the vector containing shati cDNA to examine the role of shati in DA uptake and the METH-induced decrease in DA uptake.

We used immunostaining for TH to check morphological changes of the PC12 cells after the transfection of the vector containing shati cDNA. Morphological changes to the cells were not observed after the transfection compared with mock-transfected or non-transfected PC12 cells (Fig. 4a). To confirm the transfection of the vector containing shati cDNA, we checked for immunostaining against S-4 and GFP, co-expressed with shati. No immunoreactivity for S-4 or GFP was found in the cells that were mock-transfected, which express neither shati nor GFP [Fig. 4b (i)]. The cells mock-transfected (=expression vector [pcDNA-DEST53]), which express GFP, but not shati, were immunopositive for GFP, but not S-4 [Fig. 4b (ii)]. The cells transfected with the vector containing shati cDNA, which express both shati and GFP, were immunopositive for S-4 and GFP [Fig. 4b (iii)]. The cells immunopositive for S-4 were merged with those positive for GFP. These results indicated that shati was certainly expressed in PC12 cells and transfection did not affect cell survival or morphology.

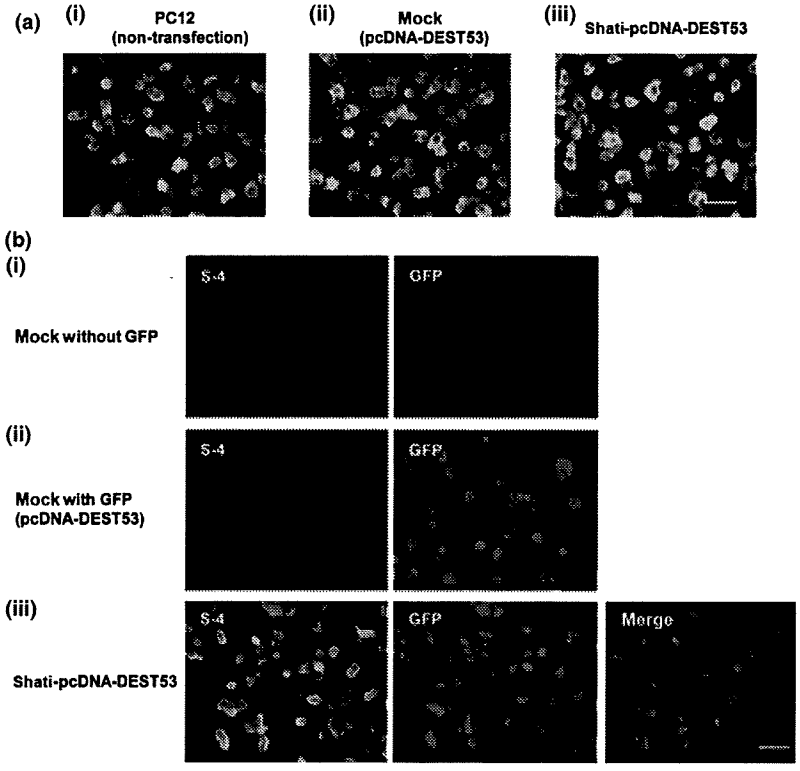



**Fig. 4** Transfection of the vector containing shati cDNA into PC12 cells. (a) The morphological changes of the PC12 cells after transfection of the expression vector (pcDNA-DEST53) (ii) or vector containing shati cDNA (iii). The expression vector alone (mock-transfection) (ii), or the vector containing shati cDNA (iii) was introduced into PC12 cells. There were no changes in survival or morphology in the transfected PC12 cells. Scale bar: 20 μm. (b) Immunostaining of shati in PC12 cells transfected with the vector containing shati cDNA. pENTR/TEV/D-TOPO (without shati recombination and green fluorescent protein (GFP) site) (i), pcDNA-DEST53 with GFP (mock-transfection) (ii), or the vector containing shati cDNA and GFP (iii), was introduced into PC12 cells. The shati-immunopositive cells (green) were colocalized with GFP-immunopositive cells (red). Double immunostaining for S-4 and GFP in PC12 cells transfected with the vector containing shati cDNA reveals overexpression of shati in PC12 cells (iii). Scale bar: 20 μm.

### Effect of over-expressed shati on DA uptake in PC12 cells

We have previously demonstrated that shati-AS, which inhibits the expression of shati mRNA, significantly potentiates the METH-induced decrease in synaptosomal and vesicular [$^3$H] DA uptake compared with that in the shati-SC or CSF-treated mice (Niwa *et al.* 2007a). Moreover, [$^3$H] DA uptake in saline-treated mice was also decreased by shati-AS compared with that in the CSF-treated mice, although shati-SC had no effect on [$^3$H] DA uptake (Niwa *et al.* 2007a). Given the results for synaptosomal and vesicular [$^3$H] DA uptake using shati-AS, we concluded that shati plays a critical role in modulating DA uptake. To address this issue, we investigated the role of shati in DA uptake in PC12 cells transfected with the vector containing shati cDNA.

Transfection of the vector containing shati cDNA increased shati mRNA expression compared with the mock-transfection, suggesting that shati was over-expressed in these cells (Fig. 5a left two columns). The increase in the levels of shati mRNA expression evoked by METH treatment (1 μM, 30 min) in mock-transfected cells was significantly potentiated by shati over-expression in PC12 cells (drug, $F_{1,28} = 20.917$, $p < 0.01$; transfection, $F_{1,28} = 247.684$, $p < 0.01$; drug × transfection, $F_{1,28} = 0.003$, $p = 0.955$; two-way ANOVA) (Fig. 5a right two columns).

We examined the *in vitro* effect of over-expressed shati on [$^3$H] DA uptake in PC12 cells. Shati-over-expressing cells themselves showed increased [$^3$H] DA uptake compared with the mock-transfected cells, suggesting that shati itself promotes DA uptake (Fig. 5b left two columns). We pre-treated PC12 cells with METH (1 μM) for 30 min, and then assayed the uptake of [$^3$H] DA. As shown in Fig. 5b, METH (1 μM, 30 min) decreased [$^3$H] DA uptake compared with the mock-transfected control cells. In the shati-over-expressing cells, the METH-induced decrease in [$^3$H] DA uptake was significantly inhibited compared with that in the mock-transfected cells (drug, $F_{1,40} = 45.807$, $p < 0.01$; transfection, $F_{1,28} = 21.551$, $p < 0.01$; drug × transfection, $F_{1,28} = 0.001$, $p = 0.971$; two-way ANOVA) (Fig. 5b right two columns). These results indicated that shati could attenuate METH-induced inhibition of DA uptake.

### Regulation of TNF-α expression by shati

TNF-α activates synaptosomal and vesicular DA uptake (Nakajima *et al.* 2004). TNF-α and its inducer diminish the METH-induced decrease in DA uptake and inhibit the METH-induced dependence (Nakajima *et al.* 2004; Niwa *et al.* 2007c, e). Moreover, given the findings on [$^3$H] DA uptake obtained using shati-AS (Niwa *et al.* 2007a) and shati-over-expressing cells (Fig. 5b), we hypothesized that shati increased DA uptake by regulating TNF-α. To address this issue, we examined expression levels of TNF-α mRNA after transfection of the vector containing shati cDNA or treatment with shati-AS.

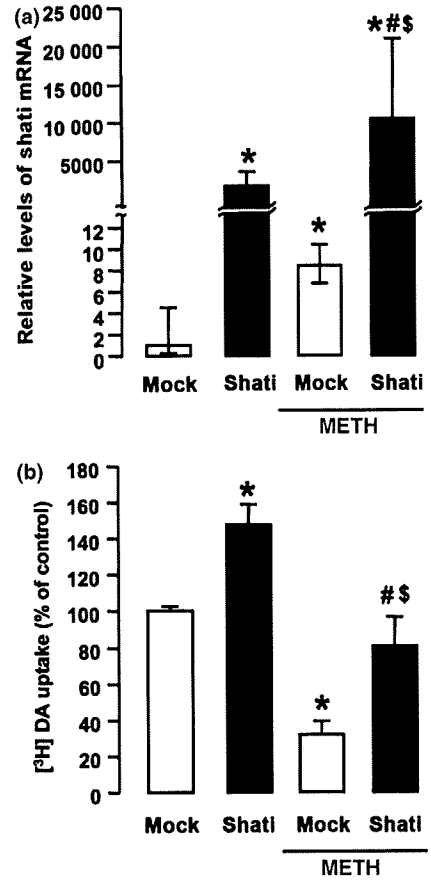
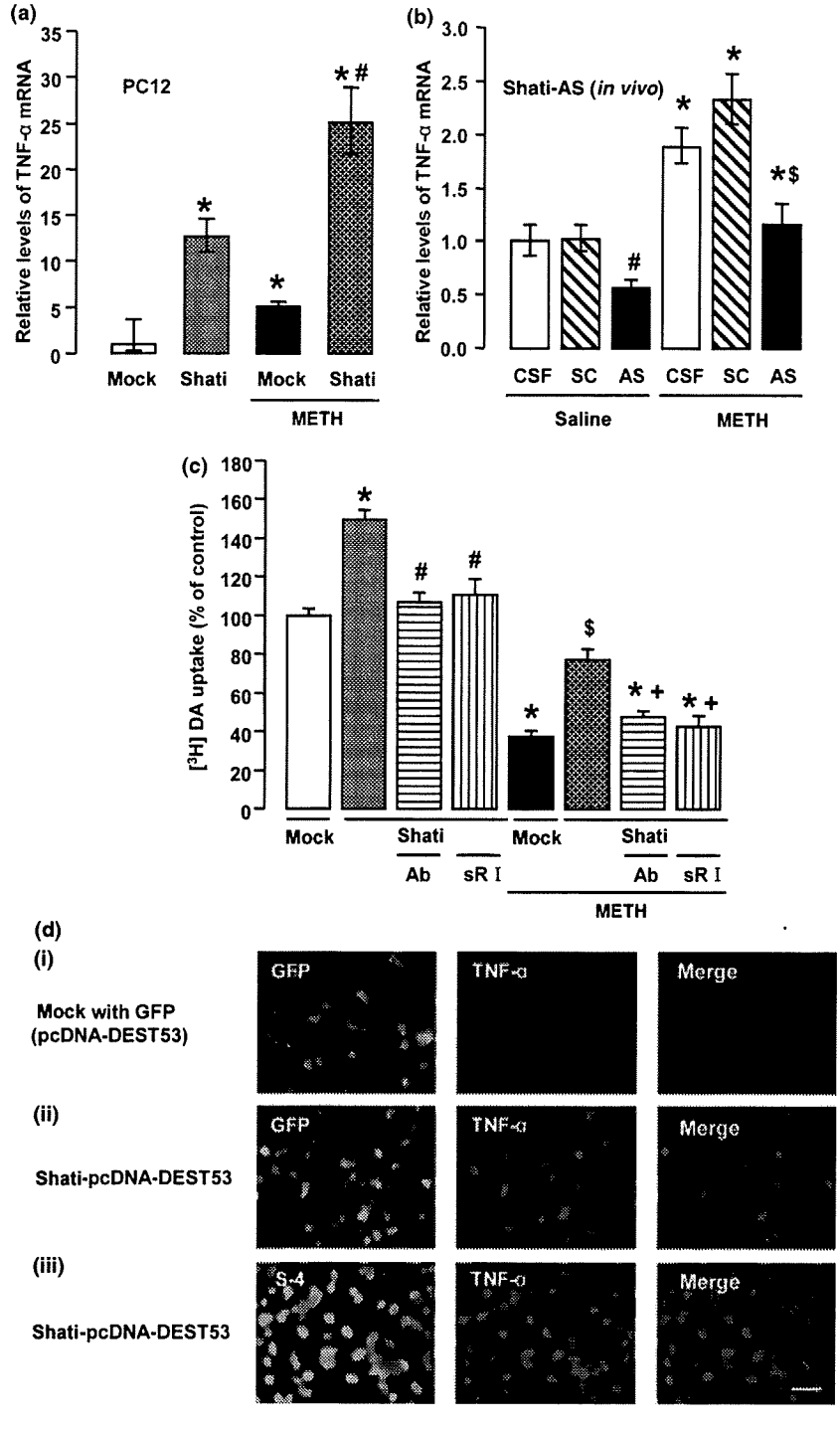



**Fig. 5** Effect of overexpression of shati on DA uptake in PC12 cells. (a) Shati mRNA expression in PC12 cells transfected with the vector containing shati cDNA. The mock construct (pcDNA-DEST53), or the vector containing shati cDNA was introduced into PC12 cells. These cells were treated with 1 μM METH for 30 min. Values are means ± SE ($n = 8$). $^*p < 0.05$ versus mock-transfected cells. $^\#p < 0.05$ versus the vector containing shati cDNA-transfected cells. $^\$p < 0.05$ versus METH + mock-transfected cells. (b) Effect of over-expression of shati on [$^3$H] DA uptake in PC12 cells. The mock construct (pcDNA-DEST53), or the vector containing shati cDNA was introduced into PC12 cells. The cells were pre-treated with 1 μM METH for 30 min, and [$^3$H] DA uptake was measured. The [$^3$H] DA uptake was 0.12 ± 0.02 pmol/10 min for the mock-transfected cells. The final concentration of [$^3$H] DA was 20 nM. Values are means ± SE ($n = 10$–12). $^*p < 0.05$ versus mock-transfected cells. $^\#p < 0.05$ versus the vector containing shati cDNA-transfected cells. $^\$p < 0.05$ versus METH + mock-transfected cells.

Shati-over-expressing cells themselves had increased TNF-α mRNA expression compared with the mock-transfected cells (Fig. 6a left two columns), suggesting that shati regulates expression of TNF-α in PC12 cells. The increase in TNF-α mRNA expression evoked by METH treatment (1 μM, 30 min) in mock-transfected cells was significantly potentiated by overexpression of shati *in vitro* (drug, $F_{1,28} = 21.000$, $p < 0.01$; transfection, $F_{1,28} = 65.860$,

$p < 0.01$; drug × transfection, $F_{1,28} = 3.557$, $p = 0.070$; two-way ANOVA) (Fig. 6a right two columns). As shown in Fig. 6b right three columns, the increase in TNF-α mRNA expression evoked by repeated METH treatment in the NAc was significantly abolished by shati-AS, although shati-SC had no effect. Moreover, TNF-α mRNA expression in the NAc of saline-treated mice was also inhibited by shati-AS, although not by shati-SC (drug, $F_{1,47} = 48.473$, $p < 0.01$; intracerebroventricular treatment, $F_{2,47} = 15.670$, $p < 0.01$; drug × intracerebroventricular treatment, $F_{2,47} = 0.239$, $p = 0.788$; two-way ANOVA) (Fig. 6b left three columns), indicating that shati-AS decreases effectively the expression of TNF-α mRNA through the down-regulation of shati mRNA expression.

As shown in Fig. 6c, right four columns, the ameliorative effect of shati on the METH-induced decrease in DA uptake



was antagonized by treatment with the TNF-αantibody (50 ng/mL) or soluble TNF receptor I (1 ng/mL). The shati-induced potentiation of DA uptake was also inhibited by the treatments in shati-over-expressing cells (drug, $F_{1,72} = 296.090$, $p < 0.01$; transfection, $F_{1,72} = 13.864$, $p < 0.01$; neutralization, $F_{1,72} = 32.930$, $p < 0.01$; drug × transfection, $F_{1,72} = 0.189$, $p = 0.665$; drug × neutralization, $F_{1,72} = 1.496$, $p = 0.225$; transfection × neutralization, $F_{1,72} = 34.828$, $p < 0.01$; drug × transfection × neutralization, $F_{1,72} = 0.003$, $p = 0.958$; three-way ANOVA) (Fig. 6c left four columns). These results suggest that over-expression of shati increased DA uptake by regulating TNF-α in PC12 cells. To confirm the relationship between shati and TNF-α, we examined immunostaining for GFP, which is co-expressed with shati, or S-4 and TNF-α. The cells mock-transfected, which express GFP, but not shati, were immunopositive for GFP, but not TNF-α. The cells transfected with the vector containing shati cDNA, which express both GFP and shati, were immunopositive for GFP or S-4 and TNF-α. The cells immunopositive for S-4 were merged with those positive for TNF-α. These results indicated that shati was expressed in TNF-α-immunopositive cells (Fig. 6d).

## Discussion

DA is the predominant catecholamine neurotransmitter in the CNS. Disruptions of DA signaling contribute to various psychiatric and neurological disorders, including drug addiction, schizophrenia, and Parkinson's disease (Self and Nestler 1995; Hyman 1996). Extracellular DA levels are primarily regulated by DAT, an integral membrane protein that is a member of the $Na^+/Cl^-$-dependent co-transporter gene family (Amara and Kuhar 1993). By removing extracellular DA and recycling it back to the neuron, DAT plays an essential role in terminating DA signaling. Pharmacological blockage of DAT by psychostimulants inhibits the reuptake of DA from the extracellular space, resulting in increased extracellular DA levels and augmented receptor stimulation (Horn 1990). Although pharmacological and genetic ablation (Grace 1995; Jones *et al.* 1998) studies indicate a critical role for DAT in the maintenance of DA neuronal homeostasis, the endogenous mechanisms regulating DAT expression and activity are poorly understood.

The PC12 cell line is derived from the rat pheochromocytoma. It is often used as an *in vitro* model to understand the physiology of central DA neurons (Roda *et al.* 1980; Tischler 2002; Fornai *et al.* 2007). A number of factors contribute to the wide use of PC12 cells: they are inexpensive as well as easy to handle, and mimic many features of central DA neurons. In fact, PC12 cells produce catecholamines (Markey *et al.* 1980; Roda *et al.* 1980; Vaccaro *et al.* 1980). In particular, they contain DA (Greene and Rein 1978) as the main catecholamine and bear DA receptors on their external membrane (Sampath *et al.* 1994). In light of the presence of DA and DA receptors, as well as DA uptake mechanisms, PC12 cell lines are considered to be closer to DA terminals than their ancestors (i.e. chromaffin cells of the adrenal medulla). This concept is reinforced by the presence of monoamine oxidase type A, which also characterizes DA neurons (Finberg and Youdim 1983), in contrast with the established prevalence of monoamine oxidase type B within chromaffin cells of the adrenal medulla (Youdim 1991).

Recently, we have demonstrated that TNF-α and its inducer play a neuroprotective role in the behavioral sensitization to and rewarding effects of METH by activating plasmalemmal and vesicular DAT as well as by inhibiting the METH-induced increase in extracellular DA levels (Nakajima *et al.* 2004; Niwa *et al.* 2007c,e). TNF-α modulates cellular responses through the ERK1/2 and NF-κB signaling pathways (van Vliet *et al.* 2005). The adaptor protein TNF receptor-associated factor 2 (TRAF2) and the serine and threonine protein kinase receptor-interacting protein are required for optimal TNF-induced signaling through ERK1/2, c-Jun N-termial kinase (JNK) and p38 mitogen-activated

**Fig. 6** Involvement of TNF-α in shati-induced increase in DA uptake in PC12 cells. (a) TNF-α mRNA expression in PC12 cells transfected with the vector containing shati cDNA. The expression vector alone (pcDNA-DEST53), or the vector containing shati cDNA was introduced into PC12 cells. The cells were treated with 1 μM METH for 30 min. Values are means ± SE ($n = 8$). $^*p < 0.05$ versus mock-transfected group. $^\#p < 0.05$ versus METH + mock-transfected group. (b) Effect of shati-AS on TNF-α mRNA expression. Mice were administered METH (1 mg/kg, s.c.) for 5 days and decapitated 2 h after the final treatment. Values are means ± SE ($n = 8$–10). $^*p < 0.05$ versus corresponding saline-treated mice. $^\#p < 0.05$ versus saline + CSF and saline + shati-SC-treated mice. $^\$p < 0.05$ versus METH + CSF and METH + shati-SC-treated mice. (c) Involvement of TNF-α in shati-induced increase in [$^3$H] DA uptake in PC12 cells. The expression vector alone (pcDNA-DEST53), or the vector containing shati cDNA was introduced into PC12 cells. The cells were pre-treated with anti-TNF-α antibody (Ab; 50 ng/mL) or soluble TNF receptor I (sR I; 1 ng/mL) 10 min before their treatment with METH (1 μM, 30 min), and assayed for [$^3$H] DA uptake. The [$^3$H] DA uptake was 0.15 ± 0.02 pmol/10 min for the mock-transfected group. The final concentration of [$^3$H] DA was 20 nM. Values are means ± SE ($n = 10$). $^*p < 0.05$ versus mock-transfected group. $^\#p < 0.05$ versus the vector containing shati cDNA-transfected group. $^\$p < 0.05$ versus METH + mock-transfected group. $^+p < 0.05$ versus METH + the vector containing shati cDNA-transfected group. (d) Immunostaining of shati and TNF-α in PC12 cells transfected with the vector containing shati cDNA. The expression vector alone (pcDNA-DEST53) (i), or the vector containing shati cDNA (ii) (iii) was introduced into PC12 cells. The GFP or shati-immunopositive cells (green) were co-localized with TNF-α-immunopositive cells (red) (ii) (iii). Double immunostaining for GFP or S-4 and TNF-α in PC12 cells transfected with the vector containing shati cDNA reveals expression of shati in TNF-α-immunopositive cells (ii) (iii). Scale bar: 20 μm.

protein kinase (p38) (Baud and Karin 2001; Devin *et al.* 2003). MEK inhibitor PD98059 significantly decreases phosphorylated ERK1/2 without affecting total ERK level, MEK-JNK, -p38, and -NF-κB, resulting in loss of DAT surface expression and DAT capacity. According these results, MEK-ERK pathway, but not MEK-JNK, -p38, or -NF-κB pathway, is important for intracellular trafficking and transport capacity of DAT (Morón *et al.* 2003). Therefore, we investigated the involvement of TNF-α in DA uptake and the METH-induced inhibition of DA uptake in PC12 cells. Moreover, we examined the involvement of MEK-ERK signaling in the effects of TNF on DA uptake. TNF-α increased DA uptake via the MEK-ERK signaling pathway in PC12 cells (Figs 1 and 2). The increase was antagonized by the anti-TNF-α antibody and soluble TNF receptor I (Fig. 1b and c), suggesting that TNF-α certainly increases DA uptake in PC12 cells. Moreover, TNF-α inhibited the METH-induced decrease in DA uptake in PC12 cells (Fig. 3b). We have previously reported that the kinetics of [$^3$H] DA uptake in the absence or presence of TNF-α (10 ng/mL). Lineweaver-burk plots show that TNF-α potentiates [$^3$H] DA uptake by increasing the affinity (Km) accompanied by reducing the maximum number of [$^3$H] DATs (Vmax) (Nakajima *et al.* 2004). We suggest that TNF-α modulates the function of DAT, although it also regulates the expression of DAT. The expression of TNF-α is induced through the activation of transcription factors such as activator protein-1 (AP-1) and NF-κB by the activation of JNK/p38 (Guha *et al.* 2000; Rahman and MacNee 2000). Further, TNF-α acts on mitochondria to generate reactive oxygen species, which are involved in the activation of AP-1 and NF-κB (Rahman and MacNee 2000). Changes in transcription factors may result in long-term changes in gene expression, thereby contributing to neuronal adaptations that underlie behavioral sensitization (Nestler 2001). Therefore, we hypothesized that TNF-α inhibits the METH-induced increase in extracellular DA levels in the NAc by promoting DA uptake and finally inhibits METH-induced sensitization and rewarding effects (Nakajima *et al.* 2004; Niwa *et al.* 2007c,e).

'Shati', named after the symbol for Nagoya castle, was identified among molecules whose expression was regulated in the NAc of mice treated with METH (Niwa *et al.* 2007a). Recently, we have demonstrated that blockage of shati expression by shati-AS potentiates the increase in extracellular DA levels in the NAc and the decrease in synaptosomal and vesicular DA uptake in the midbrain induced by repeated METH treatment (Niwa *et al.* 2007a). Both TNF-α and shati increase DA uptake and inhibit the METH-induced decrease in DA uptake (Nakajima *et al.* 2004; Niwa *et al.* 2007a). Therefore, we investigated the precise mechanism of the effects of shati on DA uptake, and the METH-induced inhibition of DA uptake in PC12 cells. Moreover, we examined the relationship between shati and TNF-α in PC12 cells. Over-expression of shati by transfection of the vector containing shati cDNA (Fig. 4) dramatically induced the expression of shati mRNA (Fig. 5a) and TNF-α mRNA (Fig. 6a) in PC12 cells. No histological or mechanical disruption was produced by transfection of the vector (Fig. 4a). Over-expression of shati (Fig. 5a), which occurs in TNF-α-immunopositive cells (Fig. 6d), potentiated DA uptake and inhibited the METH-induced decrease in DA uptake (Fig. 5b) in PC12 cells by regulating TNF-α expression (Fig. 6a), since these effects were antagonized by anti-TNF-α antibody and soluble TNF receptor I used for the neutralization of TNF-α (Fig. 6c; Barone *et al.* 1997). These findings strongly suggest that the over-expression of shati elicited by METH serves as a homeostatic mechanism that prevents behavioral sensitization and rewarding effects by attenuating the METH-induced increase in extracellular DA

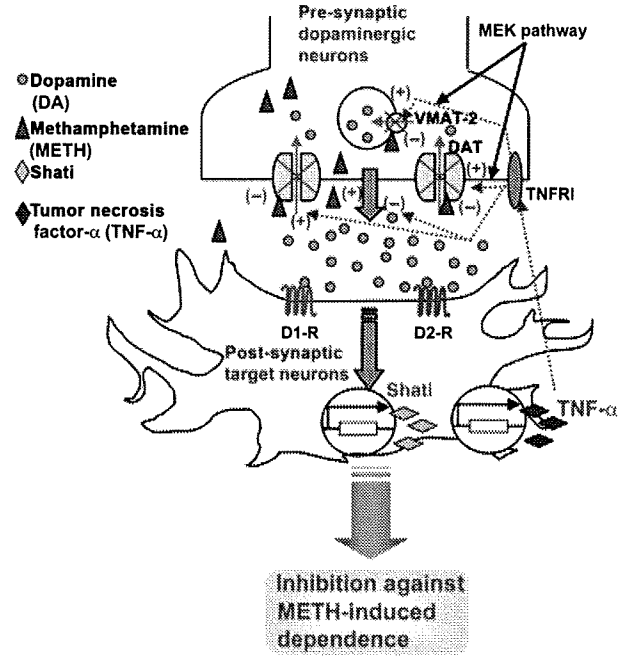



**Fig. 7** Schema for regulation of TNF-α induced by shati on METH-induced DA responses. Under basal conditions, plasmalemmal DAT is involved in the reuptake of extracellular DA into the cytosol; subsequently the cytosolic DA is stored into synaptic vesicles via VMAT-2. Treatment of METH inhibits DA uptake through DA transporter and facilitates DA's release from pre-synaptic nerve terminals. METH is associated with an increase in extracellular DA levels in the brain, resulting in potentiation of the METH-induced dependence. METH induces shati and TNF-α expression in target neurons through the activation of DA receptors. TNF-α regulated by shati inhibits the METH-induced increase in extracellular DA levels in the nucleus accumbens by promoting DA uptake via MEK pathway and finally inhibits sensitization to and the rewarding effects of METH. DA: dopamine, METH: methamphetamine, TNF-α: tumor necrosis factor-α, D1-R: dopamine D1 receptor, D2-R: dopamine D2 receptor, DAT: dopamine transporter, VMAT-2: vesicular monoamine transporter-2, TNFR I: tumor necrosis factor type I receptor.