Overexpression of Shati/Nat8l, an N-acetyltransferase, in the nucleus accumbens attenuates the response to methamphetamine via activation of group II mGluRs in mice

Yoshiaki Miyamoto1, Yudai Ishikawa1, Noriyuki Iegaki1, Kazuyuki Sumi1, Kequan Fu1, Keiji Sato1, Yoko Furukawa-Hibi2, Shin-ichi Muramatsu3, Toshitaka Nabeshima2,4, Kyosuke Uno1 and Atsumi Nitta1

1 Department of Pharmaceutical Therapy and Neuropharmacology, Faculty of Pharmaceutical Sciences, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan
2 Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan
3 Division of Neurology, Department of Medicine, Jichi Medical University, Shimotsuke 329-0498, Japan
4 Department of Regional Pharmaceutical Care and Science, Meijo University, Nagoya 468-8503, Japan

Abstract

A novel N-acetyltransferase, Shati/Nat8l, was identified in the nucleus accumbens (NAc) of mice with methamphetamine (METH) treatment. Previously we reported that suppression of Shati/Nat8l enhanced METH-induced behavioral alterations via dopaminergic neuronal regulation. However, the physiological mechanisms of Shati/Nat8l on the dopaminergic system in the brain are unclear. In this study, we injected adeno-associated virus (AAV) vector containing Shati/Nat8l into the NAc or dorsal striatum (dS) of mice, to increase Shati/Nat8l expression. Overexpression of Shati/Nat8l in the NAc, but not in the dS, attenuated METH-induced hyperlocomotion, locomotor sensitization, and conditioned place preference in mice. Moreover, the Shati/Nat8l overexpression in the NAc attenuated the elevation of extracellular dopamine levels induced by METH in vivo microdialysis experiments. These behavioral and neurochemical alterations due to Shati/Nat8l overexpression in the NAc were inhibited by treatment with selective group II metabotropic glutamate receptor type 2 and 3 (mGluR2/3) antagonist LY341495. In the AAV vector-injected NAc, the tissue contents of both N-acetylaspartate and N-acetylaspartylglutamate (NAAG), endogenous mGluR3 agonist, were elevated. The injection of peptidase inhibitor of NAAG or the perfusion of NAAG itself reduced the basal levels of extracellular dopamine in the NAc of naive mice. These results indicate that Shati/Nat8l in the NAc, but not in the dS, plays an important suppressive role in the behavioral responses to METH by controlling the dopaminergic system via activation of group II mGluRs.

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Introduction

The abuse of methamphetamine (METH) is prevalent throughout the world (Beauvais et al., 2011). However, therapeutic methods have not been established since the key molecules related to METH-induced addiction are unknown. METH induces specific behavioral responses such as hyperlocomotion, locomotor sensitization, and conditioned place preference (CPP) (Nishikawa et al., 1983), and increases extracellular dopamine (DA) levels in the brain (Giros et al., 1996; Sulzer et al., 2005). The drastic increase of DA, especially in the nucleus accumbens (NAc) and dorsal striatum (dS), results in a part of the METH-induced psychostimulative properties (Goodwin et al., 2009). The dopaminergic neuronal system that projects to the NAc from the ventral tegmental area has been implicated in drug dependence (Ikemoto, 2007). The dS is involved in motor disorders, such as Parkinson’s and Huntington’s diseases, and is related to drug addiction (Gerdeman et al., 2003). The cascading loop circuitry from the NAc to the dS plays an important role in the conditioned reinforcement of addictive drugs (Everitt and Robbins, 2005; Di Ciano et al., 2008).
Shati, a novel molecule containing a conserved sequence of the N-acetyltransferase superfamily, is expressed in the NAc in mice treated with METH (Niwa et al., 2007). Recently, Shati was shown to generate expressed in the NAc in mice treated with METH sequence of the N-acetylaspartate (NAA) from aspartate as an N-acetyltransferase 8-like protein (Nat8l) (Ariyannur et al., 2010). Shati/Nat8l mRNA expression was induced by treatment with METH in the NAc and dS (Niwa et al., 2007). Knock down of Shati/Nat8l expression by intraventricular administration of antisense oligonucleotide in mice potentiates METH-induced behavioral alterations and increases extracellular DA levels in the NAc (Niwa et al., 2007). Furthermore, NAA undergoes conversion to N-acetylaspartylglutamate (NAAG), an endogenous metabotropic glutamate receptor type 3 (mGluR3) agonist, via NAAG synthase in neurons (Becker et al., 2010). Thus, a functional role of Shati/Nat8l should be regulated by NAA and NAAG activity. However, the efficacy of Shati/Nat8l expression in specific brain regions on METH-induced responses has not been clarified. Moreover, the mechanism of Shati/Nat8l regulation of METH-induced DA elevation is unknown.

In this study, Shati/Nat8l was overexpressed in the NAc or dS to clarify its mechanism in METH addiction and its regional specificity of function. Overexpression of Shati/Nat8l in the NAc, but not in the dS, of mice attenuated METH-induced hyperlocomotion, locomotor sensitization, CPP and elevation of extracellular DA levels. Treatment with LY341495, an antagonist for group II mGluRs (mGluR2/3), inhibited those suppressive efficacies on behavioral and neurochemical alterations by Shati/Nat8l overexpression in the NAc. In addition, the tissue contents of NAA and NAAG were increased in the AAV vector-injected NAc accompanied with a decreased extracellular DA level. These results suggest that activation of group II mGluRs in the NAc is important for the suppressive function of Shati/Nat8l in METH-induced addiction.

Materials and methods

Animals

Male C57BL/6J mice (Nihon SLIC, Inc., Japan) were 8 wk old and weighed 22–27 g at the beginning of the experiments. Animals were housed in a room with 12 h light/dark cycle (lights on 08:00 hours). Food and water were available ad libitum. All experiments followed the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the committee for Animal Experiments of the University of Toyama.

Drugs

METH hydrochloride and pentobarbital were obtained from Dainippon Sumitomo Pharmaceutical Co. (Japan) and were dissolved in saline. NAAG, 2-(phosphonomethyl) pentanedioc acid (2-PMPA), and LY341495 were purchased from Tocris Bioscience (USA). NAA and 2-PMPA were dissolved in saline. LY341495 was dissolved in 0.125 M phosphate buffer (pH 8.00). NAA was purchased from Sigma-Aldrich (USA).

Production of AAV vector

The production of AAV vector was described previously (Krzyzosiak et al., 2010). Briefly, the AAV vector plasmids contained an expression cassette, consisting of a human cytomegalovirus immediate-early promoter (CMV promoter), followed by cDNA encoding either Shati/Nat8l (Shati: GenBank accession number NM_001001985) or EGFP, and a simian virus 40 polyadenylation signal sequence (SV40 poly (A)), between the inverted terminal repeats of the AAV3 genome. The recombinant AAV vectors were produced by transient transfection of HEK293 cells using the vector plasmid; an AAV2 rep and AAV1 vp expression plasmid, and the adenoviral helper plasmid, pHelper (Agilent Technologies). The recombinant viruses were purified by isolation from two sequential continuous CsCl gradients, and the viral titers were determined by qRT-PCR.

Microinjection of AAV vector

Under pentobarbital (50 mg/kg, i.p.) anesthesia, mice were fixed in a stereotactic frame (SR-5M, Narishige, Japan). AAV-Shati vector (1010 to 1012 unit/0.7 μl/side) suspension was injected bilaterally into the NAc (1.4 mm anterior and 0.6 mm lateral from bregma, 4.2 mm below skull surface) or dS (0.5 mm anterior and 2.0 mm lateral from bregma, 3.5 mm below skull surface) according to the atlas (Paxions and Franklin, 2008). Injection volume was set as in previous studies (Krzyzosiak et al., 2010). The injection was carried out at 0.05 μl/min, and the needle was left at rest in the brain for 10 min after the end of the injection. Mice were used for the experiments 3 wk later.

Quantitative RT-PCR

Whole brains were removed and divided into 1 mm thick sections using mouse brain matrix. Tissue corresponding to the NAc was collected with a 1 mm punch from the section. Similarly, dS tissue was collected using a 1 mm punch from subsequent sections. The accurate location of these brain structures was based on visual inspection of each section using a stereomicroscope and comparison with the stereotaxic atlas of mouse brain (Franklin and Paxinos, 2008). Tissue samples were placed on dry ice and kept at –80°C until use. Total RNA extraction was carried out using the RNeasy Plus Mini Kit protocol (QIAGEN, Japan). Total RNA from each tissue sample was transcribed into cDNA using the Prime Script RT reagent kit (Takara, Japan) according to the manufacturer’s
recommendation. Briefly, the reaction was carried out at 37 °C for 20 min in a total volume of 10 μl and was inactivated at 85 °C for 5 s. Twenty-times diluted cDNA was used as a template, and quantitative real-time PCR was run in a Thermal Cycler Dice Real Time System (Takara) using Power SYBR Green PCR Master Mix (Applied Biosystems, USA) with cDNA and genespecific primers (1 μM) following the manufacturer’s instructions. All of the reactions were performed in duplicate with the following cycling protocol: 10 min of heat activation of the enzyme at 95 °C, 45 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 30 s, and extension at 72 °C for 20 s. The Shati/Nat8l primers used for real-time PCR were as follows: 5'-GGATCTTCTGGCCTACCTGGA-3' (forward bp 457-477) and 5'-CCACTGTTGTTCTCCTCTCA-3' (reverse bp 616-636). 36B4 transcript was used as the internal control. 36B4 transcript amount was quantified using forward primer 5'-ACCCTGAAGTGTGCTGACATC-3' and reverse primer 5'-AGGAGGCTCTGGACTTCTTC-3'.

In situ hybridization

Adult mice were anesthetized with a lethal dose of sodium pentobarbital (50 mg/kg) and perfused through the left ventricle with 50 ml of phosphate buffered saline (PBS) to flush the blood vessels quickly, followed by 50 ml of cold 4% paraformaldehyde (PFA) in PBS. The brains were removed from the skull, postfixed in 4% PFA at 4 °C overnight, followed by cryoprotection in 30% sucrose in PBS overnight at 4 °C. Serial coronal sections of the whole brain were cut at 20 μm thickness on a cryostat. We selected this thickness to check expression of AAV vector-induced genes in the injection brain site (Leica CM 3050, Japan). To generate antisense riboprobes, mouse cDNA sequences for Shati/Nat8l (bp 1133–1557) were amplified by PCR and cloned into the pGEM-T Easy vector (Promega, USA). Digoxigenin (DIG)-labeled antisense and sense cRNA probes were synthesized from linearized plasmids using T7 and SP6 RNA polymerase (Roche, Germany), according to the instructions of the manufacturer. Brain sections from mice were hybridized to antisense and sense cRNA probes as described previously with modification (Nitta et al., 1999). Sections were covered with the hybridization buffer (10% dextran sulfate, 5× standard saline citrate (SSC), 20 μl Tris-HCl pH 8.00, 300 μl NaCl, 50% formamide, 1× Denhardt's solution and 500 ng/ml yeast tRNA) containing 25 ng/ml salmon sperm DNA at 65 °C for 1 h, and then incubated with hybridization buffer containing a DIG-labeled cRNA probe at 65 °C for 16 h. Post-hybridization washes were performed stepwise with 4× SSC at 65 °C for 20 min, 50% formamide in 2× SSC at 65 °C for 30 min, TNE buffer (500 mM NaCl, 10 mM Tris-HCl buffer pH 7.50, 1 mM EDTA) at 37 °C for 10 min, 4 ng/ml RNase A in TNE buffer at 37 °C for 30 min, TNE buffer at 37 °C for 10 min, 2× SSC at 65 °C for 30 min, 0.2× SSC at 65 °C for 30 min, buffer A (150 mM NaCl, 100 mM Tris-HCl pH 7.50) at room temperature for 10 min, and buffer B (1.5% blocking reagent in buffer C [100 mM NaCl, 100 mM Tris-HCl pH 9.50, 50 mM MgCl2]) for blocking at room temperature for 1 h. Subsequently, sections were washed with buffer B at room temperature, and then incubated with 0.75 U/ml anti-DIG-AP Fab fragments in buffer B containing 0.2% Tween-20 at 4 °C for 16 h. After washing with buffer B containing 0.2% Tween-20 at room temperature for 15 min, sections were treated with buffer C, and then with buffer C containing NBT/BCIP Stock Solution (Roche) for different periods to obtain images most appropriate for subsequent development. After washing with buffer C at room temperature for 5 min, the development was stopped by incubation in 1× TE (10 mM Tris-HCI pH 7.5, 1 mM EDTA pH 8.00). The histochemical staining signal was observed by an AxioObserver Z1 (Carl Zeiss, Germany).

Immunostaining

Sections were fixed with 4% PFA for 20 min, and incubated with 0.25% Triton X-100 for 15 min. Sections were treated with 10 μl citrate buffer (pH 6.00) for antigen retrieval at 95 °C for 10 min, washed with Tris buffered saline with Tween-20 (TBS-T), and then blocked in 10% goat serum for 1 h. Sections were incubated with rabbit antibody against GFP (1:1000, Abcam, USA) at 4 °C overnight, washed with TBS-T, and then incubated with CF™ 594 goat anti-rabbit IgG (H+L) (Biotium, USA) at room temperature for 2 h. After being washed and mounted, sections were observed.

Measurement of locomotor activity and sensitization

Mice were placed individually in a transparent acrylic cage with a black frosting Plexiglas floor (45×25×40 cm), and locomotor activity was measured every 5 min for 60 min using digital counters with infrared sensors (Scanet MV-40; MELQEST, Japan). METH (1 mg/kg s.c.) or saline was administered immediately before the measurement of locomotor activity. After repeated METH treatment for 7 d and following METH withdrawal for 5 d, the re-challenge lower dose of METH (0.3 mg/kg s.c.) for locomotor sensitization was administered immediately before the measurement of locomotor activity on Day 13.

CPP test

The place conditioning test was performed according to the method of Miyamoto et al. (2004) and Furukawa-Hibi et al. (2010). Briefly, the apparatus consisted of two compartments: transparent and black Plexiglass boxes (both 15×15×15 cm). The floors of the transparent and
black boxes were covered with white and black frosted Plexiglass, respectively. Each box could be divided by a sliding door (10×15 cm high). In the pre-conditioning, the sliding door was opened and the mouse was allowed to move freely between both boxes for 15 min once a day for 3 d. On day 3, the time that the mouse spent in the transparent and black boxes was measured using the LD mode of a Scanet MV-40 (MELQUEST). The box in which the mouse spent the most time was referred to as the ‘preferred side’, and the other box, the ‘non-preferred side’. The conditioning was performed on six successive days. The mouse was given the drug or vehicle immediately before the conditioning in the apparatus with the sliding door closed. On days 4, 6 and 8, the mouse was given METH (1 mg/kg s.c.) or saline and placed in its non-preferred side for 20 min. On days 5, 7 and 9, the mouse was given saline and placed in its preferred side (opposite to the METH-conditioning side) for 20 min. On day 10, the post-conditioning was performed without drug treatment. In the post-conditioning, the sliding door was opened, and the time that the mouse spent in the transparent and black boxes during 15 min was measured as on day 3. Place conditioning behavior was expressed by Post – Pre, which was calculated as (post value) – (pre value), where post and pre values were the difference in time spent in the METH-conditioning and the saline-conditioning sides in post-conditioning and pre-conditioning, respectively.

**In vivo microdialysis**

We performed in vivo microdialysis as described previously (Niwa et al., 2007). The cannula was placed into the NAc (1.4 mm anterior and 0.6 mm lateral from bregma, 4.2 mm below skull surface) according to the atlas (Paxions and Franklin, 2008). The next day after surgery, a dialysis probe (AI-4-1; 1 mm membrane length, EICOM) was inserted through a guide cannula and perfused with a ringer’s solution (147 mM NaCl, 4 mM KCl, 2.3 mM CaCl2) at a flow rate of 0.5 μl/min by a syringe pump (ESP-64, EICOM). Dialysates were collected in 15 min fraction and injected into the HPLC system (HTEC-500, EICOM) to quantitate extracellular DA levels. Three samples were used to establish baseline levels of extracellular DA.

**Measurement of NAA and NAAG**

The measurement of NAA and NAAG by HPLC was performed as described previously (Reynolds et al., 2005; Takanashi et al., 2012). Brain tissue was homogenized with 10 times the volume of 0.1 M perchloric acid. We applied the solution to pretreated SPEC 3 ml SAX 15 mg anion exchange columns for NAc (Agilent Technologies, USA) followed by extraction with 5.88 ml/l phosphate acid (85%). Samples were injected into the HPLC system (LC-2010CHT, Shimadzu, Japan) with ZORBAX SB-C18 columns (4.6×250 mm, Agilent Technologies). The detector was set at 215 nm. NAA and NAAG peaks in the sample were identified by their retention times compared to standards. Both NAA and NAAG gave linear standard curve and the compounds were quantified using peak height measurements.

**Statistical analysis**

All data are expressed as the mean±S.E.M. Statistical differences between the two groups were determined with a Student-t test. Statistical differences among values for individual groups were determined by analysis of variance (ANOVA), followed by the Student–Newmann–Keuls post-hoc test, when F ratios were significant (p<0.05). In the analysis of the development of locomotor sensitization and in vivo microdialysis, statistical differences were determined by ANOVA with repeated measurement, followed by Bonferroni’s post-hoc test (Prism version 5).

**Results**

**Microinjection of AAV-Shati vector enhanced the expression levels of Shati/Nat8l mRNA in the NAc and dS**

AAV vector containing only EGFP (Mock) or both Shati and EGFP (Shati) sequences (Fig. 1a) were injected into the NAc (Fig. 1b left) (NAc-Mock and NAc-Shati, respectively) or dS (Fig. 1b right) (dS-Mock and dS-Shati, respectively) of mice. Shati/Nat8l mRNA expression levels were measured by quantitative RT-PCR and were presented as relative to the expression of 36B4, the internal control (Krzyszosiak et al., 2010). Shati/Nat8l mRNA levels increased 7.0±0.69 fold in the NAc of NAc-Shati mice compared with that of NAc-Mock mice (Fig. 1c left two columns). No change was observed in the dS (Fig. 1c right two columns). On the other hand, Shati/Nat8l mRNA was elevated 13±1.9 fold only in the dS of dS-Shati mice compared with that of dS-Mock mice (Fig. 1d). As shown in both left upper panels of Fig. 1e and f, Shati/Nat8l mRNA was also detected by in situ hybridization in the NAc and dS, respectively. However, the Mock-injected site showed little detectable Shati/Nat8l mRNA (both left lower panels of Fig. 1e and f). Moreover, immunohistochemistry revealed that there was obvious protein expression of EGFP in the AAV vector injection site of the NAc and dS (both right panels of Fig. 1e and f).

**Overexpression of Shati/Nat8l in the NAc reduced METH-induced hyperlocomotion**

Figure 2a and b show locomotor activity induced by a single METH treatment (1.0 mg/kg s.c.) in mice. In Fig. 2a, both NAc-Mock and NAc-Shati mice exhibited METH-induced hyperlocomotion. However,
METH-induced hyperlocomotion in NAc-Shati mice was decreased significantly compared with that in NAc-Mock mice ($F_{3,33} = 43.16$, $p < 0.0001$). On the other hand, there were no differences of METH-induced locomotor activities between dS-Shati and dS-Mock mice (Fig. 2b).
Overexpression of Shati/Nat8l in the NAc reduced METH-induced locomotor sensitization

We examined the efficacy of overexpression of Shati/Nat8l on locomotor sensitization induced by repeated METH treatments. Mice were administrated METH (1 mg/kg/day, s.c. for 7 d) and were re-challenged with a lower dose of METH (0.3 mg/kg, s.c.) on Day 13 after withdrawal for 5 d. Locomotor activity was measured for 2 h on Day 1, 3, 5, 7, and 13. In Fig. 2c, daily METH treatment for 7 d caused a sensitization to the locomotor-stimulating effects in NAc-Mock ($F_{3,12}=4.814, p<0.05$) and NAc-Shati mice ($F_{3,12}=13.42, p<0.001$). Although there was no significant difference between NAc-Mock and NAc-Shati mice in the time course of locomotor sensitization (AAV vector treatment, $F_{1,8}=16.22, p<0.01$: time, $F_{3,24}=12.38$, $p<0.001$).
p < 0.001: AAV vector treatment × time, $F_{3,24} = 0.92$, $p = 0.4467$), METH-induced hyperlocomotion was significantly reduced in NAc-Shati mice compared with NAc-Mock mice on Day 1, 3, 5 and 7. Also in both dS-Mock ($F_{3,12} = 19.82$, $p < 0.0001$) and dS-Shati mice ($F_{3,15} = 8.562$, $p < 0.01$), repeated administration of METH resulted in the development of locomotor sensitization (Fig. 2f). There was no significant difference in the time course of METH-induced sensitization between dS-Mock and dS-Shati mice on Day 1–7 (AAV vector treatment, $F_{3,16} = 3.39$, $p = 0.0953$: time, $F_{3,30} = 8.60$, $p < 0.0001$: AAV vector treatment × time, $F_{3,30} = 1.59$, $p = 0.2211$). In NAc-Mock and NAc-Shati mice, locomotor activity after re-challenge METH treatment on Day 13 was not decreased compared with Day 1 ($F_{3,16} = 3.212$, $p = 0.0512$), although the challenge dose of METH was lower than the daily dose. Both dS-Mock and dS-Shati mice showed locomotor sensitization after re-challenge with METH on Day 13 ($F_{3,20} = 5.608$, $p < 0.01$), but there was no difference in locomotor activity of dS-Mock and dS-Shati mice on Day 13.

**Overexpression of Shati/Nat8l in the NAc reduced METH-induced CPP**

As shown in Fig. 2c, METH treatment produced place preference in NAc-Mock mice in the place conditioning paradigm. However, the preferred effect of METH in NAc-Shati mice was significantly weaker than that in NAc-Mock mice ($F_{3,15} = 5.412$, $p < 0.05$). On the other hand, the administration of METH induced CPP in both dS-Shati and dS-Mock mice, and there was no difference in the preferred effect of METH between these groups (Fig. 2f) ($F_{3,16} = 7.039$, $p < 0.01$).

The behavioral alterations in locomotor activity, locomotor sensitization and CPP described above indicate that the overexpression of Shati/Nat8l in the NAc, but not in the dS, suppressed METH-induced addictive behaviors.

**Overexpression of Shati/Nat8l in the NAc suppressed the basal levels of extracellular DA and METH-induced elevation of extracellular DA levels**

To clarify the suppressive mechanism of Shati/Nat8l overexpression in the NAc, but not in the dS, for METH-induced behavioral alterations in mice, we measured the METH-induced elevation of extracellular DA levels in the NAc of NAc-Mock or NAc-Shati mice using an in vivo microdialysis method.

Basal levels of extracellular DA in the NAc of NAc-Shati mice were significantly lower than that of NAc-Mock mice (Fig. 3a) (NAc-Mock mice, 1.39 ± 0.33 nM; NAc-Shati mice, 0.28 ± 0.07 nM). METH (1 mg/kg, s.c.)-induced DA elevation was markedly suppressed in the NAc of NAc-Shati mice compared with that of NAc-Mock mice (Fig. 3b) (AAV vector treatment, $F_{3,10} = 35.35$, $p < 0.001$: time, $F_{11,66} = 34.14$, $p < 0.0001$: AAV vector treatment × time, $F_{11,66} = 9.423$, $p < 0.0001$). The increase of extracellular DA levels by METH was observed in both NAc-Shati ($F_{3,27} = 7.121$, $p < 0.0001$) and NAc-Mock ($F_{3,27} = 22.18$, $p < 0.0001$) mice (Fig. 3b). The peak of extracellular DA levels was elevated to over 150 and 260% of the baseline levels in the NAc-Shati and NAc-Mock mice, respectively, by a single METH treatment (Fig. 3b).

**Overexpression of Shati/Nat8l in the NAc increased the tissue contents of NAA and NAAG**

Since Shati/Nat8l synthesizes NAA and its NAA is condensed with glutamate to produce NAAG in the NAc by HPLC method. The tissue contents of NAA and NAAG in the NAc are shown in Fig. 3c and d, respectively. Both NAA and NAAG levels in the NAc of NAc-Shati mice were significantly higher than those of NAc-Mock mice. However, there were no differences in tissue contents of NAA and NAAG in the dS between NAc-Shati and NAc-Mock mice (data not shown).

**The suppressive effect of Shati/Nat8l overexpression in the NAc on METH-induced behavioral alterations was inhibited by the selective group II mGluRs antagonist**

We examined the involvement with group II mGluRs on attenuated METH-induced behavioral alterations in the NAc-Shati mice, using the selective group II mGluRs antagonist LY341495. Figure 4a shows that pretreatment with LY341495 (0.1 mg/kg, i.p.) 30 min prior to METH treatment blocked the suppressive effect of Shati/Nat8l overexpression in the NAc on METH-induced hyperlocomotion ($F_{3,43} = 16.85$, $p < 0.0001$). Furthermore, the suppressive effect of Shati/Nat8l overexpression on METH-induced CPP was blocked by the same pretreatment with LY341495 (Fig. 4b, $F_{5,30} = 4.443$, $p = 0.0020$).

**The suppressive effect of Shati/Nat8l overexpression in the NAc on METH-induced DA elevation was inhibited by the selective group II mGluRs antagonist**

Next, we investigated the suppressive mechanism of Shati/Nat8l overexpression for attenuating the METH-induced elevation of extracellular DA levels in the NAc. In this experiment, we also used the selective group II mGluRs antagonist LY341495. Figure 4c shows that pretreatment with LY341495 (0.1 mg/kg, i.p.) 30 min prior to METH treatment blocked the suppressive effect of Shati/Nat8l overexpression in the NAc on METH-induced DA elevation. Therefore, two-way ANOVA with repeated measures did not reveal significant differences between NAc-Shati and NAc-Mock mice (AAV vector treatment, $F_{1,10} = 0.6003$, $p = 0.4679$: time, $F_{11,66} = 29.22$, $p < 0.0001$: AAV vector treatment × time, $F_{11,66} = 1.217$, $p = 0.2937$). Alternatively, pretreatment with 0.1 mg/kg LY341495 did not affect the METH-induced elevation of extracellular DA levels in the NAc of AAV vector non-injected mice (wild-type
mice) (Fig. 3). Furthermore, in wild-type mice, pretreatment with the higher dose of LY341495 (0.3 mg/kg) markedly increased the METH-induced elevation of extracellular DA levels (Fig. 3). The elevation of NAAG reduced the basal levels of extracellular DA and METH-induced elevation of extracellular DA levels in the NAc. NAAG is known as a highly selective agonist of mGluR3 (Neale et al., 2000). We used 2-PMPA, the selective glutamate carboxypeptidase II (GCP II) inhibitor, to prevent the degradation of endogenous NAAG. Figure 4 shows that the basal levels of extracellular DA were obviously decreased by the administration of 2-PMPA (30 mg/kg, i.p.) in the NAc of wild-type mice (F<sub>8,16</sub> = 35.01, p < 0.0001). Next, we perfused NAAG (0.1 mg/ml, 15 min) through the in vivo microdialysis probe in the NAc of wild-type mice. As shown in Fig. 4f, intra-NAc perfusion of NAAG significantly reduced the basal levels of extracellular DA in the NAc (F<sub>8,16</sub> = 10.74, p < 0.0001). Therefore, both pretreatment with 2-PMPA and preperfusion of NAAG into the NAc attenuated METH-induced elevation of extracellular DA levels in the NAc (Fig. 4e and f).

These observations in aforementioned assays suggest that the elevation of NAAG induced by Shati/Nat8l overexpression in the NAc attenuates the METH-induced elevation of extracellular DA levels via activation of mGluR3, one of the group II mGluRs.

**Discussion**

METH addiction is mediated by multiple brain regions, neurotransmitter systems and bioactive molecules. In the present study, we clarified differential roles of Shati/Nat8l, a novel aspartate N-acetyltransferase, in the NAc and dS. Our results suggest that Shati/Nat8l in the NAc mediates METH-induced behavioral and dopaminergic neuronal responses via activation of group II mGluRs.

The AAV vector is a noteworthy gene delivery tool for therapeutic approaches to neurological diseases, and the safety of this vector for clinical use in the human brain has been confirmed (Muramatsu et al., 2010; Miyazaki et al., 2012). The transfer of AAV vector is stable and long-term gene expression can be attained in neuronal function (Eberling et al., 2009). The AAV vector transduces neurons preferentially, but not glial cells (Davidson et al., 2000; Tenenbaum et al., 2004). Therefore, we used...
Fig. 4. Effect of group II mGluRs antagonist on METH-induced alterations in the NAc-Shati mice and effect of 2-PMPA and NAAG on METH-induced elevation of extracellular DA levels in the NAc in mice. (a, b) Effect of group II mGluRs antagonist on METH-induced locomotor activity (a) and conditioned place preference (b) in the NAc-Shati mice. Pretreatment with LY341495 (0.1 mg/kg, i.p. 30 min prior to METH treatment) canceled attenuated METH-induced behavioral alterations in the NAc-Shati mice. For each group, N=9. *p<0.05, ***p<0.001 vs. corresponding vehicle–saline treatment group, ††p<0.01 vs. vehicle–METH-treated NAc-Mock group (ANOVA followed by the Student–Newman–Keuls post-hoc test). (c) Effect of group II mGluRs antagonist on METH-induced elevation of extracellular DA in NAc-Shati mice. Pretreatment with LY341495 (0.1 mg/kg, i.p. 30 min prior to METH treatment) canceled attenuated METH-induced elevation of extracellular DA in the NAc of NAc-Shati mice. For each group N=4. ANOVA with repeated measures did not reveal significant differences between NAc-Mock and NAc-Shati mice. (d) Effect of group II mGluRs antagonist on METH-induced elevation of extracellular DA in the NAc of NAc-Shati mice. For each group N=4. ANOVA with repeated measures did not reveal significant differences between NAc-Mock and NAc-Shati mice. (e) Effect of GCP II inhibitor (c) and exogenous NAAG (f) on METH-induced elevation of extracellular DA in the wild-type mice. Pretreatment with 2-PMPA (30 mg/kg, i.p. 30 min prior to METH treatment) (e) and NAAG perfusion (0.1 mg/ml, 15 min) (f) inhibited METH-induced elevation of extracellular DA in the NAc of wild-type mice. For each group N=4. *p<0.05, **p<0.01 vs. vehicle–METH treatment group. (ANOVA with repeated measures followed by the Bonferroni’s post-hoc test).
AAV vector to overexpress Shati/Nat8l in the mouse brain. The AAV-Shati vector injection induced the expression of Shati/Nat8l mRNA in the NAc or dS specifically, as assayed by quantitative real-time RT-PCR and in situ hybridization methods (Fig. 1c–f). In addition, this injection also enhanced the tissue contents of NAAG in the NAc (Fig. 3c and d). Thus, it seems the inducible Shati/Nat8l by AAV vector injection possessed N-acetyltransferase activity for aspartate, at least in the NAc.

The psychostimulant, METH, causes hyperlocomotion in rodents (Kitanaka et al., 2005). METH-induced enhancement of locomotor activity was suppressed significantly in the NAc-Shati mice (Fig. 2a). Repeated treatments of METH can produce behavioral sensitization in rodents, characterized by a progressively enhanced locomotor activity (Shen et al., 2010). On Days 1–7 during METH treatments, METH-induced hyperlocomotion was reduced in the NAc-Shati mice (Fig. 2c). METH-induced CPP in mice is a popular model of drug-mediated associative learning in humans (Shen et al., 2006). In the CPP task, the potentiation of place preference by METH was not observed in the NAc-Shati mice (Fig. 2c). These results showed that the overexpression of Shati/Nat8l in the NAc inhibits METH-induced behaviors in mice. METH-induced hyperlocomotion and CPP are closely related to the activation of the dopaminergic system (Kim and Jang, 1997; Wakuda et al., 2008). The efficacy of METH depends primarily on its ability to increase extracellular DA levels in the brain (Clarke et al., 1988; Kuczenski et al., 1995; Goodwin et al., 2009). In this study, the METH-induced elevation of extracellular DA levels was significantly suppressed in the NAc of NAc-Shati mice (Fig. 3b). Thus, our results demonstrate that the attenuation of METH-induced behavioral alterations by Shati/Nat8l overexpression occurs via suppressing the increase of extracellular DA levels in the NAc.

Overexpression of Shati/Nat8l enhanced the tissue contents of not only NAAG (Fig. 3c) but also NAAG (Fig. 3d) in the NAc. NAAG is synthesized by a NAAG synthetase catalyzing the ATP-dependent condensation of NAA and glutamate (Becker et al., 2010), and is the most widely abundant distributed peptide neurotransmitter in mammalian neurons (Neale et al., 2005). It was also found to be a highly selective endogenous mGluR3 agonist (Wroblewska et al., 1997). Group II mGluRs including mGluR2 and 3 are coupled G proteins, which negatively regulate adenylate cyclase activity, and are expressed at moderate-to-high levels in brain regions implicated in drug addiction (Adewale et al., 2006). Pretreatment with the group II mGluRs antagonist LY341495 attenuated the suppressive effect of Shati/Nat8l overexpression on the METH-induced locomotor activity (Fig. 4a), CPP (Fig. 4b) and elevation of extracellular DA levels (Fig. 4c). The elevation of endogenous NAAG by its peptidase inhibitor, 2-PMPA, in naive mice resulted in a reduction in the basal levels of extracellular DA in the NAc (Fig. 4e). Moreover, exogenous NAAG perfusion significantly decreased the basal levels of extracellular DA in the NAc of naive mice (Fig. 4f). The mechanism underlying the regulation of the dopaminergic system by NAAG and mGluR3 is not fully understood. Previously, it was demonstrated that 2-PMPA lowered the basal levels of extracellular glutamate in the NAc of rats, and the effect was blocked by pretreatment with LY341495 (Xi et al., 2010). The NAc receives glutamatergic neuronal afferents from the prefrontal cortex, hippocampus and amygdala (Meredith et al., 1993), and these afferents increase DA release in the NAc (Taber and Fibiger, 1995; Chaki et al., 2006). Therefore, the activation of mGluR3 by NAAG could inhibit glutamate release from the terminals of glutamatergic afferents, which in turn reduces DA release in the NAc. Taken together, these results suggest that Shati/Nat8l may reduce indirectly the basal levels of extracellular DA by elevating NAAG, a selective endogenous mGluR3 agonist. This speculation is supported by the observation that the basal levels of extracellular DA were decreased significantly by 20% in the NAc of NAc-Shati mice (Fig. 3a), which enhanced the tissue contents of NAAG in the NAc.

Another important finding of this study was the regional specificity of the functional contribution of Shati/Nat8l in the brain. Curiously, although the expression levels of Shati/Nat8l mRNA were increased in both the dS of dS-Shati mice and the NAc of NAc-Shati mice, the overexpression of Shati/Nat8l in the NAc, but not in the dS, suppressed METH-induced abnormal behaviors. This discrepancy may be attributed to the distribution of several enzymes and amino acids involved with the functional role of Shati/Nat8l. Shati/Nat8l catalyzes the N-acetylation of aspartate to produce NAA, and then NAAG is synthesized from NAA and glutamate by NAAG synthetase I in the cytoplasm (Becker et al., 2010; Neale et al., 2011). NAAG is released from nerve terminals, most likely via synaptic vesicles, although the transporter into synaptic vesicles for NAAG has not yet been identified. Released NAAG binds to mGluR3 on the presynaptic membranes (Conn and Pin, 1997), and is also degraded by GCP II (Becker et al., 2010), liberating NAA and glutamate (Moffett, et al., 2007). Liberated NAA is translated into astrocytes and oligodendrocytes by sodium-dependent dicarboxylate (NaDC3). Another glutamate liberated by GCP II modulates glutamatergic neurotransmission (Zhou et al., 2005). The next step is to identify differences in the distribution of aspartate, glutamate, NAAG synthetase I, GCP II, and NaDC3 between in the NAc and dS, and to clarify the functional contribution of NAAG to the dopaminergic system in each brain region.

In conclusion, we hypothesize that the expression of Shati/Nat8l in the NAc inhibits METH-induced elevation in the extracellular levels of DA via mediating NAAG and mGluR3. As a result of the suppression on the extracellular levels of DA in the NAc, Shati/Nat8l inhibits...
METH-induced hyperlocomotion and CPP. Since the NAc and dS are involved in A10 and A9 neuronal cell groups, respectively, the different role of Shati/Nat8l between the NAc and dS may be dependent on distinct neuronal regulation in these cell groups. We need further study, but the difference could be a new key point to clarify the mechanisms of drug addiction. Thus, we provide evidence that Shati/Nat8l in the NAc is a key molecule to suppress METH-induced abnormal behaviors by mediating extracellular DA levels via activation of group II mGluRs, probably of mGluR3. Moreover, the Shati/Nat8l-related neuronal system may become a new target of therapy for METH addiction.

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Statement of Interest
None.

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