Inhibitory effects of Shati/Nat8l overexpression in the medial prefrontal cortex on methamphetamine-induced conditioned place preference in mice

Meriem Haddar[1], Kyosuke Uno[1], Katsunori Azuma[1], Shin-ichi Muramatsu[2,3], Atsumi Nitta[1].

[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]Division of Neurology, Department of Medicine, Jichi Medical University, Shimotsuke, Tochigi, Japan.

[3]Center for Gene & Cell Therapy, Institute of Medical Science, The University of Tokyo, Tokyo, Japan.

E-mail: nitta@pha.u-toyama.ac.jp

Running Head; Shati of mPFC attenuates CPP

Abstract

Shati/Nat8l is a novel *N*-acetyltransferase identified in the brain of mice treated with methamphetamine (METH). Shati/Nat8l mRNA is expressed in various brain areas, including the prefrontal cortex (PFC), where the expression level is higher than that in other brain regions. Shati/Nat8l overexpression in the nucleus accumbens (NAc) attenuates the pharmacological response to METH via mGluR3. Meanwhile, dopamine (DA) and glutamate dysregulations have been reported in the medial PFC (mPFC) and NAc after METH self-administration and during reinstatement. However, the mechanism the reward system and function of Shati/Nat8l in the mPFC is unclear. Here, we injected an adeno-associated virus (AAV) vector containing Shati/Nat8l into the mPFC of mice, to overexpress Shati/Nat8l in the mPFC (mPFC-Shati/Nat8l). Interestingly, the METH-induced conditioned place preference (CPP) was attenuated in the mPFC-Shati/Nat8l mice but locomotor activity was not. Additionally, immunohistochemical results from mice that were injected with AAV-GFP showed fluorescence in the mPFC and other brain regions, mainly the NAc, indicating an mPFC-NAc top-down connection. Finally, *in vivo* microdialysis experiments revealed that Shati/Nat8l overexpression in the mPFC reduced extracellular DA levels and suppressed the METH-induced DA increase in the NAc. Moreover, decreased extracellular glutamate levels were observed in the NAc. These

results indicate that Shati/Nat8l overexpression in the mPFC attenuates METH-induced CPP by decreasing extracellular DA in the NAc. In contrast, Shati/Nat8l-mPFC overexpression did not alter METH-induced hyperlocomotion. This study demonstrates that Shati/Nat8l in the mPFC attenuates METH reward-seeking behaviour but not the psychomotor activity of METH.

Key words: dopamine, drug addiction, glutamate, mPFC, NAc, Shati/Nat8l

Introduction

Methamphetamine (METH) addiction is a serious neuropsychiatric disease that does not only reduce the quality of life of addicted people but also greatly affects their health (Grant et al. 2012). However, the currently available treatments for METH-addiction are not fully effective (Courtney and Ray. 2014). Indeed, patients may experience relapse even after a long period of abstinence (Brecht and Herbeck. 2014). A novel *N*-acetyltransferase, Shati/Nat8l, which generates *N*-acetylsapartate from aspartate and acetyl-CoA, has been identified in the nucleus accumbens (NAc) of mice following exposure to METH (Niwa et al. 2007, Ariyannur et al. 2010, Wiame et al. 2010). Recent study has shown that Shati/Nat8l overexpression in the NAc attenuates the METH response (Miyamoto et al. 2014). Shati/Nat8l mRNA is expressed in various brain regions, including the prefrontal cortex (PFC), which shows a higher level of Shati/Nat8l mRNA than other brain regions (Furukawa-Hibi et al. 2012).

The mPFC is a brain region that is considered to be fundamental for psychological processes and is implicated in several psychiatric disorders. The mPFC dynamically interacts with other cortical and subcortical regions (Groenewegen et al. 1997). Thus, the mPFC is described as a control board that integrates information that is received from inputs, processes them and conveys them to other brain regions (Riga et al. 2014, Miller

and Cohen. 2001).

In mice experiments, METH induces hyperlocomotion and conditioned place preference (CPP). These behavioural alterations are mediated by an increase in the levels of DA in the NAc and striatum (STR).

After self-administration of METH and during reinstatement, dopamine (DA) and glutamate dysregulation is observed in the mPFC and NAc of rats (Parsegian et al. 2014). It has also been established that both the mPFC and NAc receive DA projections from the ventral tegmental area (VTA), another key effector brain region in drug addiction. In addition, it is known that the level of DA in the NAc is considered to have an important role in motivation behaviour (Berridge and Robinson. 1998).

Interestingly, a previous study that investigated the effects of cocaine reported that intra-mPFC injection of cocaine reinstated the cocaine seeking behaviour that is blocked by an AMPAR antagonist (Park et al. 2002). Many reports have demonstrated the involvement of glutamate in drug addiction through its action in the NAc (Scofield et al. 2016). Glutamatergic neurons of the mPFC project to the NAc core and shell (Britt et al. 2012; Suska et al. 2013).

However, the mechanism underlying mPFC control over the reward system and the function of Shati/Nat8l in the mPFC is not clear. The purpose of this study is to clarify

the function of Shati/Nat8l in the mPFC in METH addiction. We assessed the role of Shati/Nat8l overexpression in the mPFC using experimental models of METH addiction, including CPP and locomotor activity. Next, we investigated the mechanism underlying the behavioural changes by evaluating DA and glutamate in the NAc of Shati/Nat8l overexpressed mice.

Materials and Methods

Animals and drugs

Male C57BL/6 J mice (8-week-old; 22–27 g; Nihon SLC, Inc. Hamamatsu, Japan) were housed in a room with a 12 h light/dark cycle. Lights were turned on at 7 a.m. (Zeitgeber time ZT 0) and turned off at 7 p.m. (ZT 12). All procedures followed the National Institute of Health Guideline for the Care and Use of Laboratory Animals and were approved by the Animal Experiments Committee of the University of Toyama (Permission Number A2015pha-21 and A2018pha10). METH was purchased from Dainippon Sumitomo Pharmaceutical Co. Ltd. (Osaka, Japan), and was dissolved in saline (0.1 mg/mL). All other reagents were obtained from standard commercial sources.

AAV vector production and microinjection

The method used for the production and microinjection of AAV vector has been reported previously (Krzyzosiak et al. 2010; Iida et al. 2013). Briefly, the AAV vector plasmids contained an expression cassette that included a CMV promoter and cDNA encoding either 3′- 6xHis tagged Shati/Nat8l (NM_001001985) or EGFP. The recombinant AAV-Shati/Nat8l or AAV-Mock vectors were produced by co-transfection of the AAV plasmid,

AAV3 rep/AAV9 vp expression plasmid, and pHelper (Agilent Technologies) in HEK293 cells. The study was approved by the Board of Safety Committee for Recombination DNA Experiments of the University of Toyama (G2015PHA-12).

Mice were anesthetised with a combination of anaesthetics (medetomidine [0.3 mg/kg], midazolam [4.0 mg/kg], and butorphanol [5.0 mg/kg]), and were fixed in a stereotaxic frame (SR-5M, Narishige, Tokyo, Japan). AAV9 CMV 6xHis Shati/Nat8l or CMV 6xHis GFP vector were injected (10^{10} to 10^{12} unit/0.7 μl/side for mPFC-Shati/Nat8l and mPFC-Mock, respectively). The suspension was injected bilaterally into the mPFC (AP 1.7; ML +/-0.3; DV 1.5) according to the mouse brain atlas (Miyamoto et al. 2014, Paxions and Franklin, 2008). The injection volume was set similarly to that used in previous studies (Krzyzosiak et al. 2010). The injection rate was 0.05 μl/min, and the needle remained at rest at the injection site for 10 min after the end of the injection. Experiments were performed on mice 3 weeks after the injection procedure.

Quantitative RT-PCR

Whole brains were removed and divided into 1 mm thick sections using the mouse brain matrix. The tissue that corresponded to the mPFC was collected from the section. The accurate location of the brain structures was based on visual inspection of each section

using a stereomicroscope and comparison with the stereotaxic atlas of mouse brain (Paxinos and Franklin, 2008). The qRT-PCR was conducted according to a previous report (Miyamoto et al. 2014). The total RNA from the tissue sample was transcribed into cDNA using the Prime Script RT reagent kit (Takara, Otsu, Japan) according to the manufacturer's recommendation. Quantitative real-time PCR was run in a Thermal Cycler Dice Real Time System (Takara) using Thunderbird Syber qPCR Mix (Toyobo, Osaka, Japan).

Immunohistochemistry

Using the floating method, sections of 50 μm were placed in 4% PFA for 20 min, washed with PBS and incubated with 0.25% Triton X-100 for 15 min. Sections were treated with 10 mM citrate buffer (pH 6.00) for antigen retrieval at 100°C for 3 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 mouse antibody against GFP IgG (MBL) at 4°C overnight, washed with TBS-T, and then incubated with CF594 goat anti-mouse IgG (H+L) (Biotium, USA) at room temperature for 2 h. After being washed and mounted, sections were observed under a ZEISS fluorescence microscope, model BZ-X700 (Keyence Co., Osaka, Japan)

Behavioural experiments

Locomotor activity

Locomotor activity was evaluated as in previous reports (Miyamoto et al. 2014, Fu et al. 2017). Briefly, mice were placed individually in a transparent acrylic cage with a black frosting Plexiglas floor (45 × 25 × 40 cm). Locomotor activity was measured every 5 min for 60 min using digital counters with infrared sensors (Scanet MV-40; MELQEST, Japan). Mice were subcutaneously (s.c.) injected with METH (0.5 mg/kg) or saline immediately before the measurement of locomotor activity.

CPP test

The place conditioning test was performed according to a previously used method (Myamoto et al. 2014, Fu et al. 2017). Briefly, the apparatus was divided into two compartments (dark and light). The experiment was performed over three sessions, the first session consisted of a 3-day habituation period. On the third day (pre-conditioning) the time spent in each compartment was measured using Scanet MV-40 (MILQEST). The next day, the conditioning session was performed during which the mice were injected with METH (0.5 mg/Kg) or saline (day 4 to 9). Finally, on day 10 (post-conditioning

session), the time spent in each compartment was measured in the same way as in the pre-conditioning session.

In both CPP and locomotor activity, mice were randomly tested in the interval time of ZT 2-11 (between 9 a.m. and 6 p.m.).

In vivo microdialysis

DA measurement

In vivo microdialysis was performed as has been described previously (Miyamoto et al. 2014, Fu et al. 2016 and 2017). The cannula was placed into the NAc shell (1.4 mm anterior and 0.5 mm lateral from the bregma, 3.2 mm below the skull surface) according to the atlas (Paxions and Franklin, 2008). The day after surgery, a dialysis probe (AI-4-1; 1 mm membrane length, Eicom, Japan) was inserted through the guide cannula and perfused with a ringer's solution (147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$) at a flow rate of 0.5 µl/min by a syringe pump (ESP-64, EICOM). The dialysis probe was inserted from 9 a.m. (ZT 2). DA was allowed to stabilize for about 3h. DA baseline levels were measured for 1h after stabilization (ZT 5-6).

METH stimulation

After DA baseline levels assessment, mice were injected with 0.5 mg/Kg METH (s.c.) and the DA levels were measured during the 120 min after injection using *in vivo* microdialysis. DA standard was purchased from SIGMA (Switzerland).

Glutamate measurement

Extracellular glutamate levels were measured in the same position as DA microdialysis and according to the EICOM protocol with a GU-GEL column and an enzymatic column at a flow rate of 1 µL/min. Glutamate standard was purchased from Sigma (France).
We used separate groups of mice to measure DA and glutamate extracellular levels. Both groups of mice were put in the same conditions.

In these experiments we selected the dose of 0.5 mg/kg for METH treatment because we previously demonstrated that METH at the dose of 0.3mg/kg was enough to induce significant CPP (Niwa et al. 2007). Moreover, Kitanaka et al. (2006) used 0.5mg/kg to induce significant CPP .Another group reported that METH administration at 0.5mg/kg induce greater effect on CPP (Shabani et al. 2011).

Statistical Analysis

All data are expressed as mean ± standard error of the mean (S.E.M.). The statistical differences between the two groups were determined using Student's t tests. The statistical differences between the two groups after different drug administrations were determined by a two-way analysis of variance (ANOVA), followed by the Bonferroni's post-hoc tests when the F ratios were significant ($p < 0.05$). To analyse the development of *in vivo* microdialysis, statistical differences were determined by a two-way ANOVA with repeated measurement, followed by the Bonferroni's post-hoc tests (using Prism version 5).

Results

Microinjection of AAV-Shati/Nat8l vector enhanced the expression levels of Shati/Nat8l in the mPFC

Eight-week old mice were divided into two groups, in one group the mPFC was injected with AAV containing Shati/Nat8l and the other group the mPFC was injected with the GFP containing vector as a control group (mPFC-Mock). This process was described in the methods section. Three weeks after the injections, the mice brains were collected and qRT-PCR was performed for the Shati/Nat8l mRNA level, as compared to the house keeping gene 36B4 level. As expected, in the mPFC of the mice that were injected with the Shati/Nat8l AAV containing vector, the Shati/Nat8l mRNA level was more than 9-fold (9.1+/-3.6) higher than in the Mock group ($p < 0.05$; fig 1a).

Microinjection of AAV-GFP vector in the mPFC induced expression of GFP in both mPFC and NAc

Next, we attempted to confirm the injection site of the AAV vectors in the mPFC through immunostaining. The immunohistochemical results following the injection of the AAV vector containing GFP into the mPFC (mPFC-Mock) showed strong green staining in the injection site (Fig. 1b). This result confirmed the location of the injection site. Additionally, we evaluated the expression of GFP in the NAc, because the NAc has a key

role in drug addiction. As expected, green fluorescence was also clearly visible in the NAc (Fig. 1c). According to these results, the mPFC is directly connected to the NAc. This connection suggests a possible influence of the mPFC on the NAc.

Overexpression of Shati/Nat8l in the mPFC attenuated METH-induced CPP

Next, we tried to understand the role of Shati/Nat8l in the mPFC in METH drug dependence. For this purpose, we conducted a CPP test on the mPFC-Shati/Nat8l and mPFC-Mock mice, using a protocol that was described in the methods section and is outlined in Fig. 2a. In this experiment and during the conditioning session, mice were injected with 0.5 mg/kg of METH or saline immediately before the beginning of the experiment. In the post-conditioning session, CPP was induced in the mPFC-Mock group that were treated with METH (251.6+/-37.1 s). Interestingly, Shati/Nat8l overexpression in the mPFC significantly decreased the pre-post value in CPP (51.3+/-36.6 s). This means that the preference for METH was attenuated (Fig. 2b): $F_{Interaction(1,49)} = 7.96$; $p < 0.01$, $F_{Genotype(1,49)} = 4.596$; $p < 0.05$, $F_{Drug(1,49)} = 1.249$; $p > 0.05$.

Overexpression of Shati/Nat8l in the mPFC had no effect on METH-induced hyperlocomotion

After evaluating the CPP, we assessed the role of Shati/Nat8l in METH induced hyperlocomotion. A locomotor activity test was conducted using the same dose of METH

as in the previous experiment (0.5 mg/kg). The locomotor activity was significantly higher in the METH group than in the saline group in both the mPFC-Mock and the mPFC- Shati/Nat8l groups (61934.89+/- 3666.723 and 62461.3 +/- 3123.833 counts/1 h, respectively). However, the mPFC-Shati/Nat8l did not attenuate the METH induced psychomotor activity (Fig.2c and d). $F_{Interaction(1,32)} = 0.23$; $p > 0.05$, $F_{Genotype(1,32)} = 0.08$; $p > 0.05$, $F_{drug(1,32)} = 70.36$; $p < 0.0001$. In these experiments, Shati/Nat8l attenuated the METH-induced CPP but had no effect on the METH-induced hyperlocomotion.

Overexpression of Shati/Nat8l in the mPFC reduced the basal levels of extracellular DA and attenuated METH-induced elevation of extracellular DA levels

To clarify the mechanism through which Shati/Nat8l overexpression in the mPFC affects the METH-induced CPP, we performed a microdialysis of DA in the NAc. We first measured the baseline DA levels, followed by a single s.c. injection of METH (0.5 mg/g; the same dose as was used in the behavioural experiments). The DA levels were measured for 2 h after the injection. Interestingly, the DA baseline levels in the mPFC-Shati/Nat8l mice were 50% lower than those in the Mock group (Fig. 3a). The extracellular DA levels in the mPFC-Mock and mPFC-Shati/Nat8l groups were 0.49+/-0.07 pg/7.5μL and 0.25+/-0.05 pg/7.5μL, respectively.

Additionally, the increase in the level of DA after METH stimulation was also significantly alleviated in mPFC-Shati/Nat8l group (Fig. 3b) $F_{Interaction(11,204)} = 2.23$; $p < 0.05$, $F_{Genotype(11,204)} = 40.64$; $p < 0.0001$, $F_{time(11,204)} = 2.15$; $p < 0.05$. These results suggest that the mPFC not only effectively controls the NAc but that this effect could be mediated by Shati/Nat8l.

Overexpression of Shati/Nat8l in the mPFC reduced the basal levels of extracellular glutamate from NAc

To understand how Shati/Nat8l in the mPFC lowered the level of DA in the NAc, we measured the levels of glutamate in the NAc using *in vivo* microdialysis, targeting the same point that was targeted in the DA measurement. Interestingly, the microdialysis also showed that the extracellular glutamate levels in the NAc in the mPFC-Shati/Nat8l mice (glutamate level was 45.32%) were significantly lower than in the mPFC-Mock group (Fig. 3c). Extracellular glutamate levels for mPFC-Mock and mPFC-Shati/Nat8l were 0.64+/-0.13 pmol/10μL and 0.29+/-0.04 pmol/10μL, respectively.

Discussion

Shati/Nat8l is a novel molecule that has been proven to attenuate the METH response in mice. However, little is known about the mechanism that underlies this effect or the involvement of the mPFC in addiction. In this study, we demonstrated that Shati/Nat8l in the mPFC attenuated METH-induced CPP through a top down control from the mPFC to the NAc, by decreasing the DA levels in the NAc and, thus, reducing the METH-induced DA increase.

As has been previously described (Miyamoto et al. 2014), we injected an AAV vector in to the mPFC to overexpress Shati/Nat8l. After 3 weeks, we observed a significantly higher expression of Shati/Nat8l in the mPFC of mice that were injected with the AAV-Shati/Nat8l vector than in the Mock group (fig. 1a). This confirmed the effectiveness of the AAV-Shati/Nat8l vector. Neurons from the mPFC project to different brain regions that are involved in reward, including the NAc. The immunohistochemical results of this study revealed the projections from the mPFC to the NAc (Fig. 1b and c).

In the behavioural experiments, METH-induced CPP was attenuated by Shati/Nat8l overexpression (Fig. 2b). Hyperlocomotion is also induced by METH, as was demonstrated in the locomotor activity test. However, Shati/Nat8l overexpression in the mPFC had no effect on the psychomotor activity following exposure to METH (Fig. 2c).

The discrepancy in these responses might be explained by the difference between the reward and motor systems, which are distinct entities that have different pathways and mechanisms. This distinction was reported by Kelley and colleagues (Kelley et al. 1997), who used NMDA blockade to separate the reward and locomotor activity in a non-learning context. Effectively, reward is mainly controlled by the NAc while psychomotor activity is controlled by the STR (Goodwin et al. 2009). Additionally, DA terminals in the NAc shell and dorsolateral STR originate mainly from the VTA and substantia nigra, pars compacta, respectively (Haber et al. 2000). This theory is also supported by nicotine addiction experiments. Nicotine has been reported to differentially influence DA signalling in the dorsal STR and NAc shell (Zhang et al. 2009). Furthermore, another report demonstrated that systemic treatment with ceftriaxone, to upregulate the expression of the excitatory amino acid transporter 2, a glutamate transporter, effectively blocked METH-induced CPP but did not alter basal locomotor activity (Abulseoud et al. 2012). Other studies have shown that the NAc shell is mainly related to the reward system, whereas the core is related to psychomotor activity (Sellings et al. 2006).

The circadian rhythm and the time of the day (ZT 6-8 vs ZT 19-21) may affect CPP as observed in C3H/HeN mice (Clough et al. 2014) and the effect is mediated by melatonin which is a hormone mainly produced in the pineal gland. It is also shown that melatonin

affects LA in C57BL/6 mice (Hutchinson et al. 2013). In addition, melatonin in serum and pineal is different between day and dark part of the day (ZT 14 and ZT 22 respectively) as assessed in C3H/HeN mice (Masana et al. 2000). However, in our study, we conducted both CPP and LA experiments in the light part of the day between 9am to 6pm (ZT 2-11) as previously discussed in the material and methods section and performed these behavior experiments more than three times.

In our study, METH induced a psychostimulant effect by blocking the DA transporter, thus decreasing the reuptake of DA to the intracellular space and increasing the levels of DA in the synaptic cleft. Our results demonstrate that Shati/Nat8l overexpression in the mPFC decreases the baseline DA levels in the NAc shell (Fig. 3b). This suggests that the mPFC has an important role in the regulation of DA from the NAc. The top-down control of the NAc by the mPFC is not a new concept. However, in this study, we demonstrated that the mPFC can regulate the extracellular DA level in the NAc. Additionally, we demonstrated that Shati/Nat8l in the mPFC is able to decrease the DA levels in the NAc. Moreover, Shati/Nat8l overexpression diminishes the METH-induced DA increase in the NAc (Fig. 3c). The decrease in the DA level in the NAc is responsible for the reduction in the addictive effects of METH. This decrease in the DA level is caused by a modification in the mPFC that is mediated by Shati/Nat8l. This suggests that the

projections from the mPFC to the NAc are responsible for this alteration, and, as shown through *in vivo* microdialysis of glutamate in the NAc shell, a decrease in the glutamate baseline level was observed (Fig. 3d). In other words, the mechanism underlying the Shati/Nat8l-induced decrease in DA levels and the METH-induced DA increase are probably due to the decrease in glutamate levels in the NAc. Indeed, several studies have reported that the inhibition of glutamate receptors has an attenuating effect on some drugs of addiction (Allen et al. 2005; Pierce et al. 1997). Another report assessed the modulating effect of mGluR group II on DA release (Chaki et al. 2006).

We are considering that the effects of Shati/Nat8l on the reduction of dopamine and glutamate is strongly related to the enzymatic activity of Shati/Nat8l via NAAG and mGluR2/3 (Wroblewska et al. 1997). Activation of presynaptic mGluR2/3 on glutamatergic terminals in the NAc by NAAG could decrease glutamate levels and in turn reduce dopamine in the NAc. We previously assessed that mGluR2/3 antagonist, LY341495, prevented action of Shati/Nat8l on the increased dopamine induced by methamphetamine (Miyamoto et al. 2014). Effectively, Furthermore, intra-NAc perfusion of NAAG reduced basic and potentiated (with methamphetamine) dopamine level.

The hypothesised mechanism of Shati/Nat8l action on METH addiction through top-

down control of the mPFC is displayed in Fig. 4. Shati/Nat8l in the mPFC decreases the level of glutamate in the NAc as shown in this paper; which acts on MSN neurons that projects to VTA GABAergic interneurons (Xia et al. 2011). It has been established that the activation of MSNs leads to the inhibition of VTA GABA cells, and, consequently, disinhibition of DA neurons (Bocklisch et al. 2013). Thus, reducing MSNs activation will increase VTA GABA interneurons activity. In turn, boosting the inhibitory action on dopaminergic neurons that project to the NAc. Finally, this leads to a decrease in the DA levels in the NAc.

In this study, we demonstrated that the mPFC affects the NAc via glutamatergic projections that can alter METH addiction. Furthermore, this effect is mediated by Shati/Nat8l, which reduces extracellular glutamate in the NAc, and, consequently, decreases the DA levels. This mechanism of action is responsible for the attenuation of the METH-induced CPP and DA increase but not METH-induced locomotor activity.

Authors Contribution

MH, KU and AN were responsible for the study concept and design. MH and KA contributed to the acquisition of data for behavioral experiments and *in vivo* microdialysis. KU and SM designed and produced AAV vectors, respectively. KU performed the proteomics analysis. MH assisted

with data analysis and interpretation of findings. MH drafted the manuscript. AN provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved final version for publication.

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Fig. 1. The effect of AAV-Shati/Nat8l and AAV-GFP vectors microinjection in the mPFC. (a) Shati/Nat8l mRNA level expression in the mPFC of Mock and Shati/Nat8l overexpressed mice. N = 5 and 4 (mPFC-Mock and mPFC-shati/Nat8l, respectively) *p < 0.05 (Student-t test). **(b** and **c)** Tissue from the mice that were injected with the AAV-GFP vector (mPFC-Mock) were cut into 50 μm coronal slices and stained with a GFP antibody; **(b)** GFP is strongly expressed in the injected site of the mPFC; **(c)** GFP staining can also be seen in the NAc. Scale bar in (b): 1000 μm; (c)1: 500 μm; (c)2 and 3: 100 μm.

Fig. 2. Effect of overexpression of Shati/Nat8l in the mPFC on METH-induced behavioral alterations. (a) CPP paradigm protocol; mice received METH (0.5 mg/kg s.c.) or saline injections immediately before exposure to one of the light/dark box compartments. **(b)** In mPFC-Mock mice and mPFC-Shati/Nat8l mice, animals were trained for place preference with METH. Place preference data are expressed as the proportion of time spent in the drug-paired conditioning compartment. Columns indicate means ± S.E.M.; N = 13 (Mock saline), 13 (Mock METH), 15 (Shati/Nat8l saline), and 15 (Shati/Nat8l METH). *$p < 0.05$ vs saline group, $^{\#\#}$ $p < 0.01$ vs mPFC-Mock (METH) (two-way ANOVA followed by the Bonferroni's post-hoc test). (**b** and **c)** Locomotor activities in mPFC-Mock mice and mPFC-Shati/Nat8l mice were measured for 60 min after METH administration (0.5 mg/kg, s.c.). Columns indicate means ± S.E.M.; N = 8

(mPFC-Mock saline), 9 (mPFC-Mock METH), 9 (mPFC-Shati/Nat8l saline), and 10 (mPFC-Shati/Nat8l METH); ****$p < 0.0001$ vs mPFC-Mock (saline); ^{ns} $p > 0.05$ (non-significant) vs mPFC-Mock (METH) (two-way ANOVA followed by the Bonferroni's post-hoc test).

Fig. 3. Effect of overexpression of Shati/Nat8l in the NAc on basal levels of extracellular DA, glutamate and METH-induced elevation of DA. (a) Basal levels of extracellular DA in the NAc of mPFC-Mock and mPFC-Shati/Nat8l. N = 10 (mPFC-Mock) and 8 (mPFC-Shati/Nat8l). *p < 0.05 vs. Mock group (Student-t test). **(b)** METH-induced elevation of extracellular DA levels in the NAc of mPFC-Mock and mPFC-Shati/Nat8l mice. N = 10 (mPFC-Mock) and 8 (mPFC-Shati/Nat8l). *p < 0.05 and **p < 0.01 vs. METH-treated Mock group (ANOVA with repeated measures followed by the Bonferroni's post-hoc test). **(c)** *In vivo* microdialysis of glutamate in the NAc of mPFC-Mock vs mPFC-Shati/Nat8l. N = 9 (mPFC-Mock) and 7 (mPFC-Shati/Nat8l). * p<0.05 (Student-t test).

Fig. 4. Hypothesised mechanism of action of Shati/Nat8l in the mPFC during establishment of METH dependence. Shati/Nat8l overexpression in the mPFC induces a decrease in the glutamate level in the NAc. Consequently, there is a decrease in the stimulation of MSN GABAergic neurons that project to the VTA GABAergic

interneurons. This induces an increase in the inhibitory effect of VTA GABAergic interneurons on the dopaminergic neurons that project to the NAc. Finally, the DA level in the NAc is decreased.