

Title:

Induction of neuronal axon outgrowth by Shati/Nat8l via energy metabolism in mice cultured neurons

Short title:

Shati/Nat8l induces neurite outgrowth

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## Abstract

A novel N-acetyltransferase, Shati/Nat8l, was identified in the nucleus accumbens (NAc) of mice repeatedly treated with methamphetamine (METH). Shati/Nat8l has been reported to inhibit the pharmacological action induced by METH. Shati/Nat8l produces *N*-acetylaspartate (NAA) from aspartate and acetyl-CoA. Previously we reported that overexpression of Shati/Nat8l in NAc attenuates the response to METH via *N*-acetylaspartylglutamate (NAAG; which is derived from NAA)-mGluR3 signaling in the mice brain. In the present study, to clarify the type of cells that produce Shati/Nat8l, we carried out in situ hybridization for the detection of Shati/Nat8l mRNA accompanied by immunohistochemical studies using serial sections of mice brain. Shati/Nat8l mRNA was detected in neuronal cells, but not in astrocytes or microglia cells. Next, we investigated the function of Shati/Nat8l in the neuronal cells in mice brain; then, we used adeno-associated virus vector containing Shati/Nat8l for transfection and overexpression of Shati/Nat8l protein into the primary cultured neurons to investigate the contribution to neuronal activity of Shati/Nat8l. Overexpression of Shati/Nat8l in the mice primary cultured neurons induced axonal growth but not dendrite elongation at day 1.5 (DIV). This finding indicated that Shati/Nat8l contributes to neuronal development. LY341495, a selective group II mGluRs antagonist, did not abolish this axonal growth, and NAAG itself did not abolish axon outgrowth in the same cultured system. The cultured neurons overexpressing

Shati/Nat8l contained high ATP, suggesting that axon outgrowth is dependent on energy metabolism. This study shows that Shati/Nat8l in the neuron may induce axon outgrowth via ATP synthesis and not through mGluR3 signaling.

**Key Words :** Shati/Nat8l; NAA; NAAG; cultured neurons

## Introduction

Shati/Nat8l has been identified from the nucleus accumbens (NAc) of mice treated with methamphetamine (METH) using the PCR-based cDNA subtraction method [1]. The genetic locus of Shati/Nat8l is chromosome 5 (5; 5 B2), and Shati/Nat8l protein has *N*-acetyltransferase activity (Gene ID: 269642). Shati/Nat8l attenuates the pharmacological actions of methamphetamine, which are hyperlocomotion or conditioned place preference [2]. Further, Shati/Nat8l is also related to the lipid turnover and energy expenditure in brown adipocytes in mice [3]. Shati/Nat8l produces *N*-acetylaspartate (NAA) from aspartate and acetyl-CoA in central nervous system [4, 5]. NAA is expressed in the neurons at relatively high concentrations, and its level is decreased in patients with psychiatric disorders, such as schizophrenia, Alzheimer's disease, depression, and autism [6, 7, 8]. NAA is used by *N*-acetylaspartylglutamate (NAAG) synthetase to produce NAAG, a highly selective endogenous metabotropic glutamate receptor (mGluR) 3 agonist [9, 10]. mGluRs is considered to be a key receptor to clarify the physiological roles of Shati/Nat8l [9, 10]. Previously we reported that overexpression of Shati/Nat8l in the NAc attenuates the response to METH via the action of NAAG increment in mice [2]. NAA is metabolized to aspartate and acetyl-CoA by aspartoacylase, and then acetyl-CoA is converted to acetate for lipid synthesis in the oligodendrocytes [11]. NAA is produced in the mitochondria and is associated with the TCA cycle

[12]. In the mitochondria, oxaloacetate and glutamate are converted to aspartate and  $\alpha$ -ketoglutarate by aspartate amino transferase, and while aspartate is being converted to NAA by *N*-acetyltransferase,  $\alpha$ -ketoglutarate can enter the TCA cycle. Although there are many reports about NAA, the method of localization and function of Shati/Nat8l in neurons remains unclear. A review paper reported two patterns of localization of Shati/Nat8l in the brain [13]. First, Myc-tagged-Shati/Nat8l was transfected to cultured Chinese hamster ovary (CHO) cells and primary cultured neurons, which showed that Shati/Nat8l localized in the endoplasmic reticulum [4]. Alternatively, Ariyannur et al. (2010) showed that Shati/Nat8l was expressed in the mitochondria of neurons. From aforementioned results, the role of Shati/Nat8l in the neuronal systems could not be conclusively elucidated. To investigate the same, we used a different approach, to clarify the main role of Shati/Nat8l in the neuronal system.

In the present study, we transfected an adeno-associated virus (AAV) vector containing Shati/Nat8l into the primary cultured neurons. Overexpression of Shati/Nat8l in primary cultured neurons induced axonal growth but not dendrite elongation. The treatment with LY341495, a selective group II mGluRs antagonist, did not cancel Shati/Nat8l-induced axon outgrowth, and NAAG itself did not induce axon outgrowth. Overexpression of Shati/Nat8l also increased the ATP content in neurons. These results suggest that neuronal Shati/Nat8l induces axon outgrowth via ATP synthesis independently of mGluR3 signaling pathway.

## **Materials and Methods**

### *Animals*

The experiments were performed using mice (8 weeks old) and mice embryos (embryonic day 15) of the ICR strain (SLC, Japan). The animals were housed in a room with 12 h light/dark cycles (light cycle starting at 8:00 AM.). 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.

### *In situ hybridization and immunostaining of mice brains*

The mice were anesthetized with a lethal dose of sodium pentobarbital (50 mg/kg i.p.) and perfused through the left ventricle, first with 50 ml of phosphate-buffered saline (PBS) to flush the blood vessels quickly, followed by 50 ml of cold PBS containing 4% paraformaldehyde (PFA). The brains were removed, post-fixed in 4% PFA at 4°C overnight, followed by overnight cryoprotection in 30% sucrose with PBS at 4°C. The brain sections (14 µm) were treated with digoxigenin (DIG)-labeled Shati/Nat8l antisense riboprobes (accession no. NM\_001001985; nucleotides [2033 – 2845]). After hybridization, the sections were incubated

with anti-DIG-AP Fab fragments. After washing, the sections were treated with nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP). **In the pre-experiments, we did *in situ* hybridization using the brain slices of Shati/Nat8l knock out mice, any positive signal was not observed (data not shown).**

Primary antibodies were NeuN (1:200; MAB377, Millipore, USA), GFAP (1:200; **3670**, Cell Signaling Technology, USA), and Iba1 (1:200; **019-19741**, Wako, Japan). Secondary antibodies were CF<sup>TM</sup>488 goat anti-mouse IgG (H + L) (1:1000; **20018**, Biotium, USA) and CF<sup>TM</sup>594 goat anti-rabbit IgG (H + L) (1:1000; **20113**, Biotium).

#### *Primary culture of hippocampal and cortical neurons and immunostaining*

Neurons were prepared from the hippocampus and cortex of fetuses from female mice on day 15 of gestation, as previously described [14]. The **cultured neurons** were fixed with PFA in PBS and permeabilized with 0.1% Triton, and 3% normal goat serum (NGS; **G9023**) in PBS. The cells were incubated overnight with a primary antibody [anti microtubule associated protein 2 (MAP2) (1:1000; **M4403**, Sigma), and anti-green fluorescence protein (GFP) (1:1000; ab6556, Abcam), and anti-Tau-1 (1:1000; Millipore)] in PBS containing 2% NGS at 4°C. The cells were subsequently incubated with CF<sup>TM</sup>594 goat anti-mouse IgG (1:1000; **20111**, Biotium) or



CF<sup>TM</sup>488 goat anti-rabbit IgG (1:1000; 20019, Biotium) in PBS containing 2% NGS for 2 h at room temperature.

#### *Measurement of the axon outgrowth and the dendrite elongation*

The measurement of the axon outgrowth and the dendrite elongation was taken with modifications, as previously described [15]. After the cultured neurons were incubated for 1 h at 37°C, half of the medium was changed to D-MEM containing 0.2% (v/v) AAV-vector. The production of AAV vector was previously described [2]. We selected the experimental schedule described by Carlos [15]. To assess the axon outgrowth and dendrite elongation, the neurons were co-immunostained with anti-GFP and anti-Tau-1 antibodies at DIV 1.5 and with anti-GFP and anti-MAP2 antibodies at DIV 4, respectively. We randomly evaluated three different GFP positive cells per dish and considered their average to represent the score of the cultured dish. Each group was tested using 5 dishes in one experiment. We selected the doses of NAAG and LY341495, a selective group II mGluRs antagonist, following previously reports [16, 17].

#### *Quantitative RT-PCR and measurement of NAA and NAAG*

Quantitative RT-PCR was performed as previously described [2]. The measurement of NAA and NAAG by high performance liquid chromatography (HPLC) was performed as previously described [2].

#### *Measurement of ATP contents*

To measure the ATP contents during axon outgrowth, the neurons were collected in cold PBS at DIV1.5. ATP contents were measured according to the instructions of ATP assay kit (Abcam, USA).

#### *Statistical Analyses*

All data were expressed as the mean  $\pm$  standard error of mean (S.E.M.). Statistical differences between the two groups were determined with a Student-*t* test. Statistical differences among the values for individual groups were determined by analysis of variance (ANOVA) followed by Bonferroni's post-hoc test (Prism version 5).

## Results

### *Shati/Nat8l mRNA expression in mice whole brain and neuronal cells.*

We investigated Shati/Nat8l mRNA expression in the mouse brain using *in situ* hybridization (Figure 1). **Shati/Nat8l mRNA signal was observed any brain regions investigated in this study. Especially, the strong signals were detected in the cortical pyramidal cells, dentate granule cells, hippocampal pyramidal cells and cerebellar granule cells.** Neuronal cells of all brain regions were Shati/Nat8l mRNA positive. We chose to focus on the hippocampus because it contains many kind of cells, it is easy to evaluate, and it has high cell density. Hippocampus serial sections were used for both *in situ* hybridization and immunohistochemistry with neuronal and glial cell markers (Figure 1). Shati/Nat8l mRNA positive cells colocalized with NeuN (a marker of neuron) positive cells but not with GFAP (a marker of astrocyte) or Iba1 (a marker of microglia) positive cells. This result shows that Shati/Nat8l mRNA is only expressed in the neuronal cells of the mouse brain.

### *Overexpression of Shati/Nat8l affects axonal growth but not dendrite elongation of primary cultured neurons.*

To validate neuronal function of Shati/Nat8l, we measured dendrite elongation and axon outgrowth in the cultured Shati/Nat8l overexpressed neurons, since they indicate the important

role of neuronal development. We used the marker protein MAP2 for dendrite elongation evaluation (Figure 2a) and Tau-1 for axon outgrowth evaluation (Figure 2b). There was a significant difference in the axonal length between neurons overexpressing Shati/Nat8l and controls ( $t_{14} = 5.722$ ,  $p < 0.05$ ) (Figure 2c). There were no differences in the total dendrite length ( $t_{14} = 0.3095$ , n.s.), dendrite length ( $t_{14} = 0.3547$ , n.s.), number of processes ( $t_{14} = 1.144$ , n.s.), and number of brunch points ( $t_{14} = 1.602$ , n.s.) between Shat/Nat8l overexpressed neuron and mock neuron (Figure 2d-g). These results show that Shati/Nat8l induces axon outgrowth but not dendrite elongation in primary cultured neurons.

*mGluR3 signaling has no effect on Shati/Nat8l-induced axon outgrowth of primary cultured neurons.*

To investigate the contribution of group II mGluR3 signaling on Shati/Nat8l-induced axon outgrowth, we used NAAG and LY341495, a selective group II mGluRs antagonist for the cultured neurons as previously described [15, 16]. Figure3 shows that LY341495 does not affect Shati/Nat8l-induced axon outgrowth (Mock vs Shati/Nat8l,  $F_{3,56} = 5.659$ ,  $p < 0.001$  ; Shati/Nat8l vs Shati/Nat8l + LY,  $F_{3,56} = 1.605$ , n.s.) (Figure 3a, right two columns). To test whether NAAG also induces axon outgrowth, we exposed the cultured neurons to NAAG.

Figure 3b shows that NAAG does not induce axon outgrowth (Control vs NAAG 100  $\mu$ M,  $F_{3,45} = 19.76$ , n.s.). These results suggest that mGluR3 signaling does not affect axon outgrowth.

*Overexpression of Shati/Nat8l in cultured neurons increased the ATP content*

Since it is proposed that NAA synthesis is involved in mitochondrial function [5], we measured the contents of NAA and ATP in cultured neurons. Shati/Nat8l mRNA levels increased  $2.3 \pm 0.34$  folds in the AAV-Shati/Nat8l neurons compared to the AAV-Mock neurons (Figure 3c,  $t_4 = 5.502$ ,  $p < 0.05$ ). Shati/Nat8l increased the content of ATP in the cultured neurons (Figure 3d,  $t_4 = 5.431$ ,  $p < 0.01$ ). The level of NAA in AAV-Shati/Nat8l neurons was significantly higher than in AAV-Mock neurons (Figure 3e,  $t_2 = 4.419$ ,  $p < 0.05$ ), whereas there was no difference in NAAG level between these neurons (Figure 3f,  $t_2 = 1.855$ , n.s.). These results suggest that Shati/Nat8l is involved in the mitochondrial function via NAA synthesis.

## Discussion

The present study demonstrates that Shati/Nat8l is expressed in neuronal cells in every region of the mouse brain (Figure 1a) and induces axon outgrowth accompanied with ATP increment. The finding that Shati/Nat8l mRNA is expressed in neuronal cells suggests that Shati/Nat8l has a functional role in the brain (Figure 1a and b). We demonstrated that overexpression of Shati/Nat8l in neurons induced axon outgrowth but not dendrite elongation (Figure 2c). Moreover, LY341495 is unable to abolish the Shati/Nat8l induced-axon outgrowth, and NAAG didn't induce axon outgrowth by itself (Figure 3b). Overexpression of Shati/Nat8l increases ATP content in neurons (Figure 3d). Therefore, Shati/Nat8l in the neuron may be associated with the ATP synthesis pathway.

We previously reported that Shati/Nat8l is associated with the microtubule structure using Shati/Nat8l overexpression in COS7 cells and mice primary cultured neurons [18]. On the other hand, it was also reported that Shati/Nat8l colocalizes with the mitochondrial marker in SH-SH5Y cells [5], and that Shati/Nat8l is localized in the endoplasmic reticulum [4]. Thus, the localization of Shati/Nat8l required further clarification. Therefore, in the present study, we used a new method "AAV-vector expressing Shati/Nat8l" to clarify the novel and main functions of Shati/Nat8l in the neuronal cells.

NAA is predicted to be produced in the mitochondria because it is associated with the TCA cycle related to the metabolism in the cell [12]. Here, we showed that overexpression of Shati/Nat8l increases the levels of NAA and ATP in primary cultured neurons. The TCA cycle produces ATP molecules at the highest rate in terms of cell metabolism, and ATP in the growth cone is known to promote neurite elongation in cultured neurons [19]. Neuronal dendrite lengths of Shati/Nat8l knock-out mice were significantly short compared with those of the wild-type mice [16]. Taken together, Shati/Nat8l appears to play a major role in ATP induced-neurite elongation. Shati/Nat8l is an indicator for the stimulation of mGluR3. However, neither NAAG nor Ly341495, the endogenous agonist of mGluR3 and an antagonist of mGluR3, respectively, affected axon outgrowth. Therefore, Shati/Nat8l is associated with neurite elongation and ATP synthetic pathway via its function in NAA synthesis.

In conclusion, we show a unique neuronal function of Shati/Nat8l in AAV-vector harboring Shati/Nat8l cDNA to overexpress Shati/Nat8l in the neuron, we are able to show that Shati/Nat8l induces axon outgrowth with increases in ATP content in the absence of mGluR3 pathway activation. These results suggest that Shati/Nat8l is associated with axon outgrowth via the ATP synthetic pathway via its function in NAA synthesis. Role of NAA in the brain function is not clear, although NAA level is decreased in patients with various psychiatric disorders [6, 7, 8]. Further investigation of the Shati/Nat8l could provide new insights into the roles of

Shati/Nat8l and NAA in the brain function. Shati/Nat8l and/or NAA might be new target of the clinical stratagem for neuronal and psychiatric diseases. Especially NAA would be one of indicators of neuronal activity and function in brains.



## References

1. Niwa M, Nitta A, Mizoguchi H, Ito Y, Noda Y, Nagai T, et al. A novel molecule “Shati” is involved in methamphetamine-induced hyperlocomotion, sensitization, and conditioned place preference. *J Neurosci.* 2007 ; 27 : 7604 -7615.
2. Miyamoto Y, Ishikawa Y, Iegaki N, Sumi K, Fu K, Sato K, et al. Overexpression of Shati/Nat8l, an N-acetyltransferase, in the nucleus accumbens attenuates the response to methamphetamine via activation of group II mGluRs in mice. *Int J Neuropsychopharmacol.* 2014 ; 17 : 1283 - 1294.
3. Pessentheiner AR, Pelzmann HJ, Walenta E, Schweiger M, Groschner LN, Graier WF, et al. NAT8L (N-acetyltransferase 8-like) accelerates lipid turnover and increases energy expenditure in brown adipocytes. *J Biol Chem.* 2013 ; 288 : 36040 - 36051
4. Wiame E, Tyteca D, Pierrot N, Collard F, Amyere M, Noel G, et al. Molecular identification of aspartate N-acetyltransferase and its mutation in hypoacetylaspartia. *Biochem J.* 2010 ; 425 : 127 - 136.
5. Ariyannur PS, Moffett JR, Manickam P, Pattabiraman N, Arun P, Nitta A, et al. Methamphetamine-induced neuronal protein NAT8L is the NAA biosynthetic enzyme: implications for specialized acetyl coenzyme A metabolism in the CNS. *Brain Res.* 2010 ; 1335 : 1 - 13.

6. Callicott JH, Bertolino A, Mattay VS, Langheim FJ, Duyn J, Coppola R, et al. Physiological dysfunction of the dorsolateral prefrontal cortex in schizophrenia revisited. *Cereb Cortex*. 2000 ; 10 : 1078 - 1092.
7. Aoki Y, Abe O, Yahata N, Kuwabara H, Natsubori T, Iwashiro N, et al. Absence of age-related prefrontal NAA change in adults with autism spectrum disorders. *Transl Psychiatry*. 2012 ; 23 : 178.
8. Tae WS, Kim SS, Lee KU, Nam EC, Koh SH. Progressive decrease of N-acetylaspartate to total creatine ratio in the pregenual anterior cingulate cortex in patients with major depressive disorder: longitudinal 1H-MR spectroscopy study. *Acta Radiol*. 2013 ; 12 : 594 - 603.
9. Neale JH, Bzdega T, Wroblewska B. N-acetylaspartylglutamate: the most abundant peptide neurotransmitter in the mammalian central nervous system. *J Neurochem*. 2000 ; 75 : 443 - 452.
10. Neale JH, Olszewski RT, Zuo D, Janczura KJ, Profaci CP, Lavin KM, et al. Advances in understanding the peptide neurotransmitter NAAG and appearance of a new member of the NAAG neuropeptide family. *J Neurochem*. 2011 ; 118 : 490 - 498.
11. Chakraborty G, Mekala P, Yahya D, Wu G, Ledeen RW. Intraneuronal N-acetylaspartate supplies acetyl groups for myelin lipid synthesis: evidence for myelin-associated aspartoacylase. *J. Neurochem*. 2001 ; 78 : 736 - 745.

12. Madhavarao CN, Chinopoulos C, Chandrasekaran K, Namboodiri MA. Characterization of the N-acetylaspartate biosynthetic enzyme from rat brain. *J Neurochem.* 2003 ; 86 : 824 - 835.
13. Moffett JR, Arun P, Ariyannur PS, Namboodiri AM. N-Acetylaspartate reductions in brain injury: impact on post-injury neuroenergetics, lipid synthesis, and protein acetylation. *Front Neuroenergetics.* 2013 ; 5 : 11.
14. Nitta A, Ito M, Fukumitsu H, Ohmiya M, Ito H, Sometani A, et al. 4-methylcatechol increases brain-derived neurotrophic factor content and mRNA expression in cultured brain cells and in rat brain in vivo. *J Pharmacol Exp Ther.* 1999 ; 291 : 1276 – 1283.
15. Dotti CG, Sullivan CA, Banker GA. The Establishment of polarity by hippocampal neurons in culture. *J Neurosci.* 1988 ; 8 : 1454 – 1468.
16. Berent-Spillson A, Robinson AM, Golovoy D, Slusher B, Rojas C, Russell JW. Protection against glucose-induced neuronal death by NAAG and GCP II inhibition is regulated by mGluR3. *J Neurochem.* 2004 ; 89 : 90-99.
17. Wang MJ, Li YC, Snyder MA, Wang H, Li F, Gao WJ. Group II metabotropic glutamate receptor agonist LY379268 regulates AMPA receptor trafficking in prefrontal cortical neurons. *PLoS One.* 2013 ; 8 : e61787
18. Toriumi K, Ikami M, Kondo M, Mouri A, Koseki T, Ibi D, et al. SHATI/NAT8L regulates neurite outgrowth via microtubule stabilization. *J Neurosci Res.* 2013 ; 91 : 1525 - 1532.

19. Höpker VH, Saffrey MJ, Burnstock G. Neurite outgrowth of striatal neurons in vitro: involvement of purines in the growth-promoting effect of myenteric plexus explants. *Int J Dev Neurosci.* 1996 ; 14 : 439 - 451.

## Figure legends

### **Figure 1. Shati/Nat8l mRNA is expressed in the neuronal cells of the mouse brain.**

(a,b) *In situ* hybridization analyses show the expression pattern of Shati/Nat8l mRNA in the mouse brain. The small figure shows the positive-cells in detail. (b - e) NeuN, GFAP, and Iba1 were detected in the same brain region by the immunostaining. Scale bars: in the large figure = 500  $\mu\text{m}$ , in the small figure = 50  $\mu\text{m}$

### **Figure 2. Overexpression of Shati/Nat8l in the neuron induces axon outgrowth but not dendrite elongation.**

(a) The neurons were infected with AAV-Mock or AAV-Shati/Nat8l 1 h after the culture was started, and Tau-1 immunostaining shows the morphology of neuronal axons at 1.5 DIV. GFP positive-cells correspond to neurons transfected with the AAV-vector. (b) Quantitative analysis shows that overexpression of Shati/Nat8l increases axon length at 1.5 DIV (n = 5 dishes for each group). (c) MAP2 immunostaining shows the morphology of the neuronal dendrites at 4 DIV. GFP positive-cells correspond to neurons transfected with the AAV-vector. (d-g) Dendritic length, total neurite length, the number of processes, and the number of branch points per neuron were analyzed. No effects were observed in the total dendrite length, dendrite length,

the number of processes, and the number of branch points by overexpression of Shati/Nat8l. n = 5 dishes for each group, \* $p < 0.05$  vs AAV-Mock (Student's t-test).

**Figure 3. mGluR3 signaling is not needed for Shati/Nat8l-induced axon outgrowth of cultured neurons.**

(a) The neurons infected with AAV-Mock or AAV-Shati/Nat8l were treated with 100  $\mu\text{M}$  LY341495 1h after infection with the AAV vector. Tau-1 and GFP were stained at 1.5 DIV. GFP positive-cells correspond to neurons transfected with the AAV-vector. Quantitative analysis shows that inhibition of mGluR3 with 100  $\mu\text{M}$  LY341495 does not affect Shati/Nat8l-induced axon outgrowth. n = 5 dishes for each group, \*\* $p < 0.01$  vs. AAV-Mock, \*\*\* $p < 0.001$  vs. AAV-Mock (ANOVA with repeated measures followed by the Bonferroni's post-hoc test). (b) The neurons were treated with NAAG 2 h after the culture was started. Quantitative analysis shows that activation of mGluR3 with NAAG does not affect Shati/Nat8l-induced axon outgrowth. n = 5 dishes for each group. (c) Expression levels of Shati/Nat8l mRNA in the AAV-Mock and AAV-Shati/Nat8l neurons were measured at 1.5 DIV. n = 3 dishes for each group, \* $p < 0.05$  vs. AAV-Mock (Student's t-test). (d) The ATP content in the neuron was analyzed at 1.5 DIV. Quantitative analysis shows that overexpression of Shati/Nat8l significantly increases the ATP content compared with Mock. n = 5 dishes for each group, \*\* $p <$

0.01 vs AAV-Mock (Student's t-test). (e, f) The contents of NAA (e) and NAAG (f) in the AAV-Mock and AAV-Shati/Nat81 neurons were compared using HPLC at 1.5 DIV. n = 3 dishes for each group. \* $p < 0.05$  vs. AAV-Mock (Student's t-test).