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Shati/Nat8l Overexpression Improves Cognitive Decline by Upregulating Neuronal Trophic Factor in Alzheimer's Disease Model Mice

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Abstract

Alzheimer's disease (AD) is a type of dementia characterized by the deposition of amyloid β, a causative protein of AD, in the brain. Shati/Nat8l, identified as a psychiatric disease related molecule, is a responsive enzyme of N-acetylaspartate (NAA) synthesis. In the hippocampi of AD patients and model mice, the NAA content and Shati/Nat8l expression were reported to be reduced. Having recently clarified the involvement of Shati/Nat8l in cognitive function, we examined the recovery effect of the hippocampal overexpression of Shati/Nat8l in AD model mice (5XFAD). Shati/Nat8l overexpression suppressed cognitive dysfunction without affecting the Aβ burden or number of NeuN-positive neurons. In addition, brain-derived neurotrophic factor mRNA was upregulated by Shati/Nat8l overexpression in 5XFAD mice. These results suggest that Shati/Nat8l overexpression prevents cognitive dysfunction in 5XFAD mice, indicating that Shati/Nat8l could be a therapeutic target for AD.

Keywords Shati/Nat8l · Alzheimer's disease · Cognitive dysfunction · Amyloid β · BDNF · AAV vector

Introduction

Alzheimer's disease (AD) is a dementia characterized by the depositions of senile plaques and neurofibrillary tangles in the brain [1, 2]. Since there are limited effective medications for AD, the development of novel therapeutic agents is one of the most urgent problems to be solved among aging populations. Amyloid β (Aβ), a major constituent of senile plaques, is produced from amyloid precursor protein (APP) through enzymatic cleavage by β- and γ-secretases [1, 3]. Mutations in APP, which increase the amount or toxicity of Aβ, cause familial AD [4]. A nationwide genomic study conducted in Iceland revealed that a single nucleotide polymorphism, which decreases Aβ production, reduces the risk of AD onset [5]. The genetic evidence strongly indicates that Aβ plays a role in the pathogenesis of AD. This notion is supported by accumulating reports of Aβ toxicity in neurons and synapses [6–10]. In addition, novel mouse models that overproduce human Aβ using a knock-in technique exhibit cognitive dysfunction [11, 12]. Therefore, Aβ greatly contributes to the pathology that underlies AD and is a potential target for the treatment of this disease.

Shati/Nat8l was identified at our laboratory as one of the molecules upregulated in the nucleus accumbens of mice

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upon receiving methamphetamine, which are one of models for mental disorders [13]. Shati/Nat8l, expressed in neurons, is a responsive enzyme that catalyzes the synthesis of N-acetylaspartate (NAA) from aspartate and acetyl-CoA [14]. NAA is contained in a millimolar order in the brain tissue as an acetyl group donor [15]. We have previously reported on the significance of Shati/Nat8l in the pathogenesis of methamphetamine addiction and depression [16, 17]. In addition, systemic knockout of Shati/Nat8l resulted in cognitive impairment, suggesting that this protein plays a role in cognitive function [18]. Recently, studies of patients and rodent models have described an association between Shati/Nat8l and AD. Magnetic resonance spectroscopy revealed a reduction in NAA levels in the hippocampi of patients with AD [19, 20]. This is supported by the decrease in both the expression of the mRNA of Shati/Nat8l and NAA content in the hippocampi of AD model mice [21]. These reports suggest that the downregulation of Shati/Nat8l may play a pathological role in AD. Therefore, in the present study, we examined the protective effect of the recovery of Shati/Nat8l by hippocampus-specific overexpression by adeno-associated virus (AAV) against cognitive decline in AD model mice.

Materials and Methods

Animals

Hemizygous male 5XFAD mice (B6SJL-Tg (APPSwFILon, PSEN1*M146L*L286V) 6799Vas/Mmjax) (The Jackson Laboratory, ME, USA) and female B6SJF1/J mice (wild type [WT]; The Jackson Laboratory) were crossed to maintain the transgenic line. For the experiments, heterozygous 5XFAD and WT mice from the same litter genotype were determined by PCR on the tail genome using the following primer pairs: human *APP* pair, forward: 5′-AGAGTACCAACTTGCATG ACTACG-3′, reverse: 5′-ATGCTGGATAACTGCCTTCTT ATC-3′ and human *PS1* pair, forward: 5′-GCTTTTTCCAGC TCTCATTTACTC-3′, reverse: 5′-AAAATTGATGGAATG CTAATTGGT-3′. The mice were housed in plastic cages (13×15×22 cm) with 3–4 animals per cage under the following conditions: room temperature, 22±2 °C; humidity, 55±10%; and constant illumination (7:00–19:00). The mice had free access to drinking water and pellets. The animal experiments were performed in accordance with the guidelines of the National Institutes of Health, the Animal Experiment

Handling Regulations of the University of Toyama, and the Animal Experiment Regulations of the Ministry of Education, Culture, Sports, Science and Technology. The study protocol was approved by the Animal Experiment Committee of the University of Toyama (A2017INM-1, A2020INM-1) and the DNA Genetic Recombination Committee of the University of Toyama (G2016PHA-9, G2020PHA-5, G2018INM-1).

Microinjection of Mouse *Shati*-AAV Vector into the Dorsal Hippocampal CA1 Region

AAV9 vectors were produced as previously described [22, 23]. Gene expression was controlled using the cytomegalovirus promoter. The AAV vector containing only the enhanced green fluorescent protein (EGFP) sequence (denoted as mock) was prepared as a control. The mice were anesthetized with a triple anesthetic (0.01 mL/g body weight, i.p.), including 0.075 mg/mL medetomidine hydrochloride, 0.4 mg/mL midazolam, and 0.5 mg/mL butorphanol tartrate, and immobilized in a stereotaxic instrument (Narishige, Tokyo, Japan). The AAV vectors were injected into the CA1 regions of the dorsal hippocampi bilaterally using a 701 N microsyringe (Hamilton Company, NV, USA) at a dose of 0.7 μL per side (anteroposterior$= -1.6$ mm, mediolateral$= \pm 1.0$ mm, dorsoventral$= +1.2$ mm [24]) of the mice. These procedures were performed when the mice were 6 weeks old. The resultant WT mice or 5XFAD mice, which received mock injections, were represented by WT-mock or 5XFAD-mock, while those which received injections of Shati/Nat8l, were represented by WT-Shati and 5XFAD-Shati. Number of mice prepared for the behavioral analysis were 10 of WT-mock, 7 of WT-Shati, 7 of 5XFAD-mock, 8 of 5XFAD-shati.

Novel Object Recognition Test

The novel object recognition (NOR) test of cognitive function was performed at 36 weeks of age, as previously [25]. Before the test, the mice were placed in a box (30×30×35 cm) for 30 min for three consecutive days for habituation. On the fourth day, the mice were placed in and freely explored the same box, in which two similar objects (A and A′) were placed. The total time for the nose to touch each object was measured for 10 min (acquisition phase). After the measurement, the mice were returned to their home cages. Twenty-four hours after the acquisition phase, the mice were placed in and freely explored the same box with two objects, one of which was replaced with a novel object (A and B). The time was measured in the same way for 10 min (test phase). The exploratory preference (%) was calculated using the following formula:

$$\text{Exploratory preference}(\%) = \text{Time to approach object } A' (\text{or B}) \Big/ \big[\text{Time to approach object } A + \text{Time to approach object } A' (\text{or B}) \big]$$

Collection of Brain Samples from Mice

After conducting the behavioral experiments (39 weeks of age), the mice were anesthetized with a triple mixture of anesthetics (0.01 mL/g body weight, i.p.). The heart was perfused with ice-cold phosphate-buffered saline (PBS) to remove blood from the mice. After removing the brain, the right hemisphere was soaked in 4% paraformaldehyde (PFA) for immunohistochemical analysis. The left hemisphere was dissected to nine slices with two mm thick from the olfactory bulb by brain matrix, and the piece around the injection site was collected from the hippocampal CA1 region in the sixth section and stored in liquid nitrogen for real-time PCR (RT-PCR).

Quantitative RT-PCR

Hippocampal tissues were homogenized in Isogen (Nippon Gene Co. Ltd., Tokyo, Japan) using a pestle. RNA was extracted from the homogenate according to the manufacturer's instructions. After extraction, the RNA solutions were treated with DNase (Promega Corporation, WI, USA) to remove genomic DNA. The final RNA solutions were subjected to reverse transcription using the PrimeScript RT Reagent kit (Takara Bio Inc., Kusatsu, Japan) to obtain cDNA. The temperature was controlled using a thermal cycler (Takara Bio Inc.). cDNA was used as the template for real-time PCR. The reaction was performed by Thunderbird SYBR qPCR Mix (Toyobo Co. Ltd., Osaka, Japan) with Mx3000P and Mx3005p (Agilent Technologies, CA, USA) and the following specific primer pairs of the targeted genes: mouse *Shati/Nat8l* pair, forward: 5′-GTGATTCTG GCCTACCTGGA-3′, reverse: 5′-CCACTGTGTTGTCCT CCTCA-3′; mouse *Bdnf* pair, forward: 5′-GCAAACATG TCTATGAGGGTTCG-3′, reverse: 5′-ACTCGCTAATAC TGTCACACACG-3′; mouse *Ngf* pair, forward: 5′-TGT GCCTCAAGCCAGTGAAA-3′, reverse; CACTGAGGT GAGCTTGGGTC; and mouse *36B4* pair, forward: 5′-ACC CTGAAGTGCTCGACATC-3′, reverse; 5′-AGGAAGGCC TTGACCTTTTC-3′. 36B4, which is known for ribosomal protein, is used as a reference gene.

Immunohistochemistry

After fixation with 4% PFA overnight, the brains were soaked in 10%, 20%, and 30% sucrose solutions. The brains were embedded in an optimal cutting temperature compound (Sakura Finetek Inc., Tokyo, Japan) to prepare Sects. (14 μm thick) a microtome (Leica BioSystem, Wetzlar, Germany).

The brain sections were subjected to antigen activation by autoclaving in 10 mM citric acid solution (pH 6.0) at 121 °C for 20 min. After washing three times with ice-cold PBS containing 0.1% Tween 20 (PBS-T), methanol containing 0.1% hydrogen peroxide was used to inhibit endogenous peroxidases. After washing three times with ice-cold PBS-T, a blocking solution containing 10% normal goat serum (NGS) was for 1 h. Then, the primary antibody against Aβ (clone: 6E10; BioLegend, CA, USA) or NeuN (clone: A60; Merck, Darmstadt, Germany) diluted with 5% NGS was exposed overnight at 4 °C. After washing three times with ice-cold PBS-T, the secondary antibody biotinylated anti-mouse IgG (Vector Laboratories Inc., CA, USA) diluted with 5% NGS solution was exposed, and the reaction was carried out for 30 min. After washing three times with ice-cold PBS-T, the immune signals were amplified by the reaction mediated by avidin–biotin complex (VECTASTAIN ABC Standard Kit; Vector Laboratories Inc.) for 30 min. After three washes with ice-cold PBS-T, the reaction was carried out with a 3,3′-diaminobenzidine solution for 10 min. After washing with running water for 15 min, the slices were soaked in hematoxylin solution for 2 min to stain the nuclei. After washing with running water for 5 min, the cells were dehydrated with 70%, 80%, 90%, 95%, and 100% ethanol and xylene, and sealed with Fluoromount (Diagnostic BioSystems Inc., CA, USA). The level of Aβ was quantified using ImageJ version 2 (National Institutes of Health, MD, USA).

Statistical Analysis

The results are expressed as the mean ± standard error. Between-group comparisons were performed using the Student t-test. Comparisons among the four groups were performed using a two-way analysis of variance. The Bonferroni test was used for multiple comparisons. The statistical analyses were performed using GraphPad Prism version 7 (GraphPad Software, CA, USA). Statistical significance was defined as $p < 0.05$.

Results

Expression of Shati/Nat8l and Neurotrophic Factors in the Hippocampi of 5XFAD Mice

As previously reported [21], 5XFAD mice at 39 weeks of age were confirmed to exhibit downregulation of *Shati/Nat8l* in the hippocampus ($p = 0.020$), not in the cerebral cortex ($p > 0.05$) or striatum ($p > 0.05$) (Fig. 1a–c). To examine
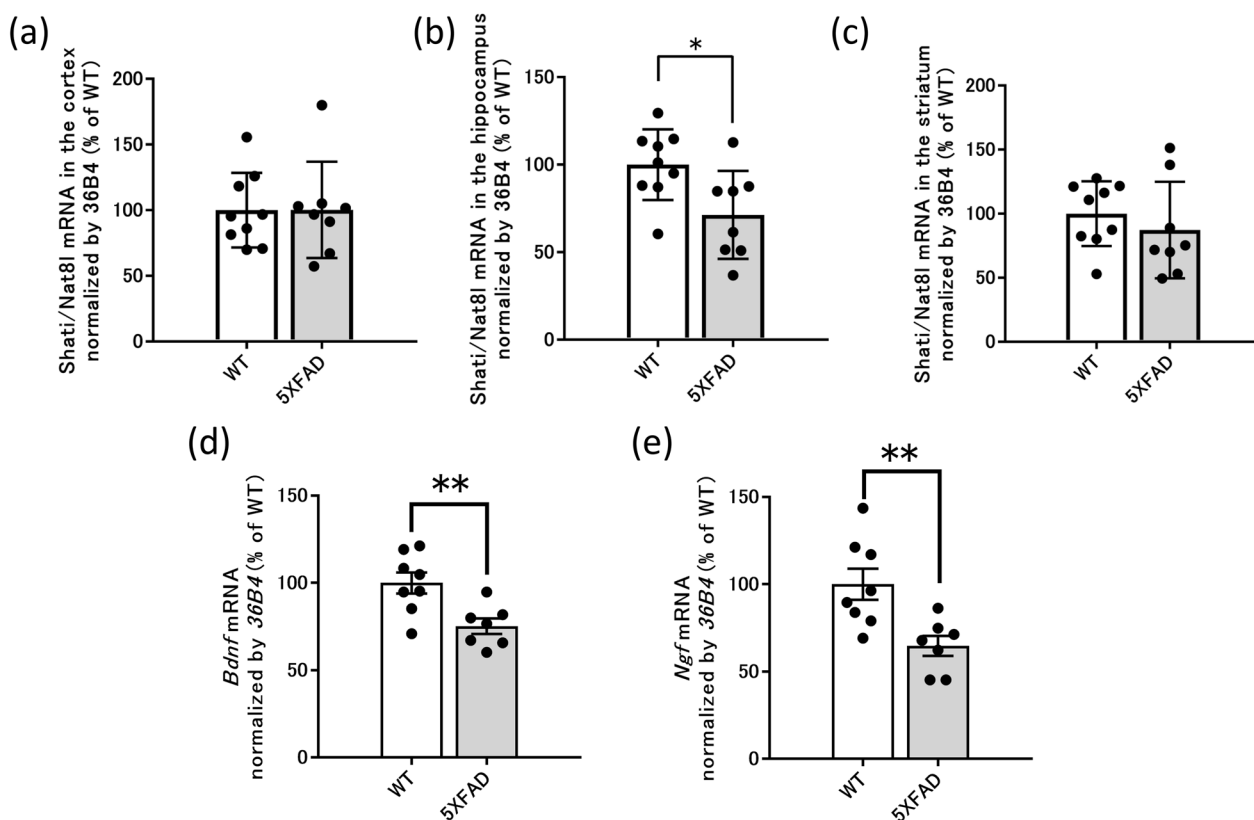
Fig. 1 Decreased gene expression in the hippocampus of 5XFAD mice. Quantitive RT-PCR analysis was performed to detect the changes of gene expression in 5XFAD mice (39 weeks of age). *Shati/Nat8l* expression normalized by *36B4* in the cerebral cortex (WT, 100.00 ± 9.45, n = 9; 5XFAD, 100.20 ± 12.97, n = 8) (**a**), the hippocampus (WT, 100.00 ± 6.74, n = 9; 5XFAD, 71.32 ± 8.90, n = 8) (**b**), and the striatum (WT, 100.00±8.44, n=9; 5XFAD, 87.23 ± 13.34, n=8) (**c**). *Bdnf* (WT, 100.00 ± 6.02, n=8; 5XFAD, 80.42 ± 4.76, n=7) (**d**), *Ngf* (WT, 100.00 ± 8.87, n=8; 5XFAD, 64.75 ± 5.74, n=7) (**e**) expression normalized by *36B4* in the hippocampal CA1 were significantly decreased in 5XFAD mice, compared to wild-type mice. Values represent the mean ± S.E.M. *$p < 0.05$, **$p < 0.01$ versus WT mice (Student's t-test)

neuronal resilience in the hippocampus, the gene expression of trophic factors was measured. The levels of the mRNA of *Bdnf* and *Ngf,* markers of neuronal resilience, were reduced in 5XFAD mice compared with WT mice (*Bdnf, p* = 0.029; *Ngf, p* = 0.0066) (Fig. 1d, e). These results are consistent with those of previous reports [26, 27].

Overexpression of Shati/Nat8l in the Hippocampal CA1 Region of 5XFAD Mice by the AAV Vector

To examine the protective effect of the recovery of Shati/Nat8l in the hippocampus of 5XFAD mice, Shati/Nat8l was overexpressed by local administration of the AAV vector of the mock or Shati/Nat8l (Fig. 2a). Microinjection was performed on WT or 5XFAD mice at 6 weeks of age to overexpress Shati/Nat8l before the start of Aβ plaque deposition. Since AD patients exhibit NAA reduction before diagnosis [19, 20], Shati overexpression on WT or 5XFAD mice started as early as 6 weeks of age. EGFP fluorescence from AAV vectors indicated proper injection in the hippocampus

(Fig. 2b). Overexpression of Shati/Nat8l mRNA was confirmed by RT-PCR ($p = 0.022$) (Fig. 2c).

Effect of Hippocampal Overexpression of Shati/Nat8l on Cognitive Dysfunction in 5XFAD Mice

To examine the effect of hippocampal-specific overexpression of Shati/Nat8l on cognitive dysfunction in 5XFAD mice, we performed the NOR test to evaluate cognitive function in the mice (WT-mock, WT-Shati, 5XFAD-mock, and 5XFAD-Shati) at 36 weeks of age. In the acquisition phase, similar patterns of approach were observed for the objects in each group (Fig. 3a). In the test phase, the 5XFAD-mock group showed a significantly lower preference for the novel object than the WT-mock group, which confirmed the phenotype of 5XFAD mice. In contrast, the 5XFAD-Shati group exhibited a significantly higher preference for the novel object than the 5XFAD mice ($F_{Interaction(1, 28)} = 7.77$, p < 0.01; $F_{genotype(1, 28)} = 3.88$, p > 0.05; $F_{Shati(1, 28)} = 3.55$, p > 0.05; WT-mock vs 5XFAD-mock, $p = 0.013$; 5XFAD-mock vs

Fig. 2 Overexpression of Shati/Nat8l by AAV vector in the hippocampal CA1. **a** Experiments were performed by following the diagram. **b** GFP fluorescence from the AAV vector in the dorsal hippocampus was observed using a fluorescence microscope. Scale bar indicates 2 mm. Dotted line represents the outline of the hippocampus. **c** Expressions of Shati/Nat8l mRNA normalized by *36B4* in dorsal hippocampus of 5XFAD-mock and 5XFAD-Shati mice (39 weeks of age) was detected (5XFAD-mock, 100.00 ± 11.59, n=5; 5XFAD-Shati, 944.92 ± 276.96, n=7). Values represent the mean $\pm$ S.E.M. $*p < 0.05$ versus 5XFAD-mock mice (Student's t-test)
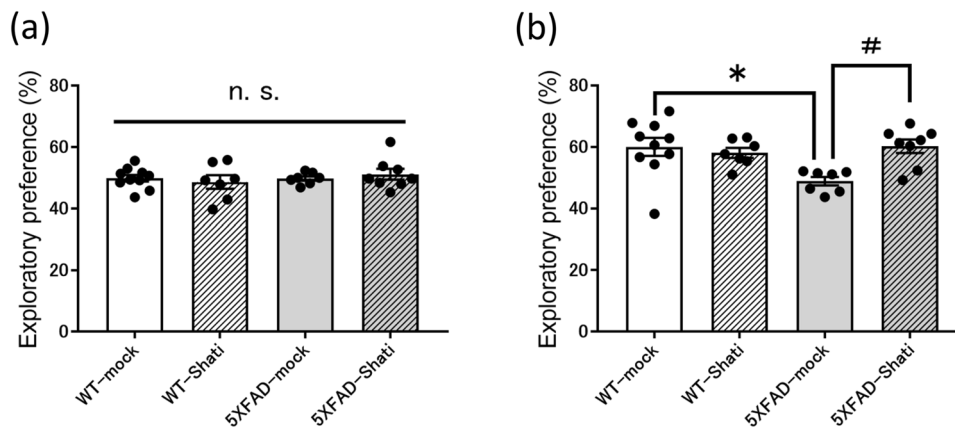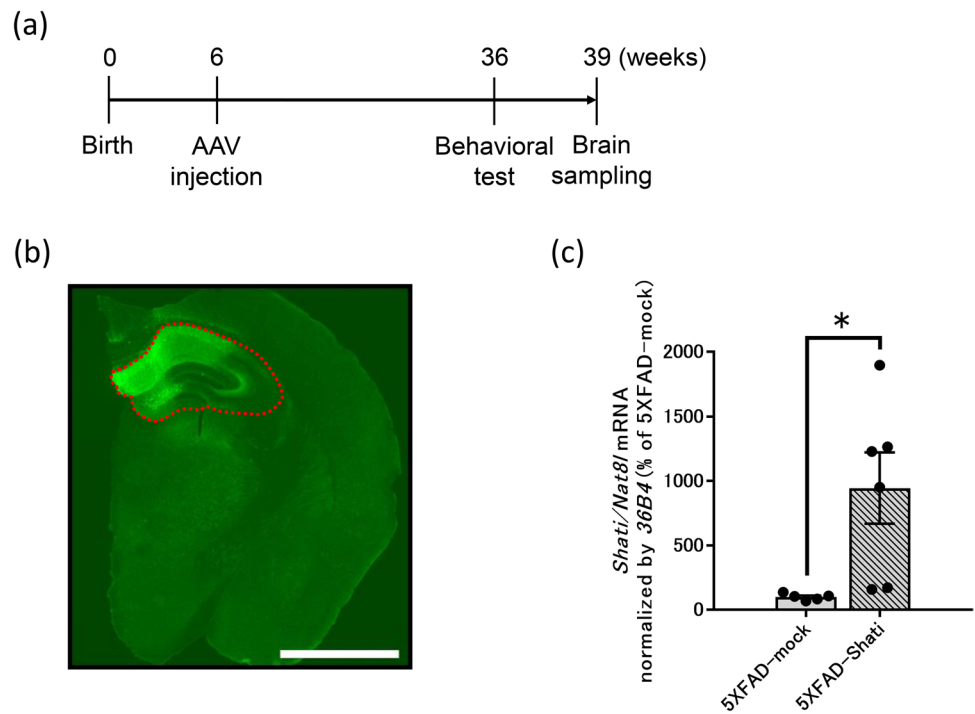

Fig. 3 Cognitive evaluation of 5XFAD mice overexpressed Shati/Nat8l in the hippocampal CA1 by NOR test. Exploratory preference of approach time was measured in NOR test. **a** In acquisition phase, mice in four groups exhibited the similar preference. **b** In test phase, WT-mock mice exhibited a preference to the novel object; 5XFAD-mock showed no preference, while 5XFAD-shati mice exhibited a preference to the novel object. (WT-mock, 60.03 ± 1.99, n=10; WT-Shati, 58.07 ± 1.35, n=7; 5XFAD-mock, 48.88 ± 1.44, n=7; 5XFAD-Shati, 60.23 ± 2.54, n=8). Values represent the mean $\pm$ S.E.M. $*p < 0.05$ versus WT-mock mice. $\#p < 0.05$ versus 5XFAD-mock mice (two-way ANOVA followed by Bonferroni's post-hoc test). "n. s." indicates no significance

5XFAD-Shati, $p = 0.017$) (Fig. 3b). These findings suggest that overexpression of Shati/Nat8l prevented cognitive decline in 5XFAD mice.

Effects of Hippocampal-Specific Overexpression of Shati/Nat8l on Aβ Pathology and Neuronal Loss

Considering that the cognitive dysfunction in 5XFAD mice is induced by the accumulation of Aβ in the brain, the Aβ burden was evaluated to clarify the mechanism underlying the suppressive effect of overexpression of Shati/Nat8l. Brain sections obtained from 5XFAD mice overexpressing the mock and Shati/Nat8l were stained with an antibody against Aβ (Fig. 4a, b). Accumulation of Aβ plaques were observed in the hippocampus of 5XFAD-mock mice. There were no significant effects in the number and area occupied in the hippocampus of 5XFAD-Shati mice compared with 5XFAD-mock mice (Fig. 4c, p > 0.05; Fig. 4d, p > 0.05). The average sizes
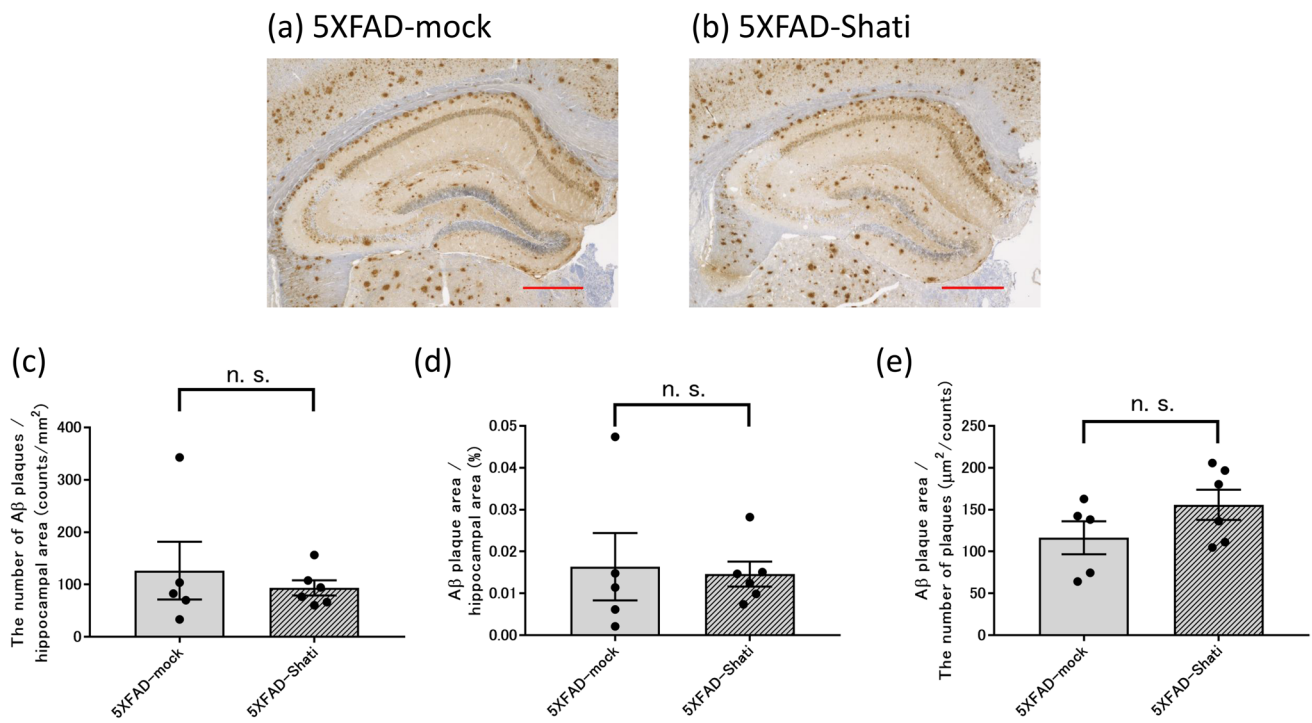
Fig. 4 Effect of Shati/Nat8l overexpression in the dorsal hippocampus on amyloid pathology in 5XFAD mice. **a, b** Representative images of immunohistochemical staining of Aβ plaque by 6E10 in the hippocampus of 5XFAD-mock (**a**), 5XFAD-Shati mice (**b**). Scale bars indicate 500 μm. **c–e** Each graph shows the average number of Aβ plaques (5XFAD-mock, 126.62 ± 55.22, n = 5; 5XFAD-Shati, 93.72 ± 14.47, n = 6) (**c**), occupying area by Aβ plaque area (5XFAD-mock, 0.016 ± 0.0080, n = 5; 5XFAD-Shati, 0.015 ± 0.0030, n = 6) (**d**), the average size of Aβ plaques (5XFAD-mock, 116.48 ± 19.70, n = 5; 5XFAD-Shati, 155.84 ± 18.00, n = 6) (**e**) in the hippocampus. Values represent the mean ± S.E.M. "n. s." indicates no significance

Fig. 5 Effect of Shati/Nat8l overexpression in the dorsal hippocampus on neuronal loss in 5XFAD mice. The neurons in the CA1 region from **a** 5XFAD-mock mice and **b** 5XFAD-shati mice were stained by anti-NeuN antibody by immunohistochemistry. The areas outlined by red dotted lines in the upper images were enlarged and shown in the lower images. The Scale bars represent 200 μm in the upper images and 100 μm in the lower images
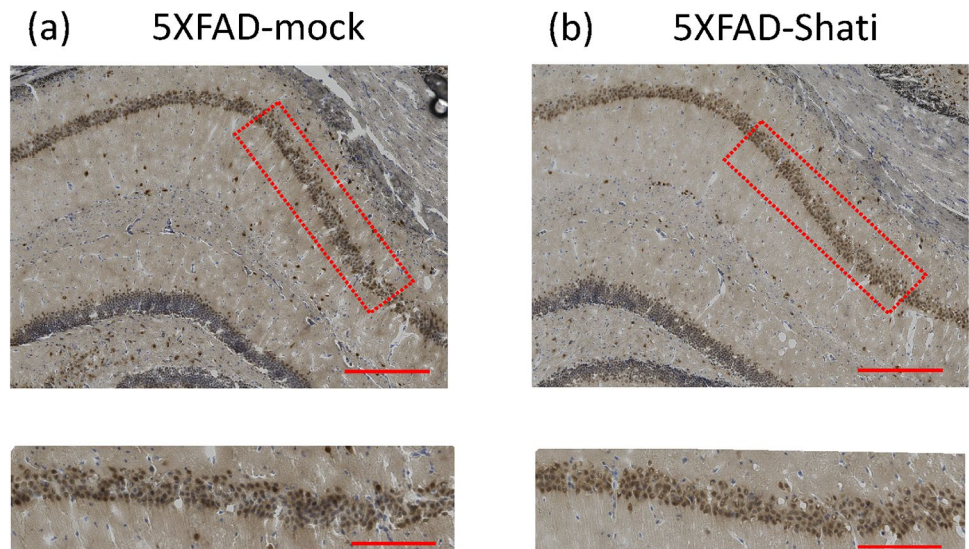
of the plaques were also unaltered in the 5XFAD-Shati mice compared with the 5XFAD-mock mice (Fig. 4e, p > 0.05). Although 5XFAD mouse are reported to exhibit neuronal loss [8], no alteration of NeuN positive cells were observed CA1 region in the hippocampus (Fig. 5). These results suggest that overexpression of Shati/Nat8l does not affect the Aβ pathology or neuronal loss in the hippocampi of 5XFAD mice.

Hippocampal-Specific Overexpression of Shati/Nat8l Alters Gene Expression in 5XFAD Mice

We recently reported that Shati/Nat8l upregulates *Bdnf* gene with histone acetylation in its promoter region under stress condition [28]. Since neurotrophic factors including brain-derived neurotrophic factor (BDNF) are involved in the regulation of neuronal activity, synapse formation and function [29–31], these gene expressions in 5XFAD-Shati mice were examined. As shown in Fig. 1, downregulation of *Bdnf* and *Ngf* was observed in 5XFAD mice compared to WT mice. In contrast, *Bdnf, not Ngf,* was significantly upregulated in the hippocampus upon overexpression of Shati/Nat8l in 5XFAD mice (Fig. 6a, p = 0.047; Fig. 6b, p > 0.05). Thus, this suggests that the recovery of neuronal resilience by the increased expression of *Bdnf* gene mediates cognitive improvement induced by Shati/Nat8l overexpression in 5XFAD mice.

Discussion

We previously identified Shati/Nat8l and clarified its physiological and pathological roles in the central nervous system. In the present study, focusing on the involvement of Shati/Nat8l in cognitive function, we investigated the effects of Shati/Nat8l overexpression on AD pathology in mouse models. Consistent with previous reports on patients and model mice, Shati/Nat8l was downregulated in the hippocampi, neither cerebral cortex nor striatum, of 5XFAD mice [20, 21]. The regional specificity of the reduction of Shati/Nat8l expression would be caused by vulnerabilities of hippocampus to the stressors. Recent report also indicated hippocampus-specific reduction of Shati/Nat8l in aged mice [32]. In a previous report, Shati/Nat8l reduction was observed prior to neurodegeneration in 5XFAD mice, suggesting that the decrease in Shati/Nat8l is not due to neuronal loss [21]. Shati/Nat8l overexpression in the hippocampi of 5XFAD mice, achieved by local injection of AAV, suppressed cognitive decline assessed using the NOR test without affecting Aβ pathology or neuronal loss. In addition, there is a possibility that Shati/Nat8l overexpression enhance the neurogenesis to suppress cognitive decline. However, activity status of neurogenesis in AD model mice is controversial. That is, increased proliferation in APP-overexpression J20 mice [33] and decreased proliferation in APPswe/PS1dE9 mice [34]. On the other hand, the expressions of the genes of neurotrophic factors in the hippocampus, which were reduced in 5XFAD mice, were upregulated by overexpression of Shati/Nat8l. These findings suggest that Shati/Nat8l
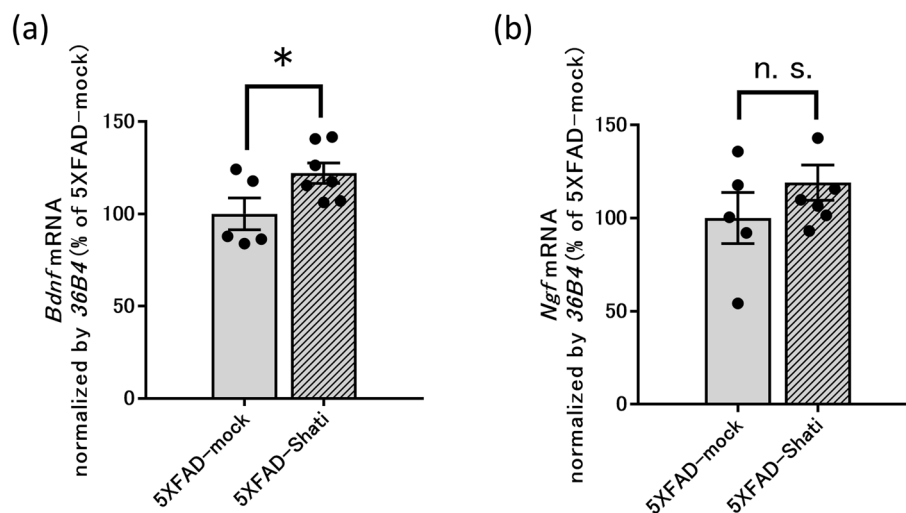

Fig. 6 Altered gene expression in dorsal hippocampus of 5XFAD mice by overexpression of Shati/Nat8l. Quantitive RT-PCR analysis was performed to detect the changes of gene expression in each group (39 weeks of age). *Bdnf* (5XFAD-mock, 100.00 ± 8.66, n = 5; 5XFAD-Shati, 122.10 ± 5.54, n = 7) (**a**) and *Ngf* (5XFAD-mock; 100.00 ± 13.70, n = 5; 5XFAD-Shati; 119.00 ± 9.51, n = 7) (**b**) expression normalized by *36B4* in the dorsal hippocampus were significantly increased in 5XFAD-Shati mice, compared with 5XFAD-mock mice. Values represent the mean ± S.E.M. *p < 0.05 versus 5XFAD-mock (Student's t-test). "n. s." indicates no significance

overexpression ameliorated cognitive dysfunction in 5XFAD mice, possibly mediated by neurotrophic factor upregulation.

Glutamatergic transmission plays an essential role in cognitive function. Two major glutamate receptors, N-methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, located on the dendritic spine, are modulated by various enzymes to regulate synaptic transmission. These receptors are primarily involved in long-term potentiation (LTP), a critical step in memory formation. LTP is a type of synaptic plasticity in which highly frequent presynaptic stimulation enhances the efficacy of synaptic transmission [35, 36]. Studies of AD model mice have clarified the deregulation of synaptic transmission, including LTP, in AD pathology [37, 38]. Aβ has been reported to decrease LTP by promoting the removal of NMDA receptors from the postsynaptic surface [39] and disturbing the transport of AMPA receptors to the postsynaptic membrane [40]. Conversely, neurotrophic factors such as BDNF and NGF have protective effects on synaptic transmission [29]. BDNF application to primary cultured neurons increases neuronal activity and synaptic transmission [30, 31]. BDNF also facilitates LTP induction in hippocampal slices [41]. These effects are mediated by the activation of NMDA receptor subunits [42] and upregulation of the AMPA receptor subunit [43]. In the present study, overexpression of Shati/Nat8l increased *Bdnf* gene expression. The preventive effect of Shati/Nat8l overexpression on cognitive dysfunction in 5XFAD mice could be mediated by the restoration of synaptic activity by BDNF.

The results of the present study indicate that Shati/Nat8l enhances *Bdnf* gene expression in the hippocampus. Shati/Nat8l synthesizes NAA, which is enzymatically converted to N-acetylaspartyl glutamate (NAAG) [14, 44]. The resultant NAAG is released into the synaptic cleft to exert an agonistic effect on metabotropic glutamate receptor 3 (mGluR3) and located on the surface of neurons and astrocytes [45]. Previously, stimulation of mGluR3 was reported to increase astrocytic BDNF expression [46] and nerve growth factor (NGF) release [47]. We have previously reported that overexpression of Shati/Nat8l significantly increases the expression of NAA and NAAG [48]. Since overexpression of Shati/Nat8l in the hippocampus was confirmed to increase regional NAA levels, the elevated expression of *Bdnf* might be mediated by the stimulation of mGluR3. Another possible mechanism underlying the upregulation of *Bdnf* is epigenetic modification. *Bdnf* gene expression is modulated in epigenetic manners, including histone methylation and acetylation, and DNA methylation [49, 50]. Our recent report indicates Shati/Nat8l regulates *Bdnf* mRNA expression by histone acetylation at H3K9 related to the *Bdnf* promotor

[28]. There are some reports that *Ngf* mRNA expression is regulated by histone acetylation such as H4 [51, 52], which are possibly different from the regulation mechanism of *Bdnf* by Shati/Nat8l. Taken together, enhanced expression of *Bdnf* by overexpression of *Shati/Nat8l* probably mediated by histone acetylation or increased levels of NAA and its metabolites.

Currently, there are limited effective medications for AD. Furthermore, it is difficult for patients with dementia to maintain fine compliance with daily medicines. An AAV vector, whose benefit is a stable treatment effect by sustained expression, has already been applied clinically to patients. Onasemnogene abeparvovec, a gene therapy medication of the AAV vector, can be used to treat spinal muscular atrophy in a single dose [53]. Shati/Nat8l overexpression by the AAV vector could be applicable for future therapeutic strategy for AD. In this study, AAV9 vectors were utilized for gene overexpression. Since AAV9 vectors were reported to robustly transduce genes to the neurons in the adult mice [54], Shati/Nat8l is probably overexpressed in the neurons. Previously, gene delivery of aromatic L-amino acid decarboxylase to the into the putamen of PD patients by AAV2 was successfully improved the clinical symptom in the phase I study [55]. Further investigations for the choice of AAV serotype is necessary for clinical applications.

Declarations

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