Inhibition of the glutamine transporter SNAT1 confers neuroprotection in mice by modulating the mTOR-autophagy system

Daisuke Yamada1, Kenji Kawabe1, Ikue Tosa1, Shunpei Tsukamoto1, Ryota Nakazato2, Miki Kou2, Koichi Fujikawa2, Saki Nakamura2, Mitsuaki Ono3, Toshitaka Oohashi3, Mari Kaneko4, Shioi Go4, Eiichi Hinoi2, Yukio Yoneda2 & Takeshi Takarada1

The pathophysiological role of mammalian target of rapamycin complex 1 (mTORC1) in neurodegenerative diseases is established, but possible therapeutic targets responsible for its activation in neurons must be explored. Here we identified solute carrier family 38a member 1 (SNAT1, Slc38a1) as a positive regulator of mTORC1 in neurons. Slc38a1floxflox and Synapsin I-Cre mice were crossed to generate mutant mice in which Slc38a1 was selectively deleted in neurons. Measurement of 2,3,5-triphenyltetrazolium chloride (TTC) or the MAP2-negative area in a mouse model of middle cerebral artery occlusion (MCAO) revealed that Slc38a1 deficiency decreased infarct size. We found a transient increase in the phosphorylation of p70S6k1 (pp70S6k1) and a suppressive effect of rapamycin on infarct size in MCAO mice. Autophagy inhibitors completely mitigated the suppressive effect of SNAT1 deficiency on neuronal cell death under in vitro stroke culture conditions. These results demonstrate that SNAT1 promoted ischemic brain damage via mTOR-autophagy system.
Functional defects in neurons cause neurodegenerative diseases such as Alzheimer disease, amyotrophic lateral sclerosis, Parkinson disease, and cerebrovascular disease. These disorders are characterized by cellular damage and death in specific cerebral neurons, which are associated with physiological disabilities of patients, including tremor, paralysis, memory impairment, and cognitive dysfunction. Autophagy is a highly programmed self-degrading system, and its dysregulation occurs in neurodegenerative diseases. The mammalian target of rapamycin (mTOR) complex 1 (mTORC1) suppresses the initiation of autophagy by phosphorylating ULK1, and mTOR activity is upregulated in Alzheimer disease, Parkinson disease, and Huntington disease. Further, mTOR inhibition exerts therapeutic effects in models of neurodegenerative disease, indicating the critical role of the mTOR-autophagy system in neuro pathological disorders.

mTOR, which is an essential subunit of mTORC1, is a serine/threonine protein kinase of the PI3K family of protein kinases. By phosphorylating multiple substrates, mTORC1 regulates intracellular events including protein translation, glycolysis, lipid synthesis, and autophagy. The small G protein RHEB, which resides on endosomal membranes, activates mTORC1, and the Tsc1/Tsc2 complex acts as a GTPase RHEB to inhibit mTORC1 activity. Inhibition of mTORC1 by Tsc1 in hippocampal neurons suppresses neuronal cell death by inducing autophagy in a model of ischemic stroke. Further, the beneficial effects of inhibiting mTOR with rapamycin and its analogues are revealed by animal models of neurodegenerative disorders. Although the pathophysiological role of mTORC1 in central nervous system is established, a therapeutic strategy that specifically targets mTORC1 in neurons is not available.

Among amino acids, L-glutamine (L-Gln) serves as a resource for energy production or protein synthesis and acts as an essential mediator of mTORC1 activation. L-Gln is a substrate of solute carrier (Slc) family transporters including Slc1, Slc7, and Slc38s, and these transporters contribute to neurodegenerative diseases. Although neurons express multiple Slc family members, L-Gln transporters that are specifically expressed by neurons, which play a critical role in neuro pathological diseases, have not been identified. Here we demonstrate that SNAT1, which is selectively expressed in neurons, acts as a positive regulator of mTORC1 in neurons and plays an important pathological role in the mechanism underlying neuronal survival after ischemia. Thus, our data provide insights into the therapeutic strategies for patients with neuro pathological diseases.

Results

High expression of SNAT1 in neurons. Certain members of the Slc38a family serve as net neutral amino acid transporters and use L-Gln as a preferred substrate. L-Gln indirectly activates mTORC1 via the simultaneous influx of essential amino acids (EAAs) and efflux of L-Gln, which are regulated by Slc7a5 or Slc7a8. To identify Slc family transporters, which are characterized by a positive regulatory role in mTORC1 activation and highly specific expression in the brain, we first compared mRNA copy numbers of systems A (Slc38a1, Slc38a2, and Slc38a4), N (Slc38a3 and Slc38a5), L (Slc7a5 and Slc7a8), and ASC (Slc1a5) transporter genes. The levels of Slc38a1 and Slc38a2 were higher compared with those of other genes tested (Fig. 1a). When the mRNA levels of each Slc transporter were compared mRNA and protein were expressed in brain segments including the cerebral cortex, hippocampus, striatum, hypothalamus, olfactory bulb, cerebellum, midbrain, and medulla-pons (Supplementary Fig. 1b, c). Immunohistochemical analysis of SNAT1 in the cerebral cortex revealed that SNAT1 was specifically expressed in NeuN-positive neurons but not in S100β-positive astrocytes or in CD11b-positive microglia (Fig. 1c). These results indicate that SNAT1 was preferentially expressed in neurons.

Targeted deletion of Slc38a1 from the genomes of neurons. We used Cre-loxP strategies to generate mutant mouse strain expressing a floxed allele of Slc38a1 (Fig. 2a). The wild-type (WT) allele yielded a 17-kb fragment, whereas the homologous targeted mutant allele yielded a 6.8-kb fragment (Fig. 2b). To investigate the function of Slc38a1 in neurons, the Synapsin 1 (Syn1)-Cre system was employed to generate mutant mice in which Slc38a1 could be selectively deleted from the genomes of Synl-positive neurons (Fig. 2a, b). Here, Slc38a1loxfllox and Syn1-Cre; Slc38a1loxfllox mice are designated as control and mutant mice, respectively. The deleted allele was only detected in mutant mice (Fig. 2c). The level of Slc38a1 mRNA was decreased, although that of Slc38a2 was unchanged throughout the whole brain (Fig. 2d). The primer set used to detect Slc38a1 mRNA recognizes exon 2 of Slc38a1, indicating that full-length Slc38a1 mRNA might be expressed in the brain except by Syn1-Cre-targeting cells of mutant mice. Consistent with this prediction, each brain segment isolated from mutant mice displayed decreased levels (<50%) of Slc38a1 compared with those of controls (Fig. 2e). Further, SNAT1 expressing neurons were undetectable in the cerebral cortex of the mutants (Fig. 2f), indicate that our system specifically deleted Slc38a1 from the genomes of neurons.

Effect of neuron-specific Slc38a1 deficiency on cerebral infarction. We employed a model of the middle cerebral artery occlusion (MCAO) to simulate neurodegenerative disease and assessed ischemic brain injury in mutant mice. When the infarct area or volume was evaluated using immunohistochemical detection of TTC (Fig. 3a), mutant mice exhibited a smaller infarct area compared with that of the control (Fig. 3b). Further, immunohistochemical analysis revealed that the NeuN- or MAP2-negative area was smaller in mutant mice (Fig. 3c, d). These results demonstrate that SNAT1 expressed in neurons positively regulated ischemic brain injury.

Critical role of mTORC1 activation via SNAT1 in ischemic brain injury. mTORC1 activation in neuropathological disorders such as ischemic brain injuries causes neuronal cell death, p70S6K1 phosphorylates ribosomal protein S6 and regulates translation. p70S6K1 is directly phosphorylated at T389 by mTORC1 via its phosphorylation level in the ipsilateral region increased 1 h after MCAO, followed by a decline to the same level detected in the contralateral region (Fig. 4a). Most NeuN- or MAP2-negative area was smaller in mutant mice (Fig. 3c, d). These results demonstrate that SNAT1 expressed in neurons positively regulated ischemic brain injury.
completely counteracted the neuroprotective effect of Slc38a1 deficiency (Fig. 4e). Consistent with previous reports,23–25, rapamycin administration also decreased infarction area (Supplementary Fig. 2). These results demonstrate that mTORC1 activation via SNAT1 caused ischemic brain injury.

Effect of Slc38a1 deficiency on mTORC1 activity. To test the possibility that L-Gln incorporation via SNAT1 promotes mTORC1 activity, primary neurons were isolated from Slc38a1fl/fl mice, and Cre recombinase (Cre) or ΔCre (inactive form of cre recombinase) was introduced using a lentiviral vector. Here, Cre- and ΔCre-introduced neurons were designated Slc38a1-null and control cells, respectively (Fig. 5a). Compared with control neurons, Slc38a1-null neurons expressed decreased levels of both mRNA and protein (Fig. 5b, c). Further, the rate of L-Gln incorporation into Slc38a1-null neurons was slower compared with that of the control (Supplementary Fig. 3a). To assess the effect of Slc38a1 deficiency on the phosphorylation of mTORC1-target molecules including p70S6K1, mTOR, and S6 cells were cultured in PBS for 3 h and then stimulated with PBS or DMEM (L-Gln[+]) for 5 h. The level of pp70S6K1(T389), pmTOR(S2448), and pS6(S235/236) was lower in Slc38a1-null neurons (Fig. 5d–f). Further, 1 h treatment with L-Gln transporter inhibitors such as MeAIB and 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) decreased the levels of pp70S6K1(T389), pmTOR(S2448), and pS6(S235/236) in primary neurons (Supplementary Fig. 3b–d). The addition of L-Gln and EAAs to PBS increased the level of pp70S6K1(T389) in the mouse neuroblastoma cell line Neuro2a, indicating that L-Gln and EAAs were required for full mTORC1 activation (Supplementary Fig. 3e). These results indicate that SNAT1 positively regulated mTORC1 activity.

Effect of Slc38a1 deficiency on autophagy in primary neurons. Autophagy, which is inhibited by mTORC1, regulates neuroprotection.1,5,26 We assessed the contribution of autophagy to the neuroprotective effect of Slc38a1 deficiency. Oxygen-glucose deprivation (OGD) serves as an in vitro model of stroke, and reflects in vivo models of ischemic stroke. Although there was no difference in the MAP2-positive area under normal culture conditions, Slc38a1-null neurons became resistant to OGD, because the MAP2-positive area was larger compared with that of the control (Fig. 6a). When cell death was assessed using PI staining, there were fewer PI+ cells in the Slc38a1-null group under the OGD (Fig. 6b). Similar results were obtained when primary neurons or Neuro2a cells were treated with MeAIB under OGD (Supplementary Fig. 4a, b). qRT-PCR analysis of autophagy-regulatory genes revealed that Slc38a1-null neurons displayed increases in the levels of Map1lc3b, Lamp, Sqstm1, Ctsb, and Ctsd mRNAs compared with those of the controls (Fig. 6c–g). Phosphorylation of p62 is specific to autophagy. Slc38a1-null neurons expressed higher levels of phosphorylated p62 compared with those of control neurons under OGD (Fig. 6h). Further, autophagy inhibitors such as bafilomycin and chloroquine decreased the inhibitory effect of Slc38a1 deficiency on OGD-induced cell death (Fig. 6i, j). Consistent with this observation, the suppressive effect of MeAIB on OGD-induced cell death was abolished by chloroquine (Supplementary Fig. 4c). These results demonstrate that SNAT1 promoted neuronal cell death by inhibiting autophagy.
Discussion

Here we show the neurotoxic effect of SNAT1 expressed by cerebral neurons in a model of ischemic infarction. Emerging evidence reveals that SLC transporters regulate the intracellular levels of nutrients and drugs and play critical roles in disease. Among SNAT family transporters, SNAT1 are upregulated in solid tumors such as hepatocellular carcinoma, breast cancer and osteosarcoma. Jin et al. used a Rett syndrome model to show that upregulation of SNAT1 expression in microglia contributes to NMDA receptor-dependent neurotoxicity. These results indicate that SNAT1 is associated with the pathogenesis of certain diseases. Here we found that SNAT1 was predominantly expressed in cerebral neurons (Fig. 1d), and Slc38a1 deficiency did not confer any behavioral disorders (Supplementary
Fig. 2 Generation of neuron-specific Slc38a1 knockout mice. **a** Targeting strategy to create the floxed Slc38a1 allele (Slc38a1^{lox}). Slc38a1 exon 2 is flanked by loxP sites. The flippase recombinase target-flanked Neo cassette was removed by crossing with CAG-FLP mice. Exon 2 was removed by crossing with SynI-Cre mice to selectively produce the Δ allele in neurons. **b** Southern blot analysis to confirm the recombination with the targeting vector at the genomic Slc38a1 locus. Genomic DNA from embryonic stem cells was digested with AfIII and hybridized with a DIG-labeled 3′ probe. **c** PCR analysis verifying the Δ allele in mutant mice. Genomic DNA was extracted from the brain of each indicated mouse, and PCR products derived from the wild-type, flox, or Δ allele were detected. **d** Quantification of Slc38a1 and Slc38a2 mRNA levels in whole brains of from mutant mice. Total RNAs were extracted from whole brains of control or mutant mice, and the mRNA levels of Slc38a1 and Slc38a2 were compared using qRT-PCR. Values were normalized to those of Gapdh (n = 3).

**e** Deletion efficiency of Slc38a1 in brain segments. Proteins were extracted from each indicated brain segment of control or mutant mice, and SNAT1 was detected using western blotting. CBB staining was used as a loading control. C and M indicate control and mutant, respectively. **f** Confirmation of neuron-specific Slc38a1 deficiency in mutant mice. Double-immunohistochemical staining using antibodies against SNAT1 and NeuN. Nuclei were counterstained with Hoechst 33342. Scale bars indicate 100 μm.

Fig. 3 Neuron-specific Slc38a1 deficiency confers resistance to ischemic brain injuries. **a, b** TTC staining of a brain section prepared from MCAO model mice. Representative images of the coronal bregma section **a** and measurements of the infarct area at each indicated point from the bregma (**b**, n = 5). **c, d** Immunohistochemical detection of the neurodegenerative effect of MCAO on cerebral neurons. Brain sections at the bregma were stained with antibodies against the neuronal markers NeuN **(c, n = 3)** or MAP2 **(d, n = 3)**, and areas with undetectable staining were measured.
mTORC1 is aberrantly activated in tumors31 and it can induce malignant phenotypes such as increased proliferative capacity and cancer stem-like properties32–34. SNAT1 in flues the malignant phenotype through activation of mTORC129,35,36. However, the functional relationship between mTORC1 and SNAT1 in neurons of the central nervous system or in ischemic brain damage is unknown. To our knowledge, this present study is the first to report a neurotoxic effect of mTORC1 activated by SNAT1 under ischemia. The ipsilateral region of MCAO model mice exhibited a transient increase in mTORC1 activity (Fig. 4a), and the neuroprotective effect of Slc38a1 deficiency was abolished by Tsc1 heterozygosity (Fig. 4e), indicating the SNAT1 promoted ischemic infarction by activating mTORC1. Consistent with our present results, Tsc1 inhibits mTORC1 and induces a neuroprotective effect on hippocampal neurons5, suggesting that hyperactivation of mTORC1 is toxic to several types of neurons.

The mechanism through which mTORC1 regulates neuronal cell death may not be easy to determine, because systemic administration of rapamycin, an mTORC1 inhibitor, has
neuroprotective effects on ischemic brain damage. However, in contrast to the results of the present study the mTORC1 activity of all cells in various tissues, including the brain, should have been inhibited in the studies cited, indicating that mTORC1 in glial cells, microglia, or other cells confer neuroprotection. SNAT1 was specifically expressed in neurons but not in glial cells or microglia (Fig. 1c). Other mechanisms may therefore induce neuroprotection by activating mTORC1 in cells exposed to ischemic stresses. Future studies are required to determine how the mTORC1 activity of each cell in brain tissues contributes to the pathogenesis of ischemic infarction or to brain damage. Further, full activation of mTORC1 requires L-Gln as well as EAAs such as leucine (Supplementary Fig. 3). Furthermore, we found that MeAIB decreased, but could not completely prevent cell death caused by OGD (Supplementary Fig. 4), suggesting that system L transporters mediate OGD-induced phenotypes.

Autophagy is a highly conserved self-degrading system that mediates stress responses. Recent studies reveal the function of autophagy in cell death, aging, tumorigenesis, metastasis, and drug resistance. Moreover, autophagy contributes to neuroprotection against ischemic damage. Autophagy is negatively regulated by mTORC1-mediated phosphorylation of ATG1 or ATG13, and its activation via inhibition of mTORC1 is associated with neuroprotective effects. Here we show that SLC38A1 deficiency decreased mTORC1 activity (Fig. 6d–f) and increased the expression levels of genes associated with autophagy (Fig. 6c–g). Further, inhibition of autophagy with bafilomycin or chloroquine decreased the suppressive effect of SLC38A1 deficiency on OGD-induced neuronal cell death (Fig. 6i, j). These results are consistent with those of a previous report demonstrating the inhibitory effect of bafilomycin on neuroprotection induced by autophagy in a model of cerebral ischemic. Activation of autophagy induced by mTORC1 inhibition or TFEB overexpression in neurons promotes neuroprotection in models of cerebral ischemia. Therefore, the neuron-selective activation of autophagy or inhibition of mTORC1 may prevent ischemic brain damage. Therefore, SNAT1 may serve as a potential therapeutic target because its expression is largely restricted to brain or central neurons (Fig. 1b, c). Although only in vitro assays were performed here to test the neuroprotective effect of autophagy activated by SLC38A1 deficiency, our data and those of previous reports support the hypothesis that administration of inhibitors of autophagy such as bafilomycin or chloroquine will inhibit the neuroprotective effects conferred by SLC38A1-deficiency during cerebral ischemic damage.

In conclusion, our study demonstrates that SNAT1 shows promise as a therapeutic target to prevent neuronal cell death caused by ischemic stress. We expect that our findings will provide insight into the neuropathological interactions between members of the SLC transporter family and other signaling pathways.

Methods

Real-time–quantitative RT-PCR (qPCR). Total RNA was extracted from cells or tissues, followed by cDNA synthesis using reverse transcriptase and oligo-dT primers. The cDNA samples were then used as templates for real-time PCR analysis using gene-specific primers (Supplementary Table 1). qPCR was performed using a MiniOpticon real-time PCR system (BioRad). The cycle parameters were as follows: denaturation at 95 °C for 30 s, annealing for 30 s at 62 °C, and elongation for 30 s at 72 °C. The expression level of each gene was calculated using the ΔΔCt method or quantification of copy number.

Immunohistochemistry. Mice were deeply anesthetized via intracardial perfusion with PBS and 4% paraformaldehyde. Brains were removed, fixed overnight, and cryoprotected in 30% sucrose. Free-floating sections (30-µm thick) were prepared using a cryostat and stored at −20 °C in cryoprotective solution (30% sucrose, 30%...
Fig. 6 Autophagy is a critical mediator of neuroprotection conferred by Slc38a1 deficiency. 

**a, b** Assessment of the neuroprotective effect against ischemic stress conferred by Slc38a1 deficiency. Control or Slc38a1-null neurons were cultured under normal (anaerobic glucose deprivation [OGD]) or OGD conditions and stained with an anti-MAP2 antibody (a, n = 3) or PI (b, n = 6) to evaluate dead neurons. Bars = 100 µm. **c–g** Increases in the mRNA levels of autophagy-related genes associated with Slc38a1 deficiency. Total RNA was extracted from control or Slc38a1-null neurons, and the mRNA levels of each indicated gene were measured using qRT-PCR. Values were normalized to those of Gapdh (n = 6). **h** Evaluation of autophagy under ischemia. Cell-free lysates of Slc38a1-null neurons cultured under OGD were subjected to western blotting using anti-phospho-p62 and anti-actin antibodies (n = 4). **i, j** Suppressive effect of autophagy inhibitors on neuroprotection induced by Slc38a1 deficiency. Control or Slc38a1-null neurons were treated with 1 nM bafilomycin i or 5 µM chloroquine j in the presence or absence of OGD. Neuronal cell death was assessed using PI staining (n = 3).
ethyleneglycol, and 1% polyvinylpyrrolidone in PBS). Sections were washed in Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated for 15 min in TBST. After washing in TBST, sections were incubated for 1 h in blocking solution (2% normal goat serum in TBST), followed by incubation at 4 °C overnight in blocking solution containing a primary antibody against SNAT1 (kindly provided by Jeffrey D. Erickson; 1:2000, Neun (MAB377 Chemicon; 1:4000), S100P (Sigma-Aldrich; 1:4000), CD11b (MCA711G Serotec; 1:200) or phospho-p70 S6 kinase (Thr389) (#9234 Cell Signaling Technology; 1:200). After rinsing in TBST, sections were incubated for 2 h at room temperature with blocking solution containing an anti-rabbit IgG antibody conjugated to Alexa 594 (Invitrogen; 1:400), an anti-mouse IgG antibody conjugated with Alexa 488 (Invitrogen; 1:400), or an anti-rat IgG antibody conjugated with Alexa 488 (Invitrogen; 1:400), rinsed in TBST, and incubated for 15 min in TBST containing Hoechst 33342 at room temperature. After washing in TBST, sections were mounted onto slides in FluorSave (Calbiochem). Confocal images were acquired using an LSM710 microscope and ZEN2009 software (Zeiss).

**Generation of mutant mice.** Three splice variants of Slc38a1, revealed by a search of the Ensemble database (http://www.ensembl.org/index.html) contain exon 2 (chromosome 15: 96,624,169–96,623,956, ENSMUSE000000621499), which encodes the start codon for the Slc38a1. Therefore, we generated conditional knockout mice with a floxed exon 2 of the Slc38a1 locus (Accession No. C008385K) 98. The targeting vectors harboring loxp sites and a neomycin resistant (Neo) cassette were used to electroporate T2 embryonic stem (ES) cells 99. DNAs were extracted from different ES cell clones after genotypic selection for the mutant allele, followed by screening using a [53]-labeling probe specific for the targeted region of genomic DNA digested with AflII. The WT and the corresponding mutant allele yielded 17-kb 6.8-kb fragments, respectively. ES cells containing the floxed allele were injected into CD-1 8-cell stage embryos to generate chimeric mice. To remove the Neo cassette, a 9-cmFRT/C Rossen (Dij-38/+ 1:200) labeled probe specific for the target region of genomic DNA digested with AflII. The WT and the corresponding mutant allele yielded 17-kb 6.8-kb fragments, respectively. ES cells containing the floxed allele were injected into CD-1 8-cell stage embryos to generate chimeric mice. To remove the Neo cassette, a 9-cmFRT/C Rossen (Dij-38/+ 1:200) labeled probe specific for the target region of genomic DNA digested with AflII. The WT and the corresponding mutant allele yielded 17-kb 6.8-kb fragments, respectively.

**Primary neuronal culture.** The protocol employed in this study met the guidelines of the Japanese Society for Pharmacology and was approved by the Committee for the Ethical Use of Experimental Animals of Kanazawa University. All efforts were made to minimize animal suffering, reduce the number of animals, and utilize alternatives to in vivo techniques. Primary cultures of cortical neuronal were obtained from 13-day embryonic mice. Briefly, the cerebral cortex was dissected and incubated with 0.25% trypsin at 37 °C for 20 min. Cells were treated with serum and 0.1 mg/ml DNAase and then mechanically dissociated using a sterile pipette tip. Dissociated cells were plated at 400,000 cells/cm² on plastic dishes coated with 7.5 μg/ml poly-l-lysine, after we performed a trypan blue dye exclusion test. The cells were maintained in Neurobasal medium (Invitro) supplemented with 2% B27 supplement (Invitrogen) for 8 days at 37 °C in a humidified atmosphere containing 5% CO2, with medium replacement every 4 days. Neurons were activated with 5 mM glutamine (glutamine) or <0.1% essential amino acid (EAA) solution (Thermo Fisher).

**Lentivirus stocks for infecting primary cultures of neurons.** Lentiviral vectors expressing Cre recombinase or ΔCre (inactive form) were kindly donated by Dr. Sudhof (Stanford University, Palo Alto, CA, USA). The lentiviral expression vector, pRSV-REV, pMD2g/pRRE, and the SVSVG expression plasmid were used to cotransfect HEK293T cells (RIKEN, Saitama, Japan) at 10, 2.5, 5, and 3 μg of DNA per 567-cm² culture area, respectively, using the calcium phosphate transfection method. After transfection (24 h), the HEK293T culture medium was replaced with DMEM, followed by culture for 24 h and subsequent collection of the culture medium. The culture medium was centrifuged at 500 × g for 5 min, and the supernatant containing lentiviruses was directly added to the culture medium. Cre or ΔCre was fused to GFP, and the lentiviral titer could therefore be determined by measuring the fluorescence emitted by EGFP. The lentiviral supernatant was added at a ratio of 1:3 of lentiviral supernatant, yielding an MOI = 10. Primary cultures of neurons infected for 12 h on day 4 and analyzed on day 8. All experiments were performed using biosafety level II conditions.

**Oxygen glucose deprivation (OGD).** We induced OGD condition using an AnesthesiPack (Mitsubishi Gas Chemical) and 50 mM 2-deoxy-glucose. Neurons were cultured for 24 h under OGD, fixed with 4% paraformaldehyde, and then reacted with anti-MAP2 antibody (M4403 Sigma-Aldrich)/Alexa Fluor 488-conjugated goat anti-mouse IgG (1:400, Invitrogen) for immunodetection. Cells were observed using a fluorescence microscope (BZ-8100; Keyence, Osaka, Japan). MAP2-positive areas were measured in four different visual fields per well, which were randomly chosen in a blinded fashion. The dead cells were analyzed using PI (10 μM) and Hoechst 33342 (10 μM) for 10 min. Stained cells were analyzed using an In Cell Analyzer 2000 (GE Healthcare). The cell death was calculated according to the ratio of PI-positive cells to Hoechst 33342-positive cells.

**Data analysis.** Data analysis was performed using Prism 7, and the data are shown as mean ± S.E. Statistical significance was determined using a two-tailed t test and an unpaired one-way or two-way ANOVA with the Tukey posthoc test. *p < 0.05, **p < 0.01, ***p < 0.001.

**In situ hybridization.** Brains were removed, fixed overnight, and cryoprotected in 30% sucrose. Brains were then dissected to prepare frozen sections (10-μm thick) using a cryostat (Leica CM 3050), and in situ hybridization was performed, as previously described 87 using DIG-labeled cRNA probes. DIG-labeled cRNA probes for Slc38a1 were prepared in vitro using T7 or SP6 RNA polymerase to transcribe Slc38a1 cDNA fragments (919 bp) subcloned into pGEM-Easy vectors (Promega). An Slc38a1 cDNA fragment was obtained from a library of mouse brain cDNAs using the forward primer 5′-TCTGAATTCGTTGGAATGAGGAAG-3′ and reverse primer 5′-CTCTTCACTGGAGGGTATAAGA-3′.

**Culture of Neuro2a cells.** Neuro2a cells derived from a mouse neuroblastoma exhibited the neuronal stem-cell like ability to differentiate into neuron-like cells in the presence of all-trans retinoic acid (ALLA). Undifferentiated Neuro2a cells were cultured in DMEM supplemented with 10% FBS and passaged at least three times before treatment with ATRA. Neuro2a cells (25,000 cells/cm²) were then plated in DMEM supplemented with 10% FBS for 24 h, followed by medium change to DMEM supplemented with 2% FBS and 20 μM ATRA to induce differentiation. Cultures were maintained at 37 °C for 2 days in a humidified atmosphere containing 5% CO2.

**L-Gln incorporation assay.** In total 4 × 10⁵ primary neurons were suspended in Neurobasal medium containing B27 and seeded into the wells of a 24-well plate. After culturing for 4 days, neurons were incubated with lentiviruses particles was directly added to the culture medium. Cre or ΔCre was fused to GFP, and the lentiviral titer could therefore be determined by measuring the fluorescence emitted by EGFP. The lentiviral supernatant was added at a ratio of 1:3 of lentiviral supernatant, yielding an MOI = 10. Primary cultures of neurons infected for 12 h on day 4 and analyzed on day 8. All experiments were performed using biosafety level II conditions.

**Data analysis.** Data analysis was performed using Prism 7, and the data are shown as mean ± S.E. Statistical significance was determined using a two-tailed t test and an unpaired one-way or two-way ANOVA with the Tukey posthoc test. *p < 0.05, **p < 0.01, ***p < 0.001.
and 5.5 mM Glucose for 30 min. Neurons were then treated with 10 μM [3H] L-Gln for 15 min at 37 °C. Reactions were stopped by washing with HKR containing 5 mM L-Gln. Cell lysates were prepared by incubating them with 0.1 N NaOH for 1 h and the neutralized with 0.1 N HCl. The radioactivities of each cell lysate were measured using a liquid scintillation counter.

Behavior analysis. The three-chamber tests for sociability were performed using a rectangular, three-chambered box with two dividing walls allowing access into each chamber. Mice were first placed in the middle chamber and allowed to explore for 10 min with free access to all parts of the arena. After habituation, an unfamiliar mouse (C57BL/6 male) was placed in the wire cage (in the right chamber); another wire cage (in the left chamber) was empty, and the test mouse was placed in the center compartment of the social test box and allowed to explore for a 10 min session, with free access into the two side chambers. The behavior of the animals was videotaped, tracked and then analyzed with the spent time in each chamber.

The elevated plus maze was constructed with 2 open- and 2 closed-square arms.

For hole board test, mice were placed on a square arena with regularly arranged holes on the floor. The frequency of spontaneous elicited hole-poking behavior are measured during 5 min.

**Reporting summary** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability** All data generated during and/or analyzed during this study are included in this published article and its supplementary information. The source data underlying graphs shown in main figures are presented in Supplementary Data 1. Full blots are shown in Supplementary Information.

Received: 29 August 2018 Accepted: 19 August 2019
Published online: 18 September 2019

### References

1. Nottegård, R. The role of autophagy in neurodegenerative disease. Nat. Med. 19, 983–997 (2013).
2. Lipton, J. O. & Sahin, M. The neurology of mTOR. Nat. Rev. Mol. cell Biol. 2, 543–558 (2011).
3. Papadakis, M. et al. Tsc1 (hamartin) confers neuroprotection against ischemia and glutamate neurotoxicity. J. Neurosci. 19, 5546–5558 (2007).
4. Zoncu, R., Efeyan, A. & Sabatini, D. M. mTOR: from growth signal integration to cancer, diabetes and ageing. Nat. Rev. Mol. cell Biol. 12, 21–35 (2011).
5. Papadakis, M. et al. Tsc1 (hamartin) confers neuroprotection against ischemia by inducing autophagy. Nat. Med. 19, 351–357 (2013).
6. Bockaert, J. & Marin, P. mTOR in brain physiology and pathologies. Physiol. Rev. 95, 1157–1187 (2015).
7. Duran, R. V. et al. Mapping of the glutaminolysis pathway: data mining of mass spectrometry datasets. Mol. Cell 47, 349–358 (2013).
8. Jewell, J. L. et al. Metabolism. Differential regulation of mTORC1 by leucine and glutamine. Science 347, 194–198 (2015).
9. Kim, S. G. et al. Metabolic stress controls mTORC1 lysosomal localization and dimerization by regulating the TTT-RUVBL1/2 complex. Mol. cell 49, 172–185 (2015).
10. Nicklin, P. et al. Bidirectional transport of amino acids regulates mTORC1 and autophagy. Cell 136, 521–534 (2009).
11. Lin, L., Yee, S. W., Kim, R. R. & Giacomini, K. M. SLC transporters as therapeutic targets: emerging opportunities. Nat. Rev. Drug Discov. 14, 543–560 (2015).
12. Pochini, L., Scalise, M., Galluccio, M. & Indiveri, C. Membrane transporters for the special amino acid glutamine: structure/function relationships and relevance to human health. Front. Chem. 2, 61 (2014).
13. Bagchi, S., Baomar, H. A., Al-Walai, S., Al-Sad, S. & Fredriksson, R. Histological analysis of SLC38A6 (SNAT6) expression in mouse brain shows selective expression in excitatory neurons with high expression in the synapses. PLoS ONE 9, e95438 (2014).
14. Haggblad, M. G. et al. Transport of L-glutamine, L-alanine, L-arginine and L-histidine by the neuron-specific Slc38a8 (SNAT8) in CNS. J. Mol. Biol. 427, 1495–1512 (2015).
15. Haggblad, M. G. et al. Identification of Slc38a7 (SNAT7) protein as a glutamine transporter expressed in neurons. J. Biol. Chem. 286, 20500–20511 (2011).
Takamura, A. et al. Autophagy-deficient mice develop multiple liver tumors. *Gene Dev.* 25, 795–806 (2011).

Zhang, Q., Bian, H., Guo, L. & Zhu, H. Pharmacologic preconditioning with berberine attenuating ischemia-induced apoptosis and promoting autophagy in neuron. *Am. J. Transl. Res.* 8, 1197–1207 (2016).

Dai, S. H. et al. Sirt3 confers protection against neuronal ischemia by inducing autophagy: Involvement of the AMPK-mTOR pathway. *Free Radics. Biol. Med.* 108, 345–353 (2017).

Shi, Z. Y. et al. Protective effect of autophagy in neural ischemia and hypoxia: Negative regulation of the Wnt/beta-catenin pathway. *Int. J. Mol. Med.* 40, 1699–1708 (2017).

Wang, L. et al. Ulk1/FUNDC1 prevents nerve cells from hypoxia-induced apoptosis by promoting cell autophagy. *Neurochem. Res.* 43, 1539–1548 (2018).

Fang, Z. et al. Neuroprotective autophagic flux induced by hyperbaric oxygen preconditioning is mediated by Cystatin C. *Neurosci. Bull.* 35, 336–346 (2018).

Guo, Y. Role of HIF-1a in regulating autophagic cell survival during cerebral ischemia reperfusion in rats. *Oncotarget* 8, 98482–98494 (2017).

Zhu, Y., Shui, M., Liu, X., Hu, W. & Wang, Y. Increased autophagic degradation contributes to the neuroprotection of hydrogen sulfide against cerebral ischemia/reperfusion injury. *Metab. brain Dis.* 32, 1449–1458 (2017).

Liu, Y. et al. Neuronal-targeted TFEB rescues dysfunction of the autophagy-lysosomal pathway and alleviates ischemic injury in permanent cerebral ischemia. *Autophagy* 15, 493–509 (2019).

Yagi, T. et al. A novel ES cell line, TT2, with high germine-differentiating potency. *Anal. Biochem.* 214, 70–76 (1993).

Zhu, Y. et al. Ablation of NF1 function in neurons induces abnormal development of cerebral cortex and reactive gliosis in the brain. *Genes Dev.* 15, 859–876 (2001).

Kwiatkowski, D. J. et al. A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells. *Hum. Mol. Genet.* 11, 525–534 (2002).

Longa, E. Z., Weinstein, P. R., Carlson, S. & Cummins, R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke; a J. Cereb. Circ.* 20, 84–91 (1989).

Takarada, T. et al. Interference by adrenaline with chondrogenic differentiation through suppression of gene transactivation mediated by Sox9 family members. *Bone* 45, 568–578 (2009).

Takarada, T. et al. An analysis of skeletal development in osteoblast-specific and chondrocyte-specific runt-related transcription factor-2 (Runx2) knockout mice. *J. Bone Min. Res.* 28, 2064–2069 (2013).

Lopatina, O. et al. Anxiety- and depression-like behavior in mice lacking the CD157/BST1 gene, a risk factor for Parkinson’s disease. *Front Behav. Neurosci.* 8, 133 (2014).