Ndufs4 ablation decreases synaptophysin expression in hippocampus

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Altered function of mitochondrial respiratory chain in brain cells is related to many neurodegenerative diseases. NADH Dehydrogenase (Ubiquinone) Fe-S protein 4 (Ndufs4) is one of the subunits of mitochondrial complex I and its mutation in human is associated with Leigh syndrome. However, the molecular biological role of Ndufs4 in neuronal function is poorly understood. In this study, upon Ndufs4 expression confirmation in NeuN-positive neurons, and GFAP-positive astrocytes in WT mouse hippocampus, we found significant decrease of mitochondrial respiration in Ndufs4-KO mouse hippocampus. Although there was no change in the number of NeuN positive neurons in Ndufs4-KO hippocampus, the expression of synaptophysin, a presynaptic protein, was significantly decreased. To investigate the detailed mechanism, we silenced Ndufs4 in Neuro-2a cells and we observed shorter neurite lengths with decreased expression of synaptophysin. Furthermore, western blot analysis for phosphorylated extracellular regulated kinase (pERK) revealed that Ndufs4 silencing decreases the activity of ERK signalling. These results suggest that Ndufs4-modulated mitochondrial activity may be involved in neuroplasticity via regulating synaptophysin expression.

Mitochondria are subcellular organelles that play vital roles in the generation of cellular energy in the form of ATP via oxidative phosphorylation, synthesis of metabolic precursors for macromolecules, calcium buffering and programmed cell death1,2. Given these essential functions, mitochondrial dysfunction in tissues with high energy demand can lead to a wide variety of diseases3. In the CNS, mitochondrial function is crucial for neurons because neurons demand high levels of ATP for cellular function such as synaptic neurotransmission and plasticity4,5. Furthermore, mitochondria provide a buffering machinery to regulate calcium concentration during signal transduction in neurons6. They are also involved in biosynthesis of iron-sulfur and heme in neurons for neurotransmitter synthesis in synapses7. Thus, altered mitochondrial function may lead to neurodegenerative diseases such as Parkinson’s disease (PD), Alzheimer’s disease (AD), Huntington’s disease, amyotrophic lateral sclerosis and Leigh syndrome8,9. For example, it has been reported that progressive accumulation of mitochondrial amyloid-β (Aβ) leads to mitochondrial dysfunction resulting in neuronal damage and cognitive decline in patients and transgenic mouse model of AD10. In another study, embryonic neurons derived from transgenic AD mouse hippocampus exhibited significantly decreased mitochondrial respiration11. Moreover, mitochondrial complex I deficiency has been detected in the substantia nigra and frontal cortex tissue of PD patients12. Recently, excitatory neurons isolated from the pluripotent stem cells of MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) patient showed impaired mitochondrial function with delayed neural maturation, reduced dendritic complexity and fewer functional excitatory synapses13. Thus, understanding the role of mitochondrial function in the development of neurodegenerative disease is quite essential.

Mitochondrial complex I (NADH: ubiquinone oxidoreductase) is the main gateway for electron entry to the electron transport chain (ETC)14. NADH Dehydrogenase (Ubiquinone) Fe-S protein 4 (Ndufs4), one of the subunits in mitochondrial complex I is involved in the assembly and stability of Complex I15. The gene encoding human NDUF54 is a hot spot for fatal pathogenic mutations that commonly results in Leigh syndrome16, an infantile onset, progressive neurodegenerative disorder clinically characterized by motor and intellectual...
retardation, seizures, respiratory insufficiency and failure to thrive leading to early death. Similarly, global Ndufs4-KO mouse showed retarded growth, loss of motor ability, breathing abnormalities, and death. Moreover, previous studies have demonstrated the importance of Ndufs4 in neuronal function. In recent study, glutamatergic neuron specific Ndufs4 loss in mice showed a significant impairment of neuronal firing, motor deficit, brain stem inflammation and region specific marked astrogliosis. Furthermore, GABAergic neurons specific Ndufs4 loss exhibited basal ganglia inflammation and severe epileptic seizures preceding death. Furthermore, dopaminergic neuron specific Ndufs4-KO mouse showed the impairment of dopamine release in striatum, amygdala without loss of neuron. These evidence suggest that Ndufs4 loss in neurons might be a key factor for the development of encephalopathy. However, the molecular biological role of Ndufs4 in neural function is still unknown.

In this study, we explored how Ndufs4 ablation affects neuron function focusing on neurite growth and synaptic plasticity and found that synaptophysin, a presynaptic protein, expression decreases significantly with a decreased activity of ERK signalling. This novel finding may provide more insights in hippocampal pathophysiology of neurodegenerative disease and may be useful in designing an effective treatment strategy.

**Results**

**Expression and localization of Ndufs4 in cerebral cortex and hippocampus.** We first explored Ndufs4 expression and localization in the brain. Western blot analysis using isolated mitochondria from brain confirmed Ndufs4 expression (Fig. 1A). To determine the expression level in different brain regions, we punched out the tissue from the cerebral cortex, hippocampus, and cerebellar cortex. qPCR analysis demonstrated that Ndufs4 was expressed in all regions of the brain although these expression levels were lower compared to the...
expression in the heart tissue (Supplemental Fig. 1A,B). Furthermore, enzyme-based immunohistochemistry also confirmed the expression of Ndufs4 in cerebral cortex and CA1, CA2 and CA3 region of hippocampus (Fig. 1B). To investigate which cell types express Ndufs4, we performed co-immunostaining of Ndufs4 with several cell markers such as neuronal nuclei (NeuN) for neuron, glial fibrillary acidic protein (GFAP) for astrocyte and myelin basic protein (MBP) for oligodendrocyte. We found that Ndufs4 co-localized with all cell markers in hippocampus and cerebral cortex with equal fluorescence (Fig. 1C and Supplemental Fig. 1C–F). Then, to explore the mitochondrial functionality, we measured complex I respiration using the isolated mitochondria from punch out hippocampus tissue and found a significant decrease in basal respiration and maximal respiration in Ndufs4-KO compared to WT, but ATP production was not changed (Fig. 1D–F).

Elevated GFAP expression was observed in Ndufs4-KO hippocampus, but not in primary cultured Ndufs4-KO astrocytes. To evaluate whether Ndufs4 deficiency alters the expression of cell markers and cell population, immunostaining and Western blot were performed using hippocampus of WT and Ndufs4-KO mice. Although there was no difference in expression of NeuN-positive neuron and MBP-positive oligodendrocyte (Fig. 2A,C,D), interestingly, an increased number of hypertrophic GFAP-positive astrocytes with increased number of processes were found in Ndufs4-KO hippocampus (Fig. 2B; Supplemental Fig. 2A,B). Elevated GFAP expression in Ndufs4-KO hippocampus was also confirmed by Western blot (Fig. 2D; Supplemental Fig. 2C). This led us to hypothesize that reactive gliosis, characterized by hypertrophy of the astrocyte cellular processes and high levels of expression of GFAP, may be occurring in Ndufs4-KO brain. As such, to investigate whether Ndufs4 deficiency in astrocytes affects the function of astrocytes, we prepared primary cultured astrocytes from Ndufs4-KO brain (Supplemental Fig. 2D), but we could not find gliosis character such as increased GFAP expression and increased cell proliferation (Fig. 2E,F; Supplemental Fig. 2E) in Ndufs4-KO astrocytes, suggesting that the gliosis character observed in Ndufs4-KO mouse brain may be due to altered neuroplasticity in hippocampus.

Ndufs4 deficiency decreased synaptophysin expression in hippocampus. To ascertain whether any neuronal dysfunction occurred in hippocampus due to Ndufs4 deficiency, we counted the number of NeuN positive cells in hippocampus, but we did not find a significant difference between WT and Ndufs4-KO mouse brain (Supplemental Fig. 3A). Because previous reports have demonstrated the presence of neuron apoptosis in the olfactory bulb of Ndufs4-KO mouse20, supportively, we evaluated neuron apoptosis, but we could not find cleaved caspase 3 positive cells in both WT and Ndufs4-KO hippocampus (Fig. 3A). Then, we investigated the synaptic protein expression in hippocampus because mitochondrial function is closely associated with the synthesis of synaptic protein25. Notably, synaptophysin expression was significantly decreased in Ndufs4-KO
hippocampus (Fig. 3B,C). However, there was no change in the expression of other proteins including PSD95, Vglut1, EAAT1, EAAT2 and EAAT3 (Fig. 3B and D–H).

Knockdown of Ndufs4 impairs neurite outgrowth and synaptophysin-positive puncta in differentiated Neuro-2a cell. To further investigate the role of Ndufs4 in synaptic change, we silenced Ndufs4 in Neuro-2a cells by siRNA treatment, then induced differentiation by treatment with retinoic acid (Supplemental Fig. 4A–C). We also confirmed that Ndufs4 silencing impaired mitochondrial function including basal respiration, maximum respiration, as well as ATP production (Supplemental Fig. 4D). Using these cells, we found that Ndufs4 silencing decreased the average neurite length compared to control (Fig. 4A,B) along with the number of synaptophysin puncta per length of β-III tubulin positive neurite (Fig. 4C,D). Furthermore, Ndufs4 silencing significantly decreased the expression level of phosphorylated-ERK signalling (Fig. 4E; Supplemental Fig. 4E). To confirm the direct relation between ERK activity and neurite growth/synaptophysin expression, we performed a rescue experiment using a pERK agonist Carbamylcholine Chloride (carbachol). We found a significantly higher neurite growth and synaptophysin expression in carbachol treated Ndufs4 knockdown cells, and even in control cells (Supplemental Fig. 5A–D). Considering that carbachol is a nonspecific cholinergic agonist and acts on nicotinic and muscarinic receptors, and as well, activates many downstream signalling pathways26–28, it may activate other cell signalling outside of the Ndufs4 cascade, but there is a strong possibility that drug-induced-increased pERK activity in Ndufs4 knockdown cells partially rescued neurite growth and synaptophysin expression. Taken together, these data suggest that Ndufs4-modulated mitochondrial function may be involved in neuroplasticity through the ERK signalling followed by alteration of neurite growth and synaptophysin expression.

Discussion
Mitochondrial function in neuron is quite essential for electrical activity, axon extension, regeneration, branching, synaptic formation, and synaptic neurotransmission29,30, and altered mitochondrial function leads to neurodegenerative diseases31–33. Ndufs4 is the gene responsible for Leigh syndrome17. It has been reported that loss of Ndufs4 reduces maximum CI dependent oxidative phosphorylation in synaptosomal mitochondria of olfactory bulb, brainstem and cerebellum19. Furthermore, striatal medium spiny neurons specific Ndufs4 loss progressively impairs the motor function without loss of neuronal number22. However, the molecular function of Ndufs4 in neuron is still not well deciphered. In this study, we first demonstrated Ndufs4 expression in hippocampal neurons in WT and then showed mitochondrial dysfunction with decreased expression of synaptophysin without loss of neuronal cells in Ndufs4 deficient mouse hippocampus.

Mitochondria are intimately involved in cell signalling pathways such as growth factor signalling, differentiation, cell death signalling, and autophagy through regulating the levels of intracellular signalling molecules such as Ca2+, ATP and ROS34. Notably, mitochondrial dysfunction promotes p38 mitogen-activated protein kinase activity, resulting in neuronal intracellular responses including inflammation and apoptosis35–37. Similarly, decreased activity of ERK signalling inhibits mitochondrial fusion and stimulates apoptotic mitochondrial
membrane permeabilization through phosphorylation of mitochondrial fusion protein, mitofusin 1, in rat primary cortical neurons thereby regulating cell death. Furthermore, pharmacological inhibition of c-Jun N-terminal kinases results in a decreased mitochondrial membrane potential in motor neurons, leading to cell degeneration. These previous studies thus suggest the involvement of mitochondria in the cell signalling cascade. In this study, although there was no difference in the phosphorylation level of ERK between WT and Ndufs4 KO hippocampus (Supplemental Fig. 4F, G), silencing of Ndufs4 decreased the levels of phosphorylation of ERK. These results may reflect the close association between Ndufs4 in neuron and ERK signalling.

It is well known that intracellular cell signalling is responsible for presynaptic formation. For example, altered ERK signalling has been shown to impair synaptic plasticity in the hippocampus. It has also been reported that ERK is responsible for the presynaptic modulation of synaptic plasticity in the hippocampal CA3 region that requires synapsin 1, a presynaptic protein. Similarly, another study also demonstrated that pharmacological blockade of ERK activity prevents stimulation-induced dendritic spine formation in hippocampal CA1 pyramidal neuron in ex vivo slice systems. In this study, we demonstrated a decreased activity of ERK signalling and decrease of synaptophysin expression, suggesting that Ndufs4 is regulating the synaptophysin expression through modulating ERK signalling. Furthermore, a rescue experiment with carbachol demonstrated that a significantly higher neurite growth and synaptophysin expression in carbachol treated Ndufs4 knockdown cells, and even in control cells. Considering that carbachol is a nonspecific cholinergic agonist and acts on nicotinic and muscarinic receptors, and as well, activates many downstream signalling pathways, it may also activate other cell signalling outside of the Ndufs4 cascade, but there is a strong possibility that drug-induced-increased pERK activity in Ndufs4 knockdown cells partially rescued the synaptophysin expression. Further experiments using a selective ERK agonist such as a mutant of ERK will clarify this issue.

Reactive gliosis is commonly observed with neuron loss or dysfunction in CNS disorders. Previous research shows a marked gliosis with significant loss of synapse in cerebral cortex of Alzheimer’s patients. Similarly, another study shows astroglial reactivity in hippocampus in a mouse model of traumatic axonal injury. Notably, it has been reported that glutamatergic neuron specific Ndufs4-KO mouse shows elevated GFAP expression in vestibular nucleus, cerebellar fastigial nucleus and inferior olive. Similarly, GABAergic neuron specific Ndufs4-KO mouse shows elevated GFAP in basal ganglia, substantia nigra pars reticulata and olfactory bulb.
Furthermore, Nestin-driven Ndufs4-KO mouse shows elevated GFAP in olfactory bulb, cerebellum, and vestibular nuclei suggesting neuron dysfunction may trigger astrogliosis. In this study, gliosis character was observed in in vivo Ndufs4-KO hippocampus with decreased synaptophysin expression with no neuron loss. In addition, primary cultured astrocytes from Ndufs4-KO hippocampus did not show any gliosis character. These results indicate that altered neuroplasticity including impaired synaptic formation may be the inducing factor of gliosis.

Further detailed study may reveal the mechanism underlying the astrocyte reactivity in Ndufs4-KO mouse.

In summary, we provide new insights on the effect of Ndufs4 loss in the function of hippocampal neurons. Our work highlighted the involvement of Ndufs4 in neuroplasticity by regulating synaptophysin expression through phosphorylation of ERK. This knowledge may be useful in understanding the pathophysiological aspect of neurodegenerative diseases but, further clear understanding of molecular mechanism of Ndufs4 is quite essential.

Materials and methods

Animals. Ndufs4-KO mice (B6.129S4-Ndufs4<sup>tm1.Rpa</sup>/J) were purchased from The Jackson Laboratory (Maine, USA). The animals were housed in standard cages in a temperature and humidity-controlled room with a 12 h light–dark cycle and ad libitum access to food and water. Animal experiments were carried out in compliance with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments). All animal care procedures were approved by the Animal Care and Use Committee of Tohoku University Graduate School of Medicine and under the law and notification of the Japanese government. Efforts were taken to minimize animal sufferings. The heterozygous mice were inter-crossed to get the homozygous KO mouse pups and then genotyped to identify the individual mouse.

Tissue collection and quantitative real-time PCR. The brain was removed quickly under anesthesia and made the serial coronal sections, followed by punching out the hippocampal tissue. Total RNA was extracted by RNeasy Mini Kit (Qiagen, Hilden, Netherlands). Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland) was used for reverse transcription. qPCR for Ndufs4 was performed using StepOnePlus Real-Time PCR system (Applied Biosystems, California, USA) using following mouse specific TaqMan Probes: Ndufs4:Mm06561766_ml, Actb: Mm02619580_gl. Actb was used as endogenous control. Relative gene expression was calculated using the 2<sup>−ΔΔCt</sup> method.

Immunostaining. The male mice at p21 were transcardially perfused with glyoxal (ALTFiX, Falma Co., Ltd. Tokyo, Japan) under anaesthesia. Brains were removed, post-fixed in glyoxal for overnight at 4 °C and subsequent dehydration by gradient concentration of alcohol, cleaned by xylene, infiltrated, and embedded in paraffin. Brain samples were cut in a serial 4 μm of coronal sections using a sliding microtome (Leica Biosystems, Wetzlar, Germany).

For enzyme-based immunohistochemistry, the sections were deparaffinized, rehydrated in phosphate buffered saline (PBS) and permeabilized with 0.3% Triton X-100 in PBS, blocked in 5% fetal bovine serum (FBS) followed by overnight incubation of primary antibody shown in Table 1. After primary antibody incubation, sections were washed with PBS and incubated with biotin conjugated goat anti mouse IgG. Then, sections were incubated with ABC reagent (VECTASTAIN Elite ABC kit, Vector Laboratories Inc., Cat. No. PK-6100, California, USA). Haematoxylin stain was used as nuclear counterstaining.

For immunofluorescence, sections were deparaffinized, hydrated followed by permeabilization with 0.3% Triton X-100. Sections were incubated in primary antibodies shown in Table 1. Respective secondary antibodies and DAPI (Thermo Fisher Scientific Inc., Massachusetts, USA) reaction were allowed. Images were acquired using a confocal laser scanning microscope (LSM780; Carl Zeiss, Oberkochen, Germany). Number of astrocytes and astrocyte processes were counted using cell counter plugin of ImageJ (NIH, USA). Actual fluorescence intensity was measured after subtracting the background intensity using ImageJ software.

For immunofluorescence using cryosection, brain was removed, post fixed with 4% paraformaldehyde (PFA) and cryoprotected in 15% sucrose followed 30% sucrose in PBS until the tissue sink. Then a cryostat (Tissue-Tek OCT Compound, Sakura Finetek, Cat. No. 4583, Osaka, Japan) was used to cut 14 μm section. Sections were blocked with 20% FBS in 0.3% Triton X-100 in PBS for 1 h followed by overnight incubation of primary antibody (Table1) in 4 °C. Respective secondary antibodies were used, and images were acquired using a confocal laser scanning microscope (LSM780).

Immunocytochemistry of synaptophysin in Neuro-2a cells neurites was performed according to described previously<sup>47</sup>. Cells were fixed with 4% PFA and permeabilized with 0.1% Triton X-100 and incubated by primary antibodies shown in Table 1. Respective secondary antibody with DAPI reaction was allowed. Images were captured by confocal laser scanning microscope LSM780. Synaptophysin stained red puncta was counted as presynapse and respective neurite length was measured using ImageJ software.

Purified astrocyte culture. Primary astrocytes were prepared from cerebral cortices and hippocampi of 0- to 1- day-old WT and littermate Ndufs4-KO mice as described previously<sup>48</sup>. Briefly, the cerebral cortices and hippocampi were isolated carefully with removing the olfactory bulbs and the meninges. Tissues were transferred in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma Aldrich, Missouri, USA) supplemented with 10% FBS, 20 mM D-glucose, 1% v/v penicillin/streptomycin and treated with trypsin (Life Technologies, California, USA) at a final concentration of 0.25% for 20 min in 37 °C. The harvested cells were resuspended in DMEM containing 10% v/v heat-inactivated FBS, 1% v/v penicillin/streptomycin and filtered using a 100 μm cell strainer (Falcon, New York, USA). Finally, the cells from individual brain were seeded in respective T-75 flasks (BD Falcon, Schaffhausen, Switzerland). Medium was replaced every three days interval. After 11–12 days, the cells...
Table 1. List of primary and secondary antibodies.

| 1st Antibody                          | Dilution | Vendor/reference               | 2nd Antibody/dilution                  |
|---------------------------------------|----------|-------------------------------|----------------------------------------|
| Rabbit polyclonal anti-Ndufs4         | WB: 1:500| Sigma Aldrich, Cat. No. HPA003884 | Goat anti-rabbit IgG-HRP conjugated (1:2000) |
| Mouse monoclonal anti-Ndufs4          | IF: 1:50 | SantaCruz, Cat. No. sc-100567 | Goat anti-mouse IgG-Alexa 488 (1:500) |
| Rat monoclonal anti-GFAP              | WB: 1:2000, IF: 1:200 | Thermo Fisher Scientific, Cat. No.13-0300 | Goat anti-rat IgG-HRP conjugated (1:2000), Goat anti-rat IgG-Alexa 568 (1:500) |
| Mouse monoclonal anti NeuN            | WB: 1:1000, IF: 1:200 | Chemicon, Cat No. MAB377 | Goat anti-mouse IgG-HRP conjugated (1:2000), Goat anti-mouse IgG-Alexa 488 (1:500) |
| Rabbit monoclonal anti-NeuN           | IF: 1:200 | Cell Signaling, Cat. No. 24367 | Goat anti-rabbit IgG-Alexa 568 (1:500) |
| Rat monoclonal anti MBP               | WB: 1:1000, IF: 1:200 | Abcam, Cat. No. ab7349 | Goat anti-rat IgG-HRP conjugated (1:2000), Goat anti-rat IgG-Alexa 568 (1:500) |
| Rabbit polyclonal anti PSD95          | WB: 1:2000, IF: 1:200 | Cell Signaling, Cat. No. 3450 | Goat anti-rabbit IgG-HRP conjugated (1:2000), Goat anti-rabbit IgG-Alexa 568 (1:500) |
| Mouse monoclonal anti Synaptophysin   | WB: 1:400, IC:1:100 | Sigma Aldrich, Cat. No. S5768 | Goat anti-mouse IgG-HRP conjugated (1:2000), Goat anti-mouse IgG-Alexa 488 (1:250) |
| Rabbit monoclonal anti β-III Tubulin  | IC: 1:00 | Cell Signaling, Cat. No. 5666P | Goat anti-rabbit IgG-Alexa 568 (1:500) |
| Rabbit polyclonal anti EAAT1          | WB: 1:1000 | Cell Signaling, Cat. No. 4166 | Goat anti-rabbit IgG-HRP conjugated (1:2000) |
| Rabbit polyclonal anti EAAT2          | WB: 1:1000 | Abcam, Cat. No. ab41621 | Goat anti-rabbit IgG-HRP conjugated (1:2000) |
| Rabbit polyclonal anti-Rat EAAC1 (EAAT3) | WB: 1:1000 | Alpha Diagnostic International, Cat. No. EAAC1-5 | Goat anti-rabbit IgG-HRP conjugated (1:2000) |
| Guinea pig polyclonal anti Vglut 1    | WB: 1:1000 | EMD Millipore, Cat. No. AB5905 | Goat anti-guinea pig IgG-HRP conjugated (1:2000) |
| Rabbit polyclonal anti p44/42 MAPK (Erk1/2) | WB: 1:1000 | Cell Signaling, Cat. No. 9102 | Goat anti-rabbit IgG-HRP conjugated (1:2000) |
| Rabbit polyclonal anti phospho-p44/42 MAPK (Erk1/2) | WB: 1:1000 | Cell Signaling, Cat. No. 9101 | Goat anti-rabbit IgG-HRP conjugated (1:2000) |
| Mouse monoclonal anti β-actin         | WB: 2000 | Santa Cruz, Cat. No. sc-47778 | Goat anti-mouse IgG-HRP conjugated (1:2000) |
| Rabbit Polyclonal anti VDAC1          | WB: 1:1000 | Abcam, Cat. No. ab15895 | Goat anti-rabbit IgG-HRP conjugated (1:2000) |
| Rabbit polyclonal anti cleaved caspase 3 | IF: 1:300 | Cell Signaling, Cat. No. 9661 | Goat anti-rabbit IgG-Alexa 568 (1:500) |

Mitochondria from tissue and cultured cells. Mitochondria were isolated from brain and heart as described before with modifications. Briefly, isolated brain and heart were trypsinized for 20 min with shaking for every 5 min. Tissue was resuspended in mitochondrial isolation buffer (0.25 M sucrose, 20 mM Tris–HCl (pH 8.0), 0.1 mM EDTA (pH 8.0) and homogenized by Dounce homogenizer with tight pestle with gentle 20 strokes. Homogenate was allowed for centrifugation at 600×g for 15 min at 4 °C. Supernatant was transferred carefully and centrifuged again at 12,000×g for 10 min at 4 °C. Finally, the pellet was dissolved in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing protease inhibitor (Roche). Protein concentration was measured by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc.) in accordance with manufacturer’s instructions.

Isolation of hippocampal mitochondria and mitochondrial complex I respiration assay. Mitochondrial complexes were isolated according to previously published report with modifications. Briefly, hippocampal tissue was collected, chopped, and washed with Dulbecco’s Phosphate-Buffered Saline followed by trypsinization. Then, mitochondria were isolated in ice cold isolation buffer (Sucrose 200 mM, Tris-MOPS 10 mM, EGTA/Tris 1 mM, final pH 7.4) using Dounce homogenizer. Homogenates were centrifuged firstly at low speed and then high speed to get the functional mitochondrial pellet. Complex I dependent mitochondrial respiration was measured using the seahorse XF24 extracellular flux analyzer (Agilent Technologies, California, USA) according to the manufacturer’s protocols and previous reports. A total of 25 μg of mitochondrial was seeded in XF24 cell culture plate with assay medium for complex I (Sucrose 70 mM, D (-) Mannitol 220 mM, KH₂PO₄ 10 mM, MgCl₂ 5 mM, HEPES–KOH 2 mM, EGTA-KOH 1 mM, fatty acid free bovine serum albumin (BSA) 0.2%, D-Malic acid 5 mM and Sodium pyruvate 10 mM, pH 7.2) and centrifuged at 2,000g for 20 min at 4 °C. OCR was monitored by sequential injections of Adenosine diphosphate (ADP), Oligomycin, Carbonyl cyanide-4- (trifluoromethoxy)phenylhydrazone (FCCP) and Antimycin A to a final concentration of 4.25 mM, 2.5 μM, 5 μM and 4 μM in complex I assay medium respectively.

Mitochondrial respiration assay for Ndufs4 silenced differentiated Neuro-2a. Oxygen consumption rate (OCR) of Neuro-2a cells was measured according to the manufacturer’s protocols and previous published article. Briefly, transfected cells were seeded in XF24 culture plate, induced differentiation and cultured in assay medium containing 25 mM glucose, 2 mM L-glutamine, 1 mM sodium pyruvate and 30 mM NaCl (pH 7.4). Mitochondrial OCR was measured with sequential injection of Oligomycin, FCCP and rotenone with
antimycin A through the ports in assay cartridge to a final concentration of 1 µg/ml, 1 µM and 5 µM, respectively. This allowed determination of the basal level of oxygen consumption, level of ATP production and maximal respiration.

**Western blot.** Protein samples were separated on SDS-PAGE and then transferred to a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Massachusetts, USA). Membrane was blocked with 5% BSA in Tris–buffered saline with Tween 20 (T-TBS) and incubated with primary antibody shown in Table 1. The protein was visualized by an enhanced chemiluminescence. Images were acquired with ChemiDoc MP Imaging System (Bio-Rad Laboratories, California, USA). Anti-β-actin and anti-VDAC1 were used as probe to ensure the equal loading of the samples. Original full-length blots/gels are shown in Supplemental Figures.

**Astrocyte proliferation assay.** WT and Ndufs4-KO primary astrocytes were seeded in 24 well plate at a density of 100,000 cells/well. Fifty microliters of Cell Count Reagent SF (Nacalai Tesque, Cat. No. 0753-44, Kyoto, Japan) was added to each well containing 500 µl of culture medium and the plates were incubated for one hour at 37 °C. Absorbance was recorded at 450 nm using the FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, California, USA).

**Ndufs4 silencing in Neuro-2a cells.** Mouse neuroblastoma Neuro-2a cells were transfected with Ndufs4 siRNA (Thermo Fisher Scientific Inc., Cat. No. 10620318) using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific Inc., Cat. No. 13778-150) according to manufacturer’s protocol. Negative control medium GC duplex (Thermo Fisher Scientific Inc, Cat. No. 465372) was used for the control. Following mouse specific primer sequences were used: siRNA1: Ndufs4MSS275954(3_RNAI) 5′–3′ GAG AAA CUG GAU AUC ACA AC ū UUA A, siRNA2: Ndufs4MSS275955(3_RNAI) 5′–3′ GAG AAU GAU AUC AČ ā UGU UCU GCC UGC CAA U.

**Evaluation of neurite outgrowth and presynapse number.** Cells were passaged after 18 h of Ndufs4 siRNA transfection. To stimulate differentiation, the cells were then incubated in medium containing 20 µM of all-trans-Retinoic acid (RA) (Sigma Aldrich, Cat. No. R2625, Tokyo, Japan) for 18 h. Live cell images were captured by phase contrast microscope. The number of neurites in the individual cells were counted and lengths were measured as the distance from the centre of the cell soma to the tip of the neurite as reported previously. Six random fields were examined from each siRNA, giving a total field of 18 and cell count was at least 70 cells/well. Each data point represents the mean of three individual wells in one experiment, and each experiment was repeated three times.

**pERK agonist carbachol treatment in Neuro-2a cells.** Neuro-2a cells were seeded at a concentration of 1 × 10⁵ cells/ml, transfected with Ndufs4 siRNA followed by induction of differentiation using RA for 12 h in serum free medium. After 36 h of transfection, media containing RA was washed with DMEM medium and treated by serum free medium containing pERK agonist Carbamylcholine Chloride (carbachol) (FUJIFILM Wako Pure Chemical Corporation, Cat. No. 036-09841, Osaka, Japan) at a final concentration of 100 µM for 8 h. Images were taken and cells were fixed by 4% followed by co-immunofluorescence staining.

**Statistical analysis.** Data were presented as mean±SEM (standard error of mean) of at least six independent experiments with at least three biological replicates. Comparisons were made by two tailed unpaired Student’s t test. Differences were considered significant at P values < 0.05. Analysis was performed using the Microsoft Excel.

Received: 31 August 2020; Accepted: 4 May 2021
Published online: 26 May 2021

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Acknowledgements

We thank Mr. Ken Hayasaka and Mr. Yosuke Otsuka for their technical assistance and Biomedical Research Unit of Tohoku University Hospital for their support.
Author contributions
S.S., Y.K. and Y.O. conceived and designed the study with comments from all authors. S.S. wrote the original manuscript and Y.K., B.U., Y.O. edited the manuscript. S.S., Y.K., B.U., F.N., H.M., Y.Y., and C.S. performed experiments. Y.K., S.K. and T.A. provided reagents and technical assistance.

Funding
This work was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI Grant (No. 20K11527 to Y.K. and No. 20K21743 to Y.O.).

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-90127-4.

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