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ORIGINAL PAPER

The Protective Effects of Levetiracetam on a Human iPSCs-Derived Spinal Muscular Atrophy Model

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Abstract

Spinal muscular atrophy (SMA) is an inherited disease characterized by progressive motor neuron death and subsequent muscle weakness and is caused by deletion or mutation of survival motor neuron (SMN) 1 gene. Protecting spinal motor neuron is an effective clinical strategy for SMA. The purpose of this study was to investigate the potential effect of an anti-epileptic drug levetiracetam on SMA. In the present study, we used differentiated spinal motor neurons (MNs) from SMA patient-derived induced pluripotent stem cells (SMA-iPSCs) to investigate the effect of levetiracetam. Levetiracetam promoted neurite elongation in SMA-iPSCs-MNs. TUNEL-positive spinal motor neurons were significantly reduced by levetiracetam in SMA-iPSCs-MNs. In addition, the expression level of cleaved-caspase 3 was decreased by levetiracetam in SMA-iPSCs-MNs. Furthermore, levetiracetam improved impaired mitochondrial function in SMA-iPSCs-MNs. On the other hand, levetiracetam did not affect the expression level of SMN protein in SMA-iPSCs-MNs. These findings indicate that levetiracetam has a neuroprotective effect for SMA.

Keywords Spinal muscular atrophy · Induced pluripotent stem cells · Levetiracetam · Motor neuron

Introduction

Spinal muscular atrophy (SMA) is an inherited disorder characterized by progressive loss of spinal motor neurons and muscle atrophy with low expression level of survival motor neuron (SMN) protein [1]. SMA is caused by deletion or mutation of *SMN1* gene [2]. In humans, SMN protein is encoded by *SMN1* gene and its highly homologous copy, *SMN2* gene [3]. SMN protein derived from *SMN1* gene is stable, but *SMN2* gene mainly produces unstable form of SMN protein [4]. Therefore, the lack or mutation of *SMN1* gene results in low expression levels of SMN protein. SMN protein is expressed ubiquitously and it has an important role in the assembly of complexes in the spliceosomes [5]. However, it is unclear why reduced level of SMN protein causes vulnerability of spinal motor neuron. SMA is divided into 4 types according to the age of onset and physical development: type I (onset within the first six months of life, unable to sit), type II (onset before the age of 3 years, unable to stand without support), type III (onset after the age of 3 years and unable to stand gradually), and type IV with adult onset [6]. Recently, nusinersen, an antisense oligonucleotide that increase SMN protein level, has been approved as the first drug for SMA treatment [7]. This breakthrough is promising for SMA therapy, but does not address all clinical problems.

To study SMA pathogenesis, animal models and cultured cell including skin fibroblasts derived from SMA patients have been used [8, 9]. However, there are fundamental differences among the species and cell strains, which

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complicates SMA study. The recent development of disease-specific stem cell-based models enable targeted transitional research for neurodegenerative diseases including SMA [10, 11]. Previously, we examined the effects of the candidate drugs, thyrotropin-releasing hormone analog and edaravone for SMA using a disease model derived from human induced pluripotent stem cells (iPSCs) [12, 13].

Levetiracetam is an anti-epileptic drug widely used for treatment of seizures. Levetiracetam has high affinity for synaptic vesicle glycoprotein 2A (SV2A) [14] and inhibits N-type Ca^{2+} channels [15]. Levetiracetam also have neuroprotective effects for central nervous system disorders such as Parkinson's disease and Alzheimer's disease [16, 17].

We previously reported edaravone had neuroprotective effects on SMA-iPSCs-MNs [13]. On the other hand, there are concerns with edaravone that may cause side effects such as renal disorders [18], and safety of edaravone for children is unknown. In terms of clinical use, levetiracetam can be used for seizures both in adults and children [19].

The aim of this study was to investigate potential effects of levetiracetam for SMA treatment. Here, we report the protective effects of levetiracetam on differentiated SMA-iPSCs-MNs.

Materials and Methods

iPSCs Culture, Spinal Motor Neuron Differentiation and Drug Assays

The iPSCs were maintained in primate ES cell medium (ReproCELL, Kanagawa, Japan) supplemented with 4 ng/mL basic fibroblast growth factor (Wako, Osaka, Japan) and 500 U/mL penicillin/streptomycin (PS, Life Technologies, Carlsbad, CA, USA). The iPSC colonies were cultured in 5% CO_2 at 37 °C and passaged every 7 days.

For spinal motor neuron differentiation from iPSCs, 9000 cells of iPSCs were seeded into a primary differentiation medium containing Dulbecco's modified Eagle medium (DMEM)/F12 (Life Technologies), 5% Knockout Serum Replacement (KSR, Life Technologies), and 500 U/mL PS with 2 μM dorsomorphin (Sigma-Aldrich, St. Louis, MO, USA), 10 μM SB431542 (SB, Cayman, San Diego, CA, USA), and 10 μM Rho-associated coiled-coil forming kinase inhibitor, Y-27632 (Wako). The medium was changed the medium supplemented with 2 μM dorsomorphin and 10 μM SB431542 after 3 days. Spheres were then plated in Matrigel-coated 24-well plates (Becton, Dickinson and Company, NJ, USA) in medium supplemented with 2 μM dorsomorphin and 10 μM SB431542 for another 7 days. The neural precursor cells were then cultured in the second differentiation medium containing DMEM/F12, 1% N2 supplement (Life Technologies), and 500 U/mL PS with 0.1 μM retinoic acid (RA, Sigma Aldrich). The cells were then cultured in the third differentiation medium with 1 μM purmorphamine (PMN, Miltenyi Biotec, Bergisch, Gladbach, Germany) and 0.1 μM RA for 7 days. Finally, the generated spinal motor neurons were cultured in the supplemented medium containing 10 ng/mL brain-derived neurotrophic factor (BDNF, R&D Systems Inc., Minneapolis, MN, USA), 10 ng/mL glial cell line-derived neurotrophic factor (GDNF, R&D), 1 μM cyclic adenosine monophosphate (cAMP, Wako) and 200 ng/mL ascorbic acid (AA, Sigma-Aldrich). During all differentiation stages, medium was changed every 2 or 3 days.

The differentiated spinal motor neurons were exposed to levetiracetam (Tokyo chemical industry, Tokyo, Japan) for 42 to 56 days of our induction protocol by changing the medium every 3 days.

Immunocytochemistry

Plated cells were washed with PBS three times. Cells were fixed in 4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan) for 20 min at 4 °C and then washed again with PBS three times. Cells were blocked with 5% donkey serum in PBS for 30 min at room temperature, and incubated with primary antibodies for 24 h at 4 °C. After washing two times with PBS, the cells were labelled with the appropriate secondary antibody-tagged fluorescent dye for 1 h. Nuclear staining was performed using Hoechst 33,342 (diluted 1:1000; Life Technologies). The following primary antibodies were used: rabbit anti- TUJ1 (βIII-tubulin) antibody (diluted 1:1000; BioLegend, San Diego, CA, USA), mouse anti-HB9 antibody (diluted 1:50; DSHB, Iowa city, IA, USA), mouse anti-SMI32 antibody (diluted 1:1000; BioLegend), mouse anti-TOM20 antibody (diluted 50:1; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse anti-HSP60 antibody (diluted 200:1, StressMarq Biosciences, Victoria, British Columbia, Canada). The secondary Alexa Fluor-labeled antibodies were 594 donkey anti-mouse IgG and 488 donkey anti-rabbit IgG (Life Technologies, the ilution for all second antibodies was 1:1000). All images were taken by BIOREVO BZ-9000 (Keyence, Osaka, Japan).

TUNEL Staining Assay

An In Situ Cell Death Detection Kit (Roche Biochemicals, Mannheim, Germany) was used to detect cell death. After washing with PBS, plated cells were fixed in 4% paraformaldehyde for 20 min at 4 °C. The fixed cells were washed again with PBS, and incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 30 min at room temperature. After washing with PBS twice, the cells were incubated with fluorescein-labeled terminal deoxyribonucleotidyl transferase at 37 °C for 1 h. The cells were then washed 3 times

with PBS. Fluorescein-labeled TUNEL-positive cells were detected by BIOREVO BZ-9000. TUNEL-positive cells were evaluated by appropriately calibrated computerized image analysis using an image processing software (Image-J, version 1.33f, National Institutes of Health, Bethesda, MD, USA).

Western Blot Analysis

Harvested cells were lysed in a cell lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 1% Igepal CA-630] containing protease (P8340; Sigma-Aldrich) and phosphatase inhibitor cocktails (P2850 and P5726; Sigma-Aldrich). The cell lysates were centrifuged at 12,000×*g* for 10 min at 4 °C. Protein concentrations were measured by comparison with a known concentration of bovine serum albumin, using a BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Sodium dodecyl sulfate (SDS) sample buffer (Wako, Osaka, Japan) was added to each sample. The samples were boiled for 5 min, and then subjected to 5–20% SDS-polyacrylamide gel electrophoresis. The separated proteins were then transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Bedford, MA, USA) and incubated with the following primary antibodies: rabbit anti-cleaved caspase-3 antibody (diluted 1:500; Cell Signaling Technology, Danvers, MA, USA), mouse anti-SMN antibody (diluted 1:1000; Abnova, Taipei city, Taiwan), and mouse anti-β-actin antibody (diluted 1:2000; Sigma-Aldrich). After incubation, the membrane was incubated with the following secondary antibodies: HRP-conjugated goat anti-rabbit IgG (Pierce Biotechnology, Rockford, IL, USA; diluted 1:2000) and HRP-conjugated goat anti-mouse IgG (Pierce Biotechnology; diluted 1:2000). The immunoreactive bands were visualized with Immunostar-LD (Wako, Osaka, Japan), and a LAS-4000 luminescent image analyzer (Fuji Film Co., Ltd., Tokyo, Japan) was used to quantify the level of antigens.

Statistical Analysis

Data are presented as mean ± S.E.M. Statistical comparisons were perfomrd using Student's *t*-test or Dunnett's test. P values less than 0.05 were considered statistically significant. All statistical analyses were performed using SPSS Statistics (IBM, Armonk, NY, USA).

Results

The Effect of Levetiracetam on Neurite Length

We previously established a human in vitro SMA model using iPSCs derived from an SMA patient [12, 13]. To assess the potential efficacy of levetiracetam, the effect of levetiracetam on the neurite length of SMA-iPSCs-MNs was evaluated. 30 μM or 100 μM levetiracetam was added to differentiated SMA-iPSCs-MNs for 14 days. We performed double immunostaining with TUJ1 and a maker of spinal motor neuron, SMI 32. Neurite length in TUJ1-positive and SMI32-positive SMA-iPSCs-MNs was evaluated. 100 μM Levetiracetam significantly restored neurite outgrowth of SMA-iPSCs-MNs (Fig. 1a, b). Levetiracetam also stimulated neurite elongation in differentiated spinal motor neurons derived from two other SMA patient-derived iPSCs (Fig. 1c).

The Effect of Levetiracetam on Apoptotic Cell Death

To evaluate the effect of levetiracetam on cell death, we performed terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) staining. TUNEL-positive MNs were significantly increased in vehicle treated SMA-iPSCs-MNs compared to that of CT-iPSCs-MNs. Levetiracetam decreased TUNEL-positive cells in SMA-iPSCs-MNs (Fig. 2a, b). However, the TUNEL method detects DNA fragmentation not only in apoptotic cells but also necrotic cells. To confirm the effect of levetiracetam on neuronal apoptosis in SMA-iPSCs-MNs, we examined the expression of cleaved caspase 3 by western blots analysis. The expression level of cleaved-caspase 3 was significantly decreased in levetiracetam treated SMA-iPSCs-MNs compared to that of vehicle treated SMA-iPSCs-MNs (Fig. 2c).

The Effect of Levetiracetam on the Expression Level of SMN Protein

Lack of SMN protein contributes degeneration of spinal motor neuron in SMA pathology, and compounds that increase SMN protein expression restore aspects of SMA pathology [7, 9]. To examine whether levetiracetam shows protective effect on SMA-iPSCs-MNs via increasing SMN protein expression, we evaluated the SMN protein level by western blot analysis. However, the expression level of SMN protein was not changed after the addition of levetiracetam (Fig. 3a, b).

The Effect of Levetiracetam on Mitochondria

To elucidate the mechanism underlying the protective effect of levetiracetam, we examined the mitochondrial function of SMA-iPSCs-MNs. Translocase of the outer membrane (TOM) 20 forms a complex with other TOM protein family members and plays a role in mitochondrial protein import [20]. We evaluated the expression level of TOM20 by immunostaining. The fluorescent intensity of TOM20 in SMA-iPSCs-MNs was decreased compared to that of

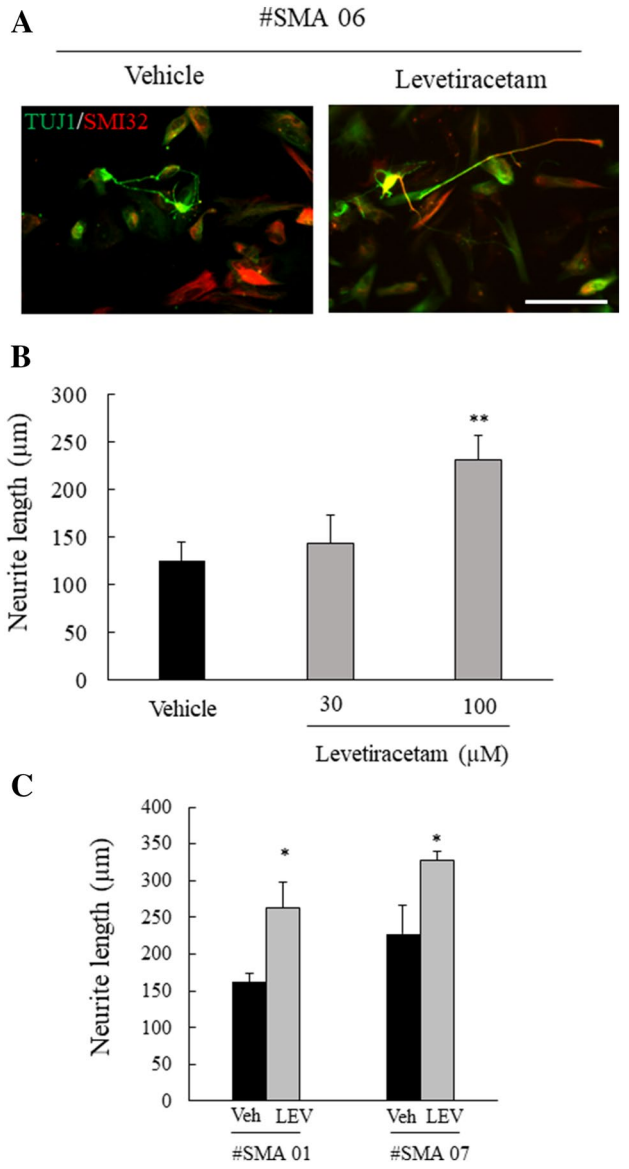

Fig. 1 Levetiracetam stimulated neurite elongation in SMA-iPSCs-MNs. **a** Representative images of SMI-32 positive neurite of SMA-iPSCs-MNs (SMA 06) after 14 days vehicle or 100 μM levetiracetam addition. The differentiated spinal motor neurons were exposed to levetiracetam for 42 to 56 days of the induction protocol. A scale bar is 100 μm. **b** Quantitative measurements of neurite length of SMA-iPSCs-MNs (SMA 06). Data are mean ± S.E.M. (vehicle treated group: n = 10; 30 μM levetiracetam treated group: n = 8; 100 μM levetiracetam treated group: n = 8). $^{**}p < 0.01$ versus vehicle group (Dunnett's test). **c** Quantitative measurements of neurite length of SMA-iPSCs-MNs after 14 days vehicle or 100 μM levetiracetam addition (SMA 01 and SMA 07). Data are means ± S.E.M. (vehicle treated SMA 01 group: n = 6; levetiracetam treated SMA 01 group: n = 7; vehicle treated SMA 07 group: n = 4; levetiracetam treated SMA 07 group: n = 4). $^{*}p < 0.05$ versus each vehicle group (Student's *t*-test). *Veh* vehicle, *LEV* levetiracetam

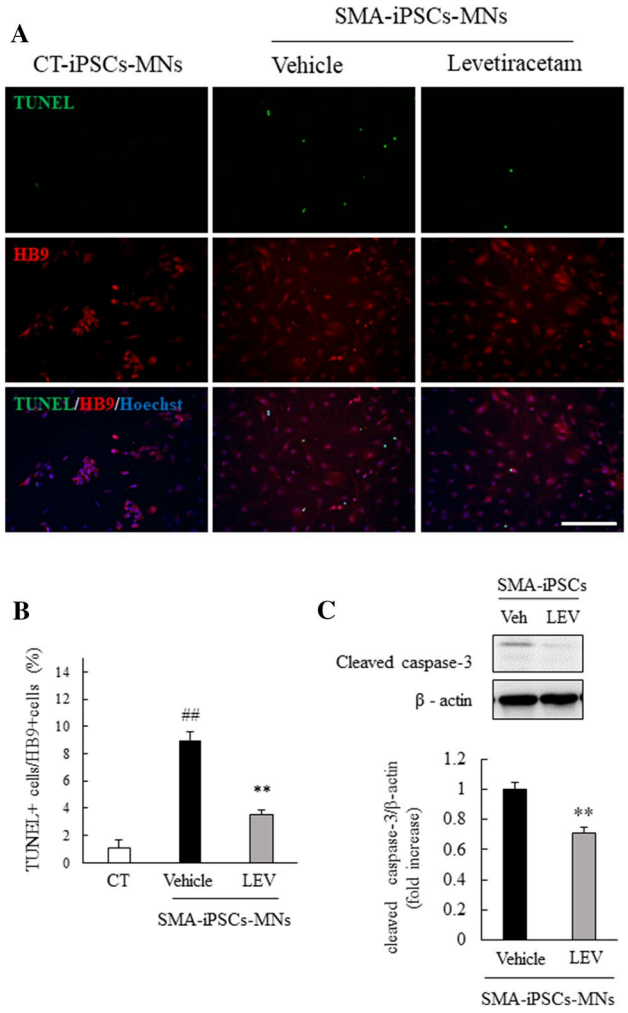

Fig. 2 Levetiracetam suppressed neuronal cell death in SMA-iPSCs-MNs. **a** Representative images of TUNEL staining of control-iPSCs-derived, vehicle-treated SMA-iPSCs-derived, and levetiracetam-treated SMA-iPSCs-derived spinal motor neurons (SMA 06). The differentiated spinal motor neurons were exposed to 100 μM levetiracetam for 42 to 56 days of the induction protocol. A scale bar is 200 μm. **b** Quantitative analysis of TUNEL-positive cells in HB9 positive mature motor neurons. Data are mean ± S.E.M. (control group: n = 4; vehicle treated group: n = 5; 100 μM levetiracetam treated group: n = 5). $^{\#\#}p < 0.01$ versus CT-iPSCs-MNs (Student's *t*-test), $^{**}p < 0.01$ versus vehicle-treated SMA-iPSCs-MNs (Student's *t*-test). **c** Western blots analysis in SMA-iPSCs-MNs (SMA 06) using anti cleaved-caspase3 antibody. Data are mean ± S.E.M. (n = 5). $^{**}p < 0.01$ versus vehicle-treated SMA-iPSCs-MNs (Student's *t*-test). *CT* control, *Veh* vehicle, *LEV* levetiracetam

CT-iPSCs-MNs, and levetiracetam increased the fluorescent intensity of TOM20 in SMA-iPSCs-MNs (Fig. 4a, b). To confirm the effect of levetiracetam on mitochondria, the expression level of another mitochondrial marker, HSP60 was evaluated. The fluorescent intensity of HSP60 in SMA-iPSCs-MNs was decreased compared to that of CT-iPSCs-MNs. Levetiracetam at 100 μM increased the fluorescent intensity of HSP60 in SMA-iPSCs-MNs (Fig. 4c, d).

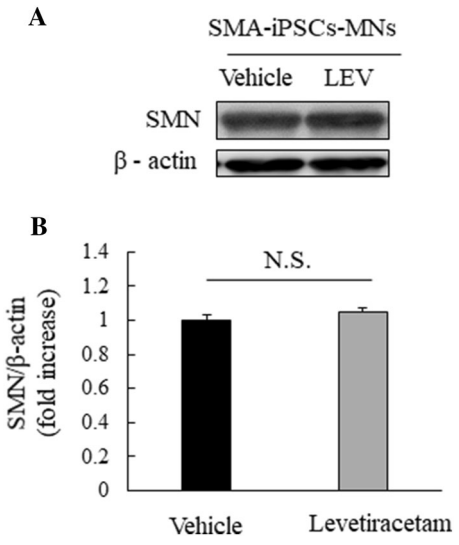

Fig. 3 Levetiracetam did not affect the expression of SMN protein in SMA-iPSCs-MNs. **a** SMN protein expression was examined by western blot analysis in SMA-iPSCs-MNs (SMA 06). The differentiated spinal motor neurons were exposed to 100 μM levetiracetam for 42 to 56 days of the induction protocol. **b** Quantitative analysis of the expression level of SMN protein. Data are means ± S.E.M. (n = 5, Student's *t*-test). *LEV* levetiracetam, *N.S.* Not significant

Discussion

In this study, we evaluated the effect of levetiracetam on SMA-iPSCS-MNs. Levetiracetam efficacy was observed by stimulation of neurite elongation, suppression of neuronal cell death, and levetiracetam restored impaired mitochondrial function in SMA-iPSCs-MNs.

Genetic loss of SMN protein leads to apoptotic cell death of spinal motor neurons [21]. In addition to *SMN* gene, the mutation or deletion of the neuronal apoptosis inhibitor protein (*NAIP*) gene, which prevents normal apoptosis, is related to SMA pathology [22]. Mutation or deletion of *NAIP* is found in about 50% of SMA type I patients, and lack of *NAIP* is also observed in SMA06 patient. Preventing apoptotic cell death in spinal motor neurons is a beneficial strategy for SMA. In this study, we observed increased TUNEL positive cells in SMA-iPSCs-MNs compared to that of CT-iPSCs-MNs, and levetiracetam decreased the number of TUNEL-positive cells in SMA-iPSCs-MNs (Fig. 2). This result suggests that levetiracetam prevented the neuronal cell death in SMA-iPSCs-MNs. Next, we evaluated the expression level of cleaved-caspase 3, activated in the final stage of apoptosis [23]. Levetiracetam decreased the cleaved-caspase 3 expression in SMA-iPSCs-MNs. These results suggest that levetiracetam suppressed apoptotic cell death in SMA-iPSCs-MNs.

To elucidate the mechanism underlying the anti-apoptotic effect of levetiracetam in SMA-iPSCs-MNs, we focused on

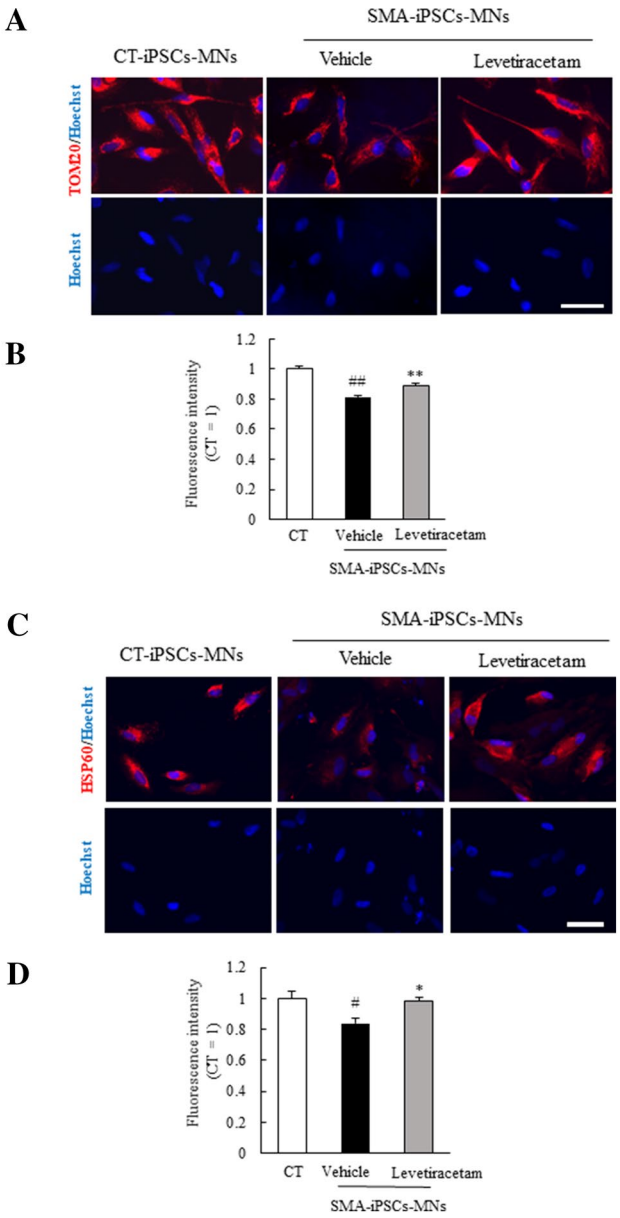

Fig. 4 Levetiracetam suppressed mitochondrial impairment in SMA-iPSCs-MNs. **a** Immunostaining of TOM20 in CT-iPSCs-MNs, vehicle-treated SMA-iPSCs-MNs and levetiracetam-treated SMA-iPSCs-MNs (SMA 06). The differentiated spinal motor neurons were exposed to 100 μM levetiracetam for 42 to 56 days of the induction protocol. A Scale bar is 50 μm. **b** Quantitative analysis of the fluorescence intensity of TOM20. Data are means ± S.E.M. (control group: n = 5; vehicle treated group: n = 5; 100 μM levetiracetam treated group: n = 4). $^{\#\#}p < 0.01$ versus CT-iPSCs-MNs (Student's *t*-test), $^{**}p < 0.01$ versus vehicle-treated SMA-iPSCs-MNs (Student's *t*-test). **c** Immunostaining of HSP60 in CT-iPSCs-MNs, vehicle-treated SMA-iPSCs-MNs and levetiracetam-treated SMA-iPSCs-MNs (SMA 06). A Scale bar is 50 μm. **d** Quantitative analysis of the fluorescence intensity of HSP60. Data are means ± S.E.M. (n = 3). $^{\#}p < 0.05$ versus CT- iPSCs-MNs (Student's t-test), $^{*}p < 0.05$ versus vehicle-treated SMA-iPSCs-MNs (Student's *t*-test). *CT* control

SMN protein expression because increasing SMN protein leads to an anti-apoptotic effect [21]. Many antiepileptic drugs represented by valproic acid could inhibit histone deacetylase [24] and the inhibition of histone deacetylase increases SMN protein expression [9]. Therefore, we hypothesized that levetiracetam shows an anti-apotoic effect in SMA-iPSCs-MNs via increasing SMN protein. However, levetiracetam did not affect the expression level of SMN protein in SMA-iPSCs-MNs (Fig. 3). This result suggests that the protective effect of levetiracetam is independent of SMN protein expression.

Mitochondria plays an essential role for maintaining neuronal survival and function. The important mitochondria functions are ATP production, Ca^{2+} homeostasis, reactive oxygen production (ROS), and regulation of apoptotic signaling [25–27]. Mitochondrial dysfunction occurs in various neurodegenerative diseases including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis [28–30]. Previously, Acsadi et al. found decreased SMN protein level in NSC-34 cells, mouse motor neuron-like cells, using siRNA resulted in increased mitochondrial membrane potential and increased free radical production [31], and Miller et al. reported ultrastructural mitochondria abnormalities in motor neurons in SMA model mice [32]. In addition, we recently found mitochondrial ROS level was upregulated in SMA-iPSCs-MNs [13]. Olesoxime, a mitochondrial-targeted drug, showed efficacy in SMA mouse model [33]. Therefore, mitochondrial dysfunction is considered to be related to SMA pathology and mitochondria may be an effective therapeutic target for SMA. In the present study, we conformed TOM20 expression in SMA-iPSCs-MNs was decreased compared to that of CT-iPSCs-MNs (Fig. 4). This finding demonstrated the dysregulation in mitochondrial protein import. Here, we observed the upregulation of TOM20 in SMA-iPSCs-MNs by levetiracetam treatment (Fig. 4). This result suggests that levetiracetam has a protective effect on mitochondria in SMA-iPSCs-MNs. Depletion of TOM20 enhances cytochrome C release from mitochondria and subsequent apoptotic cell death [34]. Therefore, the anti-apoptotic effect of levetiracetam in SMA-iPSCs-MNs may be because of the upregulation of TOM20. In addition, HSP60 deficiency causes progressive deficit in motor functions in mice [35]. Levetiracetam upregulated the expression of HSP60 in the present study (Fig. 4). These results suggest that levetiracetam improves mitochondrial function in SMA-iPSCs-MNs. Levetiracetam has a high affinity for SV2A [14]. Stockburger et al. revealed that SV2A is expressed in not only synaptic vesicles but also mitochondria, and levetiracetam showed a protective effect on an in vitro Alzheimer's disease model by binding to mitochondrial SV2A [17]. Taken together, levetiracetam may show a protective effect on SMA-iPSCs-MNs via mitochondrial SV2A.

In conclusion, levetiracetam showed a protective effect in SMA-iPSCs-MNs by suppressing neuronal apoptotic cell death, which suggest that levetiracetam may be a potential candidate drug for SMA.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

Ethics Statement The procedures used in the experiments on human induced pluripotent stem cells (Control iPSCs; 201B7 line) adhered to the tenets of the Declaration of Helsinki. The 201B7 line was generated from adult human dermal fibroblasts which was purchased from Cell Applications, Inc (San Diego, CA, USA) [36]. The somatic cells collection from the patients, the establishment of patient-derived iPSCs, and pathological analysis of differentiated iPSCs including evaluation of the potential drug efficacy were planned in this study. These planed protocols were approved by the Ethics Review Committee of the National Hospital Organization, Nagara Medical Center (Approval Number: 26-15), and informed consents were obtained from the parents of our pediatric patients. Informed consents were obtained from the subjects after explanation of the nature and possible consequences of the study.

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