

Regulation of nuclear transport of the transcriptional factor REST improves axon regeneration in peripheral nerves

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Abstract. The expression of repressor element 1 silencing transcription factor (REST) in peripheral nerves increases with age, which leads to a decline in axon regeneration. However, the detailed mechanisms behind the decline in axon regeneration with age remain to be elucidated. The present study investigated the mechanism of nuclear transport of REST using hydrogen (H₂), which has neuroprotective effects. First, aged mice as an animal model and REST-overexpressed (REST-OE) cells generated from the NIH-3T3 fibroblast cell line as a cellular model were treated with H₂ to examine REST expression and growth-associated protein 43 (GAP43) as an axon regeneration marker. Subsequently, to examine differences in the localization of REST expression, *in vitro* cell lines were fractionated into cytoplasmic and nuclear fractions for immunofluorescence staining and western blotting, and REST expression was quantified. Furthermore, to investigate the mechanisms of nuclear transport, REST nuclear transport proteins (REST-interacting LIM domain protein, Huntingtin and dynactin subunit 1/p150^{Glued}) and the autophagy-related protein LC3 were semi-quantified by western blotting. REST expression was decreased, and GAP43 expression was increased following H₂ administration in animal models and REST-OE cells. REST intracellular localization analysis revealed that REST expression was significantly increased in the cytoplasm and significantly decreased in the nucleus in the REST-OE + H₂ group compared with the REST-OE group.

Furthermore, the present findings revealed that the addition of H₂ resulted in a significant decrease in several REST nuclear transport proteins, subsequently suppressing the nuclear translocation of REST. These findings suggest that regulation of the nuclear transport of REST by H₂ improves the decline in axon regeneration.

Introduction

Various musculoskeletal disorders develop with age. In bones, the progressive increase in receptor activator of NF-κB ligand and sclerostin levels (1,2), as well as a decline in normal type I collagen (3), lead to a reduction in bone mass and density that ultimately results in osteoporosis (4). In cartilage, an increase in matrix metalloproteinases and disintegrin and metalloproteinase with thrombospondin motifs, coupled with a reduction in normal type II collagen, contributes to cartilage degeneration, leading to osteoarthritis (5,6). In peripheral nerves, axonal regenerative capacity is reported to decline with age; however, the pathology in peripheral nerves remains to be elucidated (7).

Repressor element 1 silencing transcription factor (REST) is a transcriptional factor that regulates neuron-specific gene expression in the nervous system (8). Our previous studies demonstrated that REST expression increases with age in peripheral nerves, leading to a decline in axonal regenerative capacity (9,10). However, the role of REST in peripheral nerve axonal regeneration with age remains to be elucidated. Nuclear transport mechanisms are essential for transcription factors to perform their functions within cells (11). Under healthy aging, REST is transported to the nucleus in brain cells, where it provides neuroprotection against oxidative stress and apoptosis (12,13). On the other hand, oxidative stress has been shown to induce abnormal modifications in nuclear proteins such as histones, leading to immune responses associated with disease (14). Moreover, REST is not transported to the nucleus in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (12,13). Thus, understanding the role of

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REST in peripheral nerves requires elucidation of its nuclear transport mechanism.

Several studies have reported that hydrogen (H_2) has neuroprotective effects. In mice, oral administration of H_2 -rich water (HRW) suppresses oxidative stress markers and provides protective effects against cognitive impairment (15). A previous study using a cerebral ischemia mouse model reported that intraperitoneal administration of HRW promoted neuronal recovery through autophagy (16). Furthermore, using a diabetic rat model with peripheral neuropathy, intraperitoneal administration of HRW improved axonal degeneration (17). However, the mechanism of neuroprotective effects of H_2 administration on peripheral nerves remains to be elucidated. This study investigated the effects of H_2 administration on the decline in axonal regenerative capacity with aging. Additionally, to elucidate the pathology of age-related decline in axonal regeneration, the molecular mechanisms involved in REST nuclear transport were analyzed.

Materials and methods

Animals. This study was approved by the Animal Care Committee of Juntendo University, Tokyo, Japan (2-1-1 Hongo, Bunkyo-ku, Tokyo, 113-8421, Japan; Registration No. 1555; Approval No. 2024183, Date of Approval: March 12th, 2024).

Eighteen male C57BL/6J mice (Young group: 8-week-old mice, $n=6$; Aged group: 70-week-old mice, $n=6$; Aged + H_2 group: 70-week-old mice, $n=6$) for quantitative polymerase chain reaction (qPCR) and western blotting, and 15 male C57BL/6J mice (Young group: 8-week-old mice, $n=5$; Aged group: 70-week-old mice, $n=5$; Aged + H_2 group: 70-week-old mice, $n=5$) for immunofluorescence staining were purchased from JAPN SLC, Inc. (Shizuoka, Japan). The body weight of the mice at the start of the experiment was 40.0 ± 6.4 g. Mice were housed in sterile cages with five animals per cage, under controlled conditions of $22 \pm 2^\circ\text{C}$, 40–60% humidity, and a 12-h light/dark cycle. The mice were given water that was CRF-1 gamma-ray-irradiated (15 kGy) (Oriental Yeast Co. Ltd., Tokyo, Japan) *ad libitum*. As estrogen levels are known to influence peripheral neuropathy and naturally decrease with age, male mice with less estrogen fluctuations and susceptibility to estrogen were used in this study. The health status and behavior of the animals were monitored daily throughout the experiment period. Humane endpoints were defined as a loss of 20% of body weight, difficulty breathing, coughing, wheezing, severe diarrhea, vomiting, flaccid or spastic paralysis, convulsions, coupled with body temperature significantly below normal, and continuously assessed to determine whether animals should be euthanized before the scheduled end of the experiment. No animals reached these humane endpoints during the study.

Hydrogen treatment. H_2 -containing physiological saline was prepared using the H_2 gas generator Sulive® SS-150 (SUIISO JAPAN Co. Ltd., Osaka, Japan). The Aged + H_2 group received daily intraperitoneal injections of H_2 -containing physiological saline (10 ml/kg, H_2 concentration: 800 ppb) for 2 weeks. The Aged group received an equivalent volume of normal saline (10 ml/kg).

Analysis of REST and GAP43 expression in young and aged mice. The Young group ($n=6$), the Aged group ($n=6$), and the Aged + H_2 group ($n=6$) were sacrificed by cervical dislocation and their sciatic nerves (SN) were collected. The expression levels of REST and growth-associated protein 43 (GAP43), a neuronal protein known for its important role in axon regeneration, in SN were measured by qPCR, western blotting, and immunofluorescence staining and compared among the three groups.

Cell culture. Mouse embryonic fibroblast cell line NIH3T3 (Cell line service, Eppelheim, Germany) was cultured in a humidified incubator with 5% CO_2 at 37°C . The culture medium consisted of Dulbecco's modified Eagle's medium/Ham's F-12 (Sigma-Aldrich), supplemented with 10% fetal bovine serum and 100 U/ml penicillin. H_2 -containing culture medium was prepared using Sulive® SS-150. H_2 concentration was confirmed to be 800 ppb at the start of administration and 500 ppb after 2 h according to our preliminary experience (data not shown). Based on the results of these preliminary experiments, the cell culture time in the H_2 -containing culture medium was set to 2 h.

Construction of REST-overexpressed cells. Using cultured cell lines, REST-overexpressed (REST-OE) cells were constructed (REST-OE group). REST was overexpressed using a lentiviral vector (VectorBuilder Inc., Chicago, IL, USA; vector ID: VB900006-3284rup), while a mock plasmid served as the negative control (Control group). The plasmids were amplified in *Escherichia coli* DH5 α , and plasmid DNA was purified from the amplified lentiviral vectors using the QUIAGEN® Plasmid Maxi kit. To make REST-OE cells, cells were transfected with the REST plasmid using Lipofectamine 3000 (Thermo Fisher Scientific), according to the manufacturer's instructions. REST-OE cells were cultured in H_2 -containing medium (H_2 concentration: 800 ppb) for 2 h in the cells of the REST-OE + H_2 group.

Quantitative PCR. Total RNA was extracted from SN of animal models and from cultured cell lines using *in vitro* models with the RNeasy Micro kit (Qiagen, Tokyo, Japan), following the manufacturer's instructions. Complementary DNA was synthesized using the PrimeScript™ RT Reagent Kit (Takara, Shiga, Japan). Next, qPCR was performed with SYBR Green real-time PCR assay (Thermo Fisher Scientific) according to the $\Delta\Delta\text{CT}$ method. The expression levels of targets (REST and GAP43) were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). qPCR reactions were performed in triplicate, and the $\Delta\Delta\text{Ct}$ method was used for relative quantification (18). The primer sequences used are listed in Table I.

Western blotting. Proteins were extracted from SN of animal models and from cultured cell lines using 1 x radio immunoprecipitation assay (RIPA) buffer. Equal amounts of protein were loaded for each sample as determined by BCA assay and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membranes by Trans-Blot Turbo system (BIORAD, CA, USA). Non-specific binding sites were blocked with PVDF Blocking Reagent (Toyobo Co. Ltd., Osaka, Japan)

Table I. Primer sequences used for quantitative PCR in the present study.

Gene	Forward primer sequences (5'-3')	Reverse primer sequences (5'-3')
<i>Rest</i>	ACCTGCAGCAAGTGCAACTA	CCGCATGTGTCGCGTTAGA
<i>Gap43</i>	AAGGCAGGGGAAGATACCAC	TTGTTCAATCTTTTGGTCCTCAT
<i>Gapdh</i>	TGTGTCCGTCGTGGATCTG	TTGCTGTTGAAGTCGCAGG

Rest, repressor element 1 silencing transcription factor; *Gap43*, growth-associated protein 43.

for 1 h at room temperature. The membranes were then washed three times with Tris-buffered saline containing 0.1% Tween 20 (TBST), 10 min per wash. Subsequently, membranes were incubated overnight at 4°C with primary antibodies diluted in Solution 1 (Toyobo Co. Ltd.), according to the manufacturer's instructions. Although each target protein and its corresponding loading control were detected on separate membranes, equal amounts of protein (20 µg) were loaded per lane as determined by BCA assay, and all membranes were quantified based on relative band intensities using standardized loading across samples.

The following primary antibodies were used: rabbit polyclonal anti-REST (1:1,000, 22242-1-AP; ProteinTech), rabbit polyclonal anti-GAP43 (1:1,000, 16971-1-AP; ProteinTech), rabbit polyclonal anti-PRICKLE1 (RILP) (1:1,000, 22589-1-AP; ProteinTech), rabbit monoclonal anti-Huntingtin (1:5,000, ab109115; Abcam), rabbit monoclonal anti-DCTN1/p150Glued (1:1,000, ab246505; Abcam), rabbit polyclonal anti-LC3 (1:1,000, 14600-1-AP; ProteinTech), mouse monoclonal anti-GAPDH (1:2,000, sc-32233; Santa Cruz, CA, USA), and rabbit polyclonal anti-Lamin B1 (1:5,000, 12987-1-AP; ProteinTech).

After incubation, membranes were washed with TBST and incubated with appropriate secondary antibodies for 2 h at room temperature. Following additional washes, signals were detected using the Amersham Imager 680 system (GE Healthcare Life Sciences, IL, USA). Image Lab 3.0 software was used to measure the relative optical density of the protein bands. GAPDH was used as a loading control for the total cell and cytoplasmic fractions, and Lamin B1 as a loading control for the nuclear fractions.

Immunofluorescence staining. Immunofluorescence staining was performed to assess the expression of REST and GAP43 in both animal models and cultured cell lines. SN was fixed in 4% paraformaldehyde at room temperature for 72 h and subsequently embedded in paraffin. Tissue sections were prepared by slicing cutting the harvested SN into 3 µm-thick sections. Samples were deparaffinized and subjected to antigen retrieval by autoclaving at 121°C for 10 min. Cell lines were cultured using the Nunc™ Lab-Tek™ II Chamber Slide™ System to create three group. Then, the cultured medium was discarded, and were fixed in 4% paraformaldehyde at room temperature for 15 min. To suppress autofluorescence sections were treated with True View™ (SP-8400, Vector, CA, USA), followed by blocking in PBS containing 0.05% Tween 20 and 2% bovine serum albumin (BSA) (A2153, Sigma-Aldrich, MO, USA) for 30 min.

Sections were then incubated with primary antibodies against the target proteins at 4°C for 15 h. After washing with TBST, a goat anti-mouse IgG antibody conjugated to Alexa Fluor 488 (A11001, Thermo Fisher Scientific) was used as the secondary antibody. A rabbit IgG monoclonal antibody was used as a negative control. Fluorescence intensity was visualized using a fluorescence imaging microscope (Leica, TCSSP5, Wetzlar, Germany) in photon-counting mode. The primary antibodies were rabbit polyclonal anti-REST (1:100, 22242-1-AP, ProteinTech, IL, USA), and rabbit polyclonal anti-GAP43 (1:100, 16971-1-AP, ProteinTech) in this study.

Fluorescence intensity was measured at 10 randomly selected sites within the perikaryon in the region of interest (ROI) showing fluorescence emission. The mean fluorescence intensity was then calculated. Fluorescence levels detected by each antibody were compared among the groups: for the *in vivo* experiment (Young group, Aged group, Aged + H₂ group) and for the *in vitro* experiment (Control group, REST-OE group, and REST-OE + H₂ group).

Isolation of cytoplasmic and nuclear fractions from REST-regulated cells. To demonstrate that REST, functioning as a transcriptional regulator, is transported to the nucleus from the cytoplasm, we used NE-PER® Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific), following the manufacturer's instructions. Using these fractions, REST and the proteins reported to be involved in REST nuclear transport, including RILP, Huntingtin, and DCTN1/p150^{Glued}, and LC3 were quantified by western blotting.

Statistical analysis. Data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Sidak's multiple comparisons test for pairwise group comparisons, with Prism 7 software (GraphPad Software, San Diego, CA, USA). A P-value of less than 0.05 was considered statistically significant.

Results

REST expression and GAP43 in SN. To examine differences in the expression of REST and GAP43 among the Young group, the Aged group, and the Aged + H₂ group, their expression in SN were quantified by qPCR, western blotting, and immunofluorescence staining.

qPCR analysis revealed a significant upregulation of REST in the Aged group compared with the Young group (Young group: 1.00±0.00-fold; Aged group: 2.37±0.35-fold, P=0.0014),

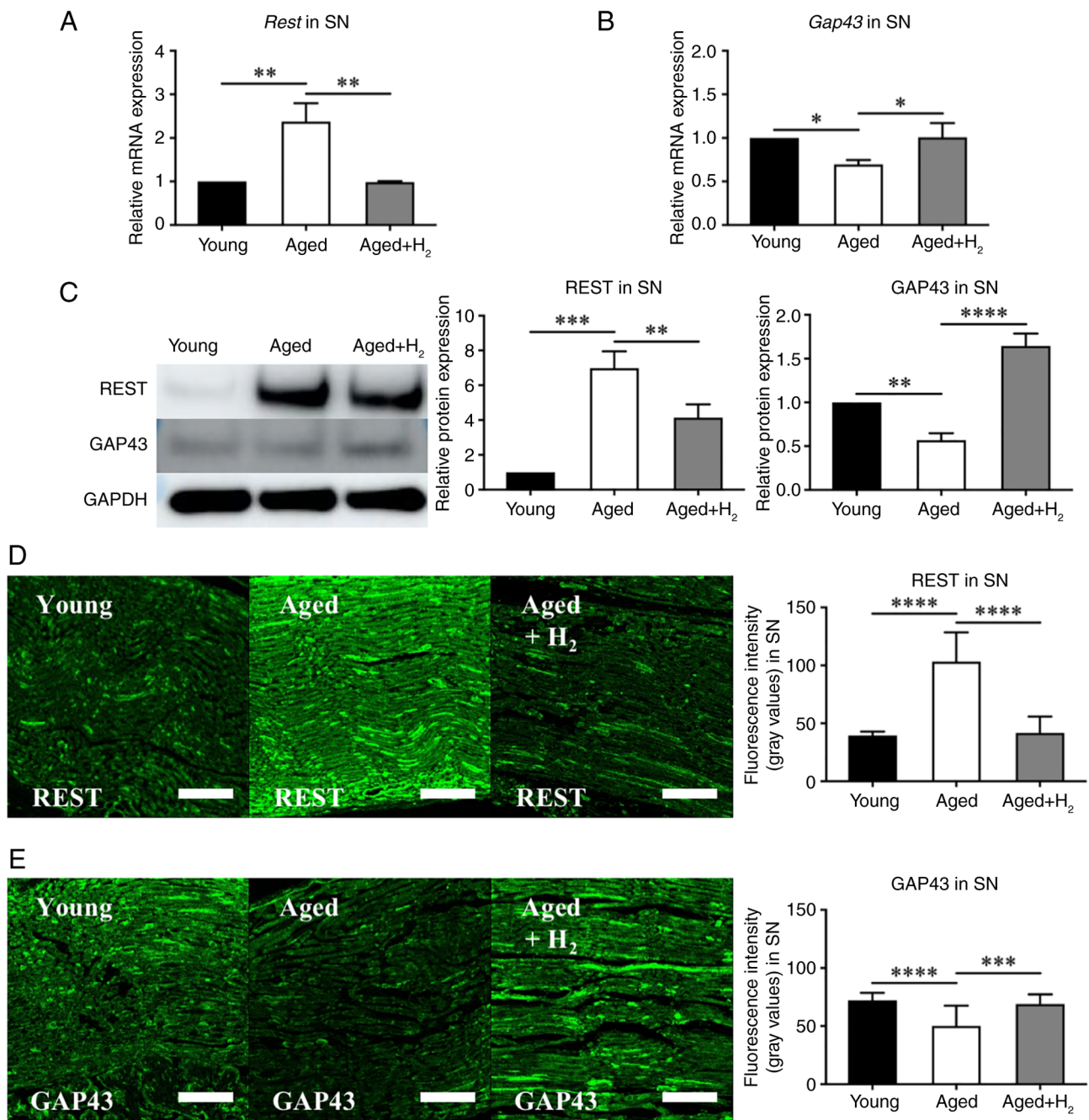


Figure 1. REST and GAP43 expression in SN of the young, aged and aged + H₂ groups. The aged group was compared with the young group, and the aged + H₂ group was compared with the aged group. The graphs show the quantification of relative protein and mRNA abundance. Mean $\pm$ SD, n=6 mice per group. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 (one-way ANOVA). (A) qPCR analysis of *Rest* expression. (B) qPCR analysis of *Gap43* expression. (C) Western blot analysis of REST and GAP43 expression. (D) Histochemical assessment of REST expression by immunofluorescence staining. Scale bar, 100 μ m. (E) Histochemical assessment of GAP43 expression by immunofluorescence staining. Scale bar, 100 μ m. GAP43, growth-associated protein 43; H₂, hydrogen; qPCR, quantitative PCR; REST, repressor element 1 silencing transcription factor; SN, sciatic nerves.

whereas it was significantly decreased in the Aged + H₂ group compared with the Aged group (Aged group: 2.37 ± 0.35 -fold; Aged + H₂ group: 0.98 ± 0.02 -fold, $P=0.0013$) (Fig. 1A). GAP43 expression in SN was significantly decreased in the Aged group compared with the Young group (Young group: 1.00 ± 0.00 -fold; Aged group: 0.70 ± 0.04 -fold, $P=0.0274$), whereas it was significantly increased in the Aged + H₂ group compared with the Aged group (Aged group: 0.70 ± 0.04 -fold, $P=0.0274$; Aged + H₂ group: 1.00 ± 0.13 -fold, $P=0.0249$) (Fig. 1B).

Western blotting further supported these results. REST expression was markedly increased in the Aged group compared with the Young group (Young group: 1.00 ± 0.00 -fold; Aged group: 6.98 ± 0.79 -fold, $P=0.0001$), whereas it was significantly decreased in the Aged + H₂ group compared with the Aged group (Aged group: 6.98 ± 0.79 -fold; Aged + H₂ group: 4.14 ± 0.62 -fold, $P=0.0082$) (Fig. 1C). GAP43 expression in SN was significantly decreased in the Aged group compared with the Young group (Young group: 1.00 ± 0.00 -fold; Aged group: 0.57 ± 0.06 -fold, $P=0.0040$), whereas it was

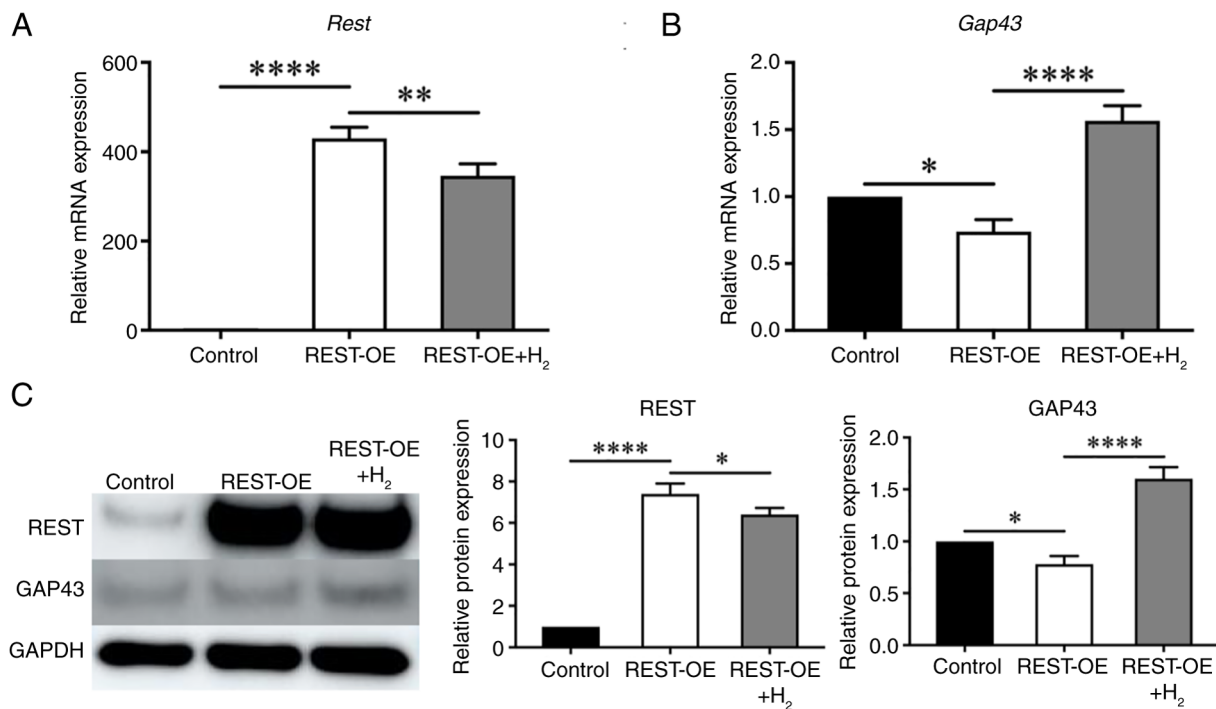


Figure 2. REST and GAP43 expression in the control, REST-OE and REST-OE + H₂ groups. The REST-OE group was compared with the control group, and the REST-OE + H₂ group was compared with the REST-OE group. The graphs show the quantification of relative protein and mRNA abundance. Mean ± SD, n=3 per group. *P<0.05, **P<0.01 and ****P<0.0001 (one-way ANOVA). (A) qPCR analysis of *Rest* expression. (B) qPCR analysis of *Gap43* expression. (C) Western blot analysis of REST and GAP43 expression. GAP43, growth-associated protein 43; H₂, hydrogen; qPCR, quantitative PCR; REST, repressor element 1 silencing transcription factor; REST-OE, REST-overexpressed.

significantly increased in the Aged + H₂ group compared with the Aged group (Aged group: 0.57±0.06-fold; Aged + H₂ group: 1.64±0.12-fold, P<0.0001) (Fig. 1C).

Immunofluorescence staining revealed that the fluorescence intensity of REST in SN was significantly increased in the Aged group compared with the Young group (Young group: 39.57±3.19; Aged group: 103.30±6.48, P<0.0001), whereas it was significantly decreased in the Aged + H₂ group compared with the Aged group (Aged group: 103.30±6.48; Aged + H₂ group: 41.75±3.64, P<0.0001) (Fig. 1D). On the other hand, the fluorescence intensity of GAP43 in SN was significantly decreased in the Aged group compared with the Young group (Young group: 72.15±6.26; Aged group: 50.17±4.51, P<0.0001), whereas it was significantly increased in the Aged + H₂ group compared with the Aged group (Aged group: 50.17±4.51; Aged + H₂ group: 68.96±2.17, P=0.0002) (Fig. 1E).

These results suggest that REST is upregulated with aging and inhibits axonal regeneration capacity; however, H₂ may have the potential to recover this reduced capacity for axonal regeneration in mice.

REST expression and GAP43 expression in REST-OE cells. To verify whether the results obtained in SN can be reproduced in cultured cells, REST-OE cells were constructed and compared among the Control, REST-OE, and REST-OE + H₂ groups.

qPCR showed that REST expression was markedly increased in the REST-OE group compared with the Control group (Control group: 1.00±0.00-fold; REST-OE group: 430.0±20.51-fold, P<0.0001), whereas it was significantly

decreased in the REST-OE + H₂ group compared with the REST-OE group (REST-OE group: 430.0±20.51-fold; REST-OE + H₂ group: 346.6±21.95-fold, P=0.0059) (Fig. 2A). On the other hand, GAP43 expression was significantly decreased in the REST-OE group compared with the Control group (Control group: 1.00±0.00-fold; REST-OE group: 0.74±0.07-fold, P=0.0248), whereas it was significantly increased in the REST-OE + H₂ group compared with the REST-OE group (REST-OE group: 0.74±0.07-fold; REST-OE + H₂ group: 1.60±0.09-fold, P<0.0001) (Fig. 2B).

At the protein level, REST expression followed a similar pattern. REST expression was significantly increased in the REST-OE group compared with the Control group (Control group: 1.00±0.00-fold; REST-OE group: 7.40±0.41-fold, P<0.0001), whereas it was significantly decreased in the REST-OE + H₂ group compared with the REST-OE group (REST-OE group: 7.40±0.41-fold; REST-OE + H₂ group: 6.40±0.26-fold, P=0.0240) (Fig. 2C). On the other hand, GAP43 expression was significantly decreased in the REST-OE group compared with the Control group (Control group: 1.00±0.00-fold; REST-OE group: 0.78±0.06-fold, P=0.0409), whereas it was significantly increased in the REST-OE + H₂ group compared with the REST-OE group (REST-OE group: 0.78±0.06-fold; REST-OE + H₂ group: 1.60±0.09-fold, P<0.0001) (Fig. 2C).

These results are consistent with those observed in mice, suggesting that these cells are a suitable model for evaluating changes in SN of animal models.

Differences in the localization of REST expression between the cytoplasm and the nucleus. H₂ reduces the intracellular

expression of REST (Fig. 2A and C); therefore, to examine differences in the localization of REST expression, *in vitro* cell lines were separated into cytoplasmic and nuclear fractions, and REST expression was investigated by immunofluorescence staining and western blotting.

Immunofluorescence staining revealed that the fluorescence intensity of REST in the cytoplasm was significantly elevated in the REST-OE group compared to controls (52.01 ± 12.40 in Control vs. 119.33 ± 24.81 in REST-OE, $P < 0.0001$), and further increased following H_2 treatment (119.33 ± 24.81 in REST-OE vs. 148.08 ± 27.25 in REST-OE + H_2 , $P = 0.0226$) (Fig. 3A and B). On the other hand, the fluorescence intensity of REST in the nucleus was significantly increased in the REST-OE group compared with the Control group (Control group: 65.39 ± 16.39 ; REST-OE group: 135.03 ± 15.61 , $P < 0.0001$), whereas it was significantly decreased in the REST-OE + H_2 group compared with the REST-OE group (REST-OE group: 135.03 ± 15.61 ; REST-OE + H_2 group: 113.86 ± 17.26 , $P = 0.0144$) (Fig. 3A and C).

Western blotting supported these findings. REST protein levels in the cytoplasm were significantly increased in the REST-OE group compared to controls (1.00 ± 0.00 -fold in Control vs. 2.78 ± 0.11 -fold in REST-OE, $P = 0.0053$), and further increased with H_2 treatment (2.78 ± 0.11 -fold in REST-OE vs. 4.07 ± 0.62 -fold in REST-OE + H_2 , $P = 0.0238$) (Fig. 3D). On the other hand, REST expression in the nucleus was significantly increased in the REST-OE group compared with the Control group (Control group: 1.00 ± 0.00 -fold; REST-OE group: 3.45 ± 0.24 -fold, $P < 0.0001$), whereas it was decreased in the REST-OE + H_2 group compared with the REST-OE group (REST-OE group: 3.45 ± 0.24 -fold; REST-OE + H_2 group: 2.66 ± 0.32 -fold, $P = 0.0281$) (Fig. 3E). Furthermore, the ratio of REST expression in the nucleus to that in the cytoplasm was calculated. As a result, the REST-OE group showed a significant increase compared to controls (Control group: 1.00 ± 0.00 -fold; REST-OE group: 1.31 ± 0.08 -fold, $P = 0.0355$) (Fig. 3F). In contrast, the REST-OE + H_2 group showed a significant decrease compared to the REST-OE group (REST-OE group: 1.31 ± 0.08 -fold; REST-OE + H_2 group: 0.75 ± 0.13 -fold, $P = 0.0021$) (Fig. 3F).

Although H_2 reduces REST expression in the whole cell (Fig. 2A and C), it increases REST expression in the cytoplasm and decreases it in the nucleus in REST-OE cells. This suggests that H_2 affects the degradation within the cytoplasm and nuclear transport of REST.

REST nuclear transport proteins and autophagy-related protein LC3 in the cytoplasm and nucleus of REST-OE cells.

REST in the cytoplasm is either degraded by autophagy or transported into the nucleus. To investigate these processes, REST nuclear transport proteins, such as REST-interacting LIM domain protein (RILP), Huntingtin, and DCTN1/p150^{Glued}, and the autophagy-related protein, light chain 3 protein (LC3), were quantified by western blotting.

RILP levels were significantly higher in the REST-OE group compared with the Control group (Control group: 1.00 ± 0.00 -fold; REST-OE group: 1.42 ± 0.04 -fold, $P = 0.0003$); however, it was significantly decreased following H_2 treatment (1.42 ± 0.04 -fold in REST-OE vs. 1.16 ± 0.08 -fold in REST-OE + H_2 , $P = 0.0040$) (Fig. 4A). In contrast, there was no

significant difference in the expression of Huntingtin between the REST-OE group and the Control group (Control group: 1.00 ± 0.00 -fold; REST-OE group: 1.17 ± 0.18 -fold, $p = 0.7587$), as well as between the REST-OE + H_2 group and the REST-OE group (REST-OE group: 1.17 ± 0.18 -fold; REST-OE + H_2 group: 1.15 ± 0.37 -fold, $P = 0.9970$) (Fig. 4B). There was no significant difference in the expression of DCTN1/p150^{Glued} between the REST-OE group and the Control group (Control group: 1.00 ± 0.00 -fold; REST-OE group: 0.97 ± 0.07 -fold, $P = 0.9126$), as well as between the REST-OE + H_2 group and the REST-OE group (REST-OE group: 0.97 ± 0.07 -fold; REST-OE + H_2 group: 0.95 ± 0.09 -fold, $P = 0.9421$) (Fig. 4C). The expression of LC3 was significantly decreased in the REST-OE group compared with the Control group (Control group: 1.00 ± 0.00 -fold; REST-OE group: 0.70 ± 0.08 -fold, $P < 0.0001$), whereas it was significantly increased in the REST-OE + H_2 group compared with the REST-OE group (REST-OE group: 0.70 ± 0.08 -fold; REST-OE + H_2 group: 0.91 ± 0.08 -fold, $P = 0.0022$) (Fig. 4D).

To further evaluate the effects of H_2 on REST in nuclear transport and cytoplasm degradation, the expression of RILP, Huntingtin, DCTN1/p150^{Glued}, and LC3 in cytoplasmic and nuclear extracts was quantified by western blotting. In the cytoplasm, the expression of RILP in the cytoplasm was significantly increased in the REST-OE group compared with the Control group (Control group: 1.00 ± 0.00 -fold; REST-OE group: 1.43 ± 0.04 -fold, $P < 0.0001$), whereas it was significantly decreased in the REST-OE + H_2 group compared with the REST-OE group (REST-OE group: 1.43 ± 0.04 -fold; REST-OE + H_2 group: 0.97 ± 0.06 -fold, $P < 0.0001$) (Fig. 5A). The expression of Huntingtin in the cytoplasm was significantly increased in the REST-OE group compared with the Control group (Control group: 1.00 ± 0.00 -fold; REST-OE group: 1.54 ± 0.16 -fold, $P = 0.0105$), whereas it was significantly decreased in REST-OE + H_2 group compared with the REST-OE group (REST-OE group: 1.54 ± 0.16 -fold; REST-OE + H_2 group: 0.77 ± 0.16 -fold, $P = 0.0018$) (Fig. 5B). The expression of DCTN1/p150^{Glued} in the cytoplasm was not significantly different between the REST-OE group and the Control group (Control group: 1.00 ± 0.00 -fold; REST-OE group: 0.92 ± 0.07 -fold, $P = 0.4667$), and between the REST-OE + H_2 group and the REST-OE group (REST-OE group: 0.92 ± 0.07 -fold; REST-OE + H_2 group: 1.02 ± 0.08 -fold, $P = 0.3248$) (Fig. 5C). The expression of LC3 in the cytoplasm was significantly decreased in the REST-OE group compared with the Control group (Control group: 1.00 ± 0.00 -fold; REST-OE group: 0.68 ± 0.04 -fold, $P = 0.0017$), whereas it was significantly increased in the REST-OE + H_2 group compared with the REST-OE group (REST-OE group: 0.68 ± 0.04 -fold; REST-OE + H_2 group: 1.03 ± 0.08 -fold, $P = 0.0011$) (Fig. 5D). In the nucleus, there was no significant difference between the expression of RILP, Huntingtin, DCTN1/p150^{Glued}, and LC3 between the REST-OE group and the Control group (RILP: 1.19 ± 0.09 -fold, $P = 0.0827$; Huntingtin: 1.20 ± 0.26 -fold, $P = 0.5167$; DCTN1/p150^{Glued}: 1.02 ± 0.27 -fold, $p = 0.9953$; LC3: 0.98 ± 0.10 -fold, $P = 0.9079$), as well as between the REST-OE + H_2 group and the REST-OE group (RILP: 1.10 ± 0.08 -fold, $P = 0.4874$; Huntingtin: 1.18 ± 0.16 -fold, $P = 0.9923$; DCTN/p150^{Glued}: 0.94 ± 0.10 -fold, $P = 0.8998$; LC3: 0.99 ± 0.06 -fold, $P = 0.9458$) (Fig. 6A-D).

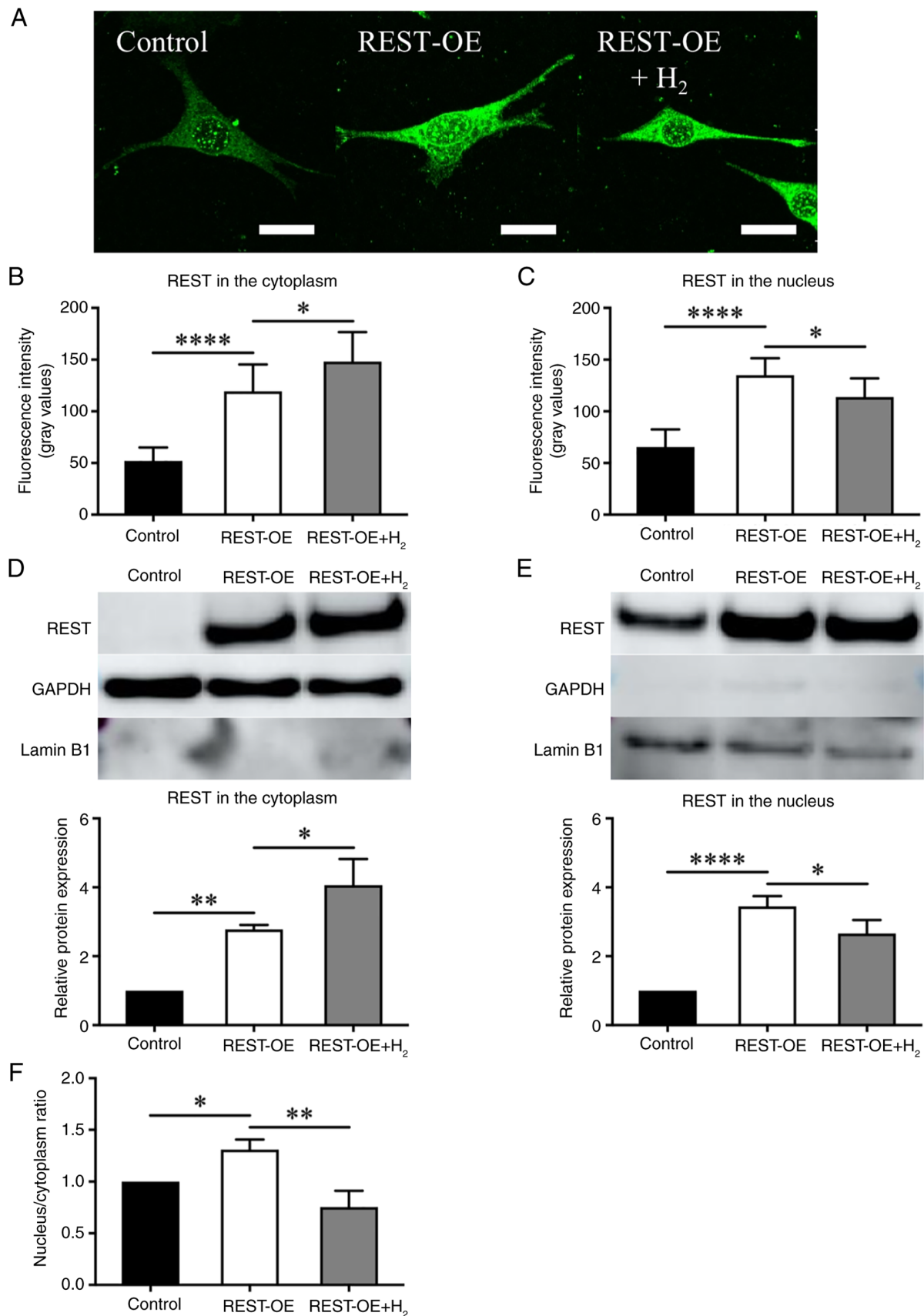


Figure 3. REST expression in the cytoplasm and nucleus of the control, REST-OE and REST-OE + H₂ groups. The REST-OE group was compared with the control group, and the REST-OE + H₂ group was compared with the REST-OE group. Mean ± SD, n=30 cells for (A-C), n=3 per group for (D-F). *P<0.05, **P<0.01 and ****P<0.0001 (one-way ANOVA). (A) Immunofluorescence staining of REST expression. REST is labeled in green. Scale bar, 50.0 μm. (B) Histochemical assessment of REST expression in the cytoplasm by immunofluorescence staining. (C) Histochemical assessment of REST expression in the nucleus by immunofluorescence staining. (D) Western blot analysis of REST expression in the cytoplasm. (E) Western blot analysis of REST expression in the nucleus. (F) Ratio of REST expression in the nucleus to that in the cytoplasm. H₂, hydrogen; REST, repressor element 1 silencing transcription factor; REST-OE, REST-overexpressed.

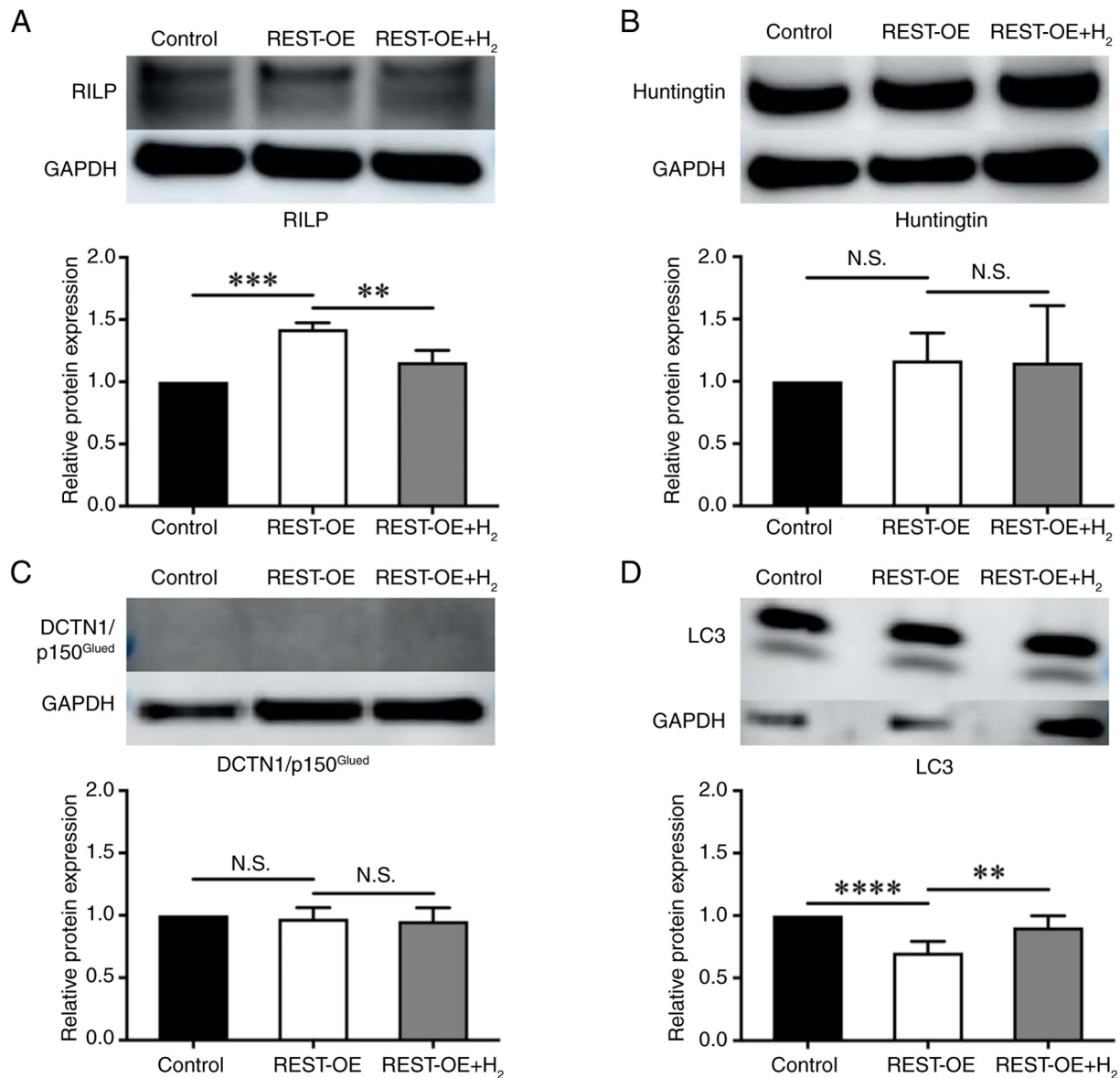


Figure 4. RILP, Huntingtin, DCTN1/p150^{Glued} and LC3 expression in the control, REST-OE and REST-OE + H₂ groups evaluated by western blotting. The REST-OE group was compared with the control group, and the REST-OE + H₂ group was compared with the REST-OE group. The graphs show the semi-quantification of relative protein expression. Mean $\pm$ SD, n=3 per group. **P<0.01, ***P<0.001 and ****P<0.0001 (one-way ANOVA). (A) RILP expression. (B) Huntingtin expression. (C) DCTN1/p150^{Glued} expression. (D) LC3 expression. DCTN1, dynactin subunit 1; H₂, hydrogen; N.S., no significant difference; REST, repressor element 1 silencing transcription factor; REST-OE, REST-overexpressed; RILP, REST-interacting LIM domain protein.

Together these results suggest that under REST-OE conditions, REST nuclear transport proteins RILP and Huntingtin were upregulated but autophagy was suppressed in the cytoplasm, which promotes the nuclear transport of REST from the cytoplasm. On the other hand, H₂ inhibited the nuclear transport of REST due to suppression of REST nuclear transport-related proteins such as RILP and Huntingtin.

Discussion

In this study, REST expression was decreased, and GAP43 expression was increased following H₂ administration to aged animal models and REST-OE cells (Figs. 1 and 2). Next, we examined the intracellular localization of REST expression. We found that REST expression was increased in the cytoplasm and decreased in the nucleus following H₂ addition in

REST-OE cells (Fig. 3). Furthermore, the REST expression in the cytoplasm was investigated, focusing on nuclear transport mechanisms and autophagy. We found that H₂ addition led to a decrease in several REST nuclear transport proteins, and nuclear translocation of REST was suppressed (Fig. 5A-C). H₂ administration restored the impaired autophagic capacity (Fig. 5D).

Various types of stress promote nuclear transport of proteins from the cytoplasm to the nucleus. Hikeshi, which is not a member of the importin family, is increased during heat shock stress and promotes the nuclear transport of heat shock protein 70s (19). On the other hand, *in vitro* studies using human bronchial epithelial-like cells found that the antioxidant vitamin E inhibits the nuclear transport of phospho-signal transducer and activator of transcription 3 and suppresses the induction of inflammatory cells (20). These findings suggest

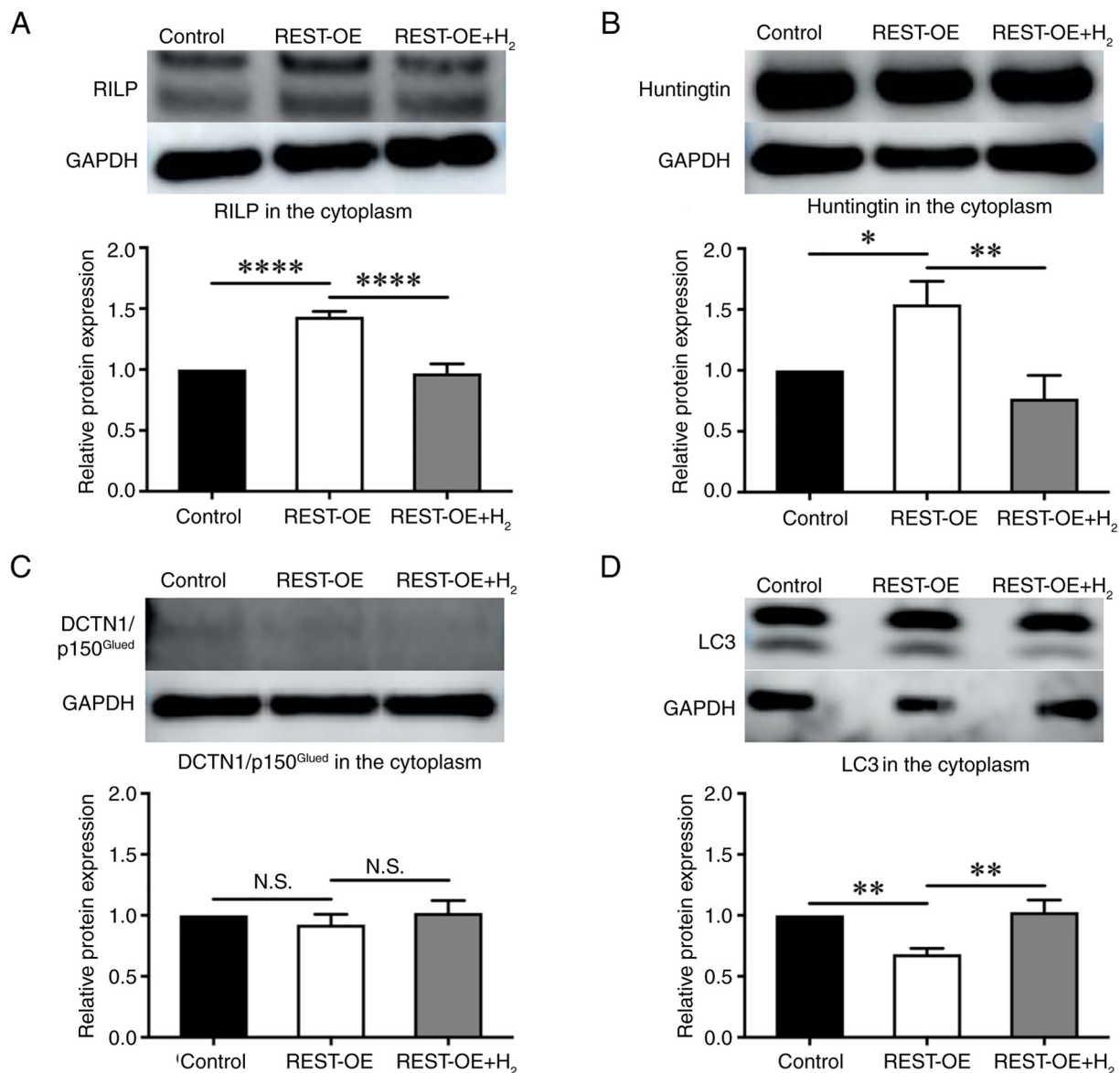


Figure 5. RILP, Huntingtin, DCTN1/p150^{Glued} and LC3 expression in the cytoplasm of the control, REST-OE and REST-OE + H₂ groups evaluated by western blotting. The REST-OE group was compared with the control group, and the REST-OE + H₂ group was compared with the REST-OE group. The graphs show the semi-quantification of relative protein expression. Mean ± SD, n=3 per group. *P<0.05, **P<0.01 and ****P<0.0001 (one-way ANOVA). (A) RILP expression. (B) Huntingtin expression. (C) DCTN1/p150^{Glued} expression. (D) LC3 expression. DCTN1, dynactin subunit 1; H₂, hydrogen; N.S., no significant difference; REST, repressor element 1 silencing transcription factor; REST-OE, REST-overexpressed; RILP, REST-interacting LIM domain protein.

that the nuclear transport of transcriptional factors depends on biological stress. Previous studies reported that H₂ has a neuro-protective effect through antioxidant ability (17). Furthermore, it may affect nuclear transport of transcriptional factors via its antioxidant ability. Since both cytoplasmic and nuclear expression of REST were changed by H₂ administration in this study, our findings suggest that H₂ influences the nuclear transport of REST. Moreover, H₂ administration led to an increase in the expression of axon regeneration markers through suppression of the nuclear transport of REST. Therefore, nuclear transport of REST was investigated through a molecular approach.

REST is transported into the nucleus by forming a complex with RILP, Huntingtin, and DCTN1/p150^{Glued} (21). In this study, under high REST expression conditions, the expression of RILP and Huntingtin in the cytoplasm were increased, promoting the nuclear transport of REST (Fig. 5A and B). On

the other hand, the expression of RILP and Huntingtin was decreased, and the nuclear transport of REST was suppressed by H₂ addition (Fig. 5A and B). It is reported that RILP is activated by reactive oxygen species (ROS) and inhibited by the antioxidant N-acetylcysteine (22,23). Melatonin is an antioxidant that provides protective effects against Huntington's disease (24). Additionally, melatonin decreases with aging, leading to a decrease in antioxidant activity and an increase in the expression of Huntingtin (24). It is likely that the antioxidant ability of H₂ led to a decrease in the expression of RILP and Huntingtin. This study reveals new findings about the regulatory roles of RILP and Huntingtin in the nuclear transport of REST. On the other hand, among the REST nuclear transport proteins, only DCTN1/p150^{Glued} in the cytoplasm remained unaffected by changes in REST expression. DCTN1/p150^{Glued} has other functions than the nuclear transport

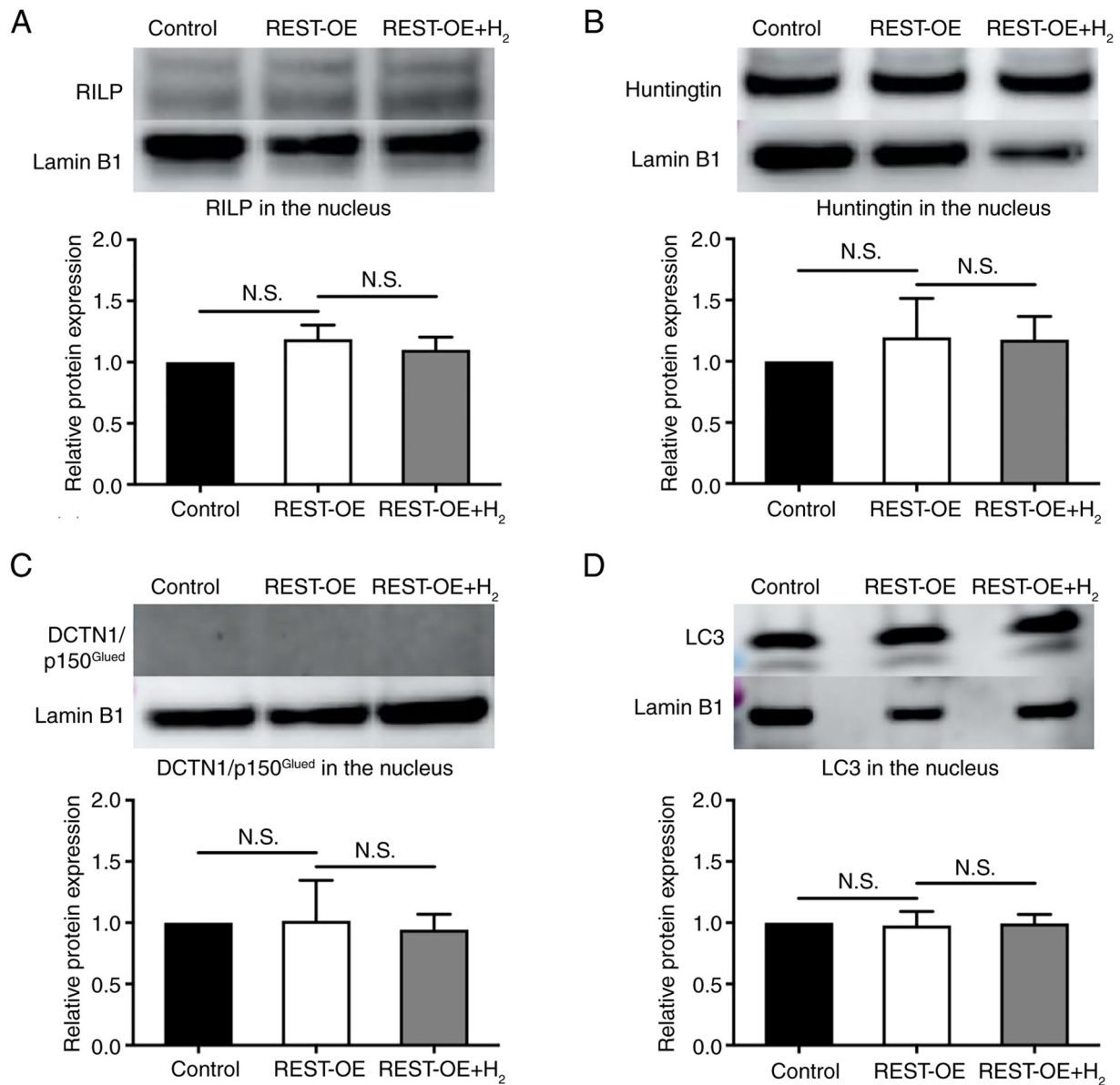


Figure 6. RILP, Huntingtin, DCTN1/p150^{Glued} and LC3 expression in the nucleus of the control, REST-OE and REST-OE + H₂ groups evaluated by western blotting. The REST-OE group was compared with the control group, and the REST-OE + H₂ group was compared with the REST-OE group. The graphs show the semi-quantification of relative protein expression. Mean $\pm$ SD, n=3 per group. The data were analyzed using one-way ANOVA. (A) RILP expression. (B) Huntingtin expression. (C) DCTN1/p150^{Glued} expression. (D) LC3 expression. DCTN1, dynactin subunit 1; H₂, hydrogen; N.S., no significant difference; REST, repressor element 1 silencing transcription factor; REST-OE, REST-overexpressed; RILP, REST-interacting LIM domain protein.

of REST, such as retrograde axonal transport, and microtubule stabilization (25,26). Therefore, the change in the expression of DCTN1/p150^{Glued} in the cytoplasm was not detectable, although the nuclear transport of REST was suppressed.

Autophagy is an important process for maintaining cellular homeostasis (27); however, its activity decreases with aging (28,29). Furthermore, REST expression in neurons increases with age, leading to decreased autophagy activity (30). On the other hand, myocardial protective effects of H₂ on sepsis-induced cardiomyopathy via autophagy activation (31). Thus, H₂ may have the capacity to promote autophagy activity according to these reports. In this study, the expression of LC3 in both whole-cell and cytoplasmic fractions are

increased in REST-OE cells by H₂ addition (Figs. 4D and 5D). Therefore, H₂-induced changes in autophagy activity in the cytoplasm may be upregulated.

This study has some limitations. First, a comprehensive analysis of the nuclear transport proteins related to REST, which have various functions in neurons, was not performed. RILP, Huntingtin, and DCTN1/p150^{Glued} were selected as they form a complex with REST and that protein complex plays essential roles in the nuclear transport of REST (21). Second, the optimal duration of H₂ administration and addition was not evaluated. The primary methods of H₂ administration include inhalation of H₂ gas, drinking HRW, and injection of H₂-containing physiological saline (32). In this study, the

duration of intraperitoneal injection of H₂-containing physiological saline was determined based on a study evaluating autophagy in a rat neurodegeneration model (33). Additionally, in the *in vitro* study using H9C2 cells in a hypoxia-reoxygenation model, the H₂ administration period was set to 2 h based on a previous study that assessed cell survival (34). Third, REST-OE cells used in this study were generated by fibroblasts, which are non-neuronal cells. Although Schwann cells were initially considered due to their physiological relevance to the peripheral nervous system, stable overexpression of REST in these cells was not achieved. This limitation was likely attributable to a combination of REST-induced growth inhibition and cytotoxic effects associated with Lipofectamine 3000-based transfection. Consequently, fibroblasts were selected as an alternative model due to their genetic stability and widespread use in studies of nuclear-cytoplasmic transport mechanisms. Although primary cultured neurons can be maintained for a limited period, their application in long-term experiments is constrained. In fact, Xue *et al* (30) considered a 10-day culture duration as 'long-term' in studies using neuronal cell. Furthermore, gene delivery efficiency in primary neurons is generally low, and specialized transfection methods are often required (35). On the other hand, a previous study reported the use of NIH3T3 cells, the cells used in the present study, in peripheral nerve research (36). Furthermore, the nuclear transport mechanism of megakaryoblastic leukemia 1 protein, a co-activator of the transcription factor serum response factor, has been analyzed using the NIH3T3 cell line (37). Thus, the use of NIH3T3 cells is considered scientifically valid for studying the nuclear transport mechanisms of cytoplasmic proteins. Nevertheless, this approach has a limitation in physiological relevance, as fibroblasts differ from neuronal cells and cannot replicate neuron-specific functions or the neural microenvironment (38). Consequently, the current findings may not fully reflect the *in vivo* role of REST. To address this limitation, future studies are planned using peripheral nerve-specific conditional REST knockout mice. *In vivo* axonal regeneration will be evaluated using a sciatic nerve crush model, and axon regeneration will be assessed in primary cultured dorsal root ganglion neurons. These approaches are expected to complement the current *in vitro* results and provide a more comprehensive understanding of the physiological role of REST in peripheral nerve regeneration.

Previous studies reported that axon regenerative capacity declines with aging, however, the underlying pathology remains unclear. In this study, to elucidate the pathology of age-related decline in axonal regeneration, the molecular mechanisms involved in REST nuclear transport were analyzed. The results of this study suggest that the regulation of nuclear transport of REST may improve the decline in age-related axon regeneration.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

KN, DK and YU conceptualized the study. TS, TNK and YY designed the methodology. TS performed most of the experiments. SK, NI and KK assisted with the experiments. TS, KN, NH and MI analyzed and interpreted the data. TS and KN prepared the original draft, while TS, KN and MI reviewed and edited the manuscript. KN secured funding. TS, KN, DK and NH confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Animal Care Committee of Juntendo University, Tokyo, Japan (registration no. 1555; approval no. 2024183; date of approval, March 12, 2024).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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