Glycoprotein 130 improves repressor element-1 silencing transcription factor-related axon regenerative capacity in peripheral nerves with aging

SO KAWAKITA[1,2], KIYOHITO NAITO[1-3], DAISUKE KUBOTA[1,2], YUJI UENO[4], TAKAKO NEGISHI-KOGA[2,3], YASUHIRO YAMAMOTO[2], TAKAMARU SUZUKI[1,2], NORIZUMI IMAZU[1,2], KENJIRO KAWAMURA[1,2], NOBUTAKA HATTORI[5] and MUNEAKI ISHIJIMA[1-3]

[1]Department of Medicine for Orthopedics and Motor Organ, Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan; [2]Department of Orthopedics, Juntendo University Faculty of Medicine, Tokyo 113-8421, Japan; [3]Department of Community Medicine and Research for Bone and Joint Diseases, Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan; [4]Department of Neurology, Graduate School of Medical Sciences, University of Yamanashi, Chuo, Yamanashi 409-3898, Japan; [5]Department of Neurology, Juntendo University Faculty of Medicine, Tokyo 113-8421, Japan

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Abstract. Axon regenerative capacity diminishes with aging and differences in the condition of peripheral nerves between young and elderly individuals have been reported. However, the underlying pathology remains unclear. The expression of repressor element-1 silencing transcription factor (REST) increases with age and is reported to suppress axon regeneration. The present study investigated the pathology and potential treatment of reduced axon regenerative capacity using REST-regulated cells and a mouse model. This study examined the molecular expression of the janus kinase 1 (JAK1)/signal transducer and activator of transcription 3 (STAT3) pathway, which is involved in growth-associated protein 43 (GAP43) expression. In REST-overexpressed (REST-OE), glycoprotein 130 (GP130), JAK1 and phosphorylated STAT3 (p-STAT3) expression was decreased compared with the control (GP130, P=0.004; JAK1, P=0.038; pSTAT3, P=0.015). On the other hand, in REST-low expressed (siREST), GP130, JAK1 and pSTAT3 expression was increased compared with the control (GP130, P=0.004; JAK1, P=0.003; pSTAT3, P=0.033). It suggested that GP130 plays an important role. Therefore, GP130 agonist was administered to REST-OE and aged mice and resulted in a significant increase in GAP43 expression (REST-OE: Protein P=0.018, mRNA P=0.040; aged mice: Protein P=0.016, mRNA P=0.013). The results of this study suggest that the pathology of reduction in peripheral nerve axon regenerative capacity is inhibited by age-related increase in REST expression, which leads to decreased GP130 expression and inhibition of JAK1/STAT3 pathway activity. These findings suggest that regulating GP130 expression may improve axon regenerative capacity by aging.

Correspondence to: Dr Kiyohito Naito, Department of Orthopedics, Juntendo University Faculty of Medicine, 2-1-1 Hongo, Bunkyo, Tokyo 113-8421, Japan
E-mail: knaito@juntendo.ac.jp

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Introduction

In recent years, the global population has aged rapidly (1). The incidence of entrapment neuropathy increases with age; thus, its prevalence is expected to increase in an aging society (2). In clinical practice, treatments for entrapment neuropathy do not always yield satisfactory outcomes (3). Therefore, it is necessary to consider ways to improve the outcomes of entrapment neuropathy. Treatment outcomes for entrapment neuropathy are worse in elderly patients than young patients (4). In carpal tunnel syndrome, elderly patients showed less improvement in numbness and distal latency after carpal tunnel release than young patients (5,6). These outcomes suggest that there are differences in the condition of the peripheral nerves between young and elderly patients. Previous studies reported chronic macrophage infiltration of peripheral nerves and elevated inflammatory cytokine expression in the peripheral nerve (7), and disorganization and degeneration of the myelin sheath in age-related peripheral nerves (8). The pathophysiology of axon regeneration, which may be a therapeutic target for peripheral neuropathy, remains to be elucidated.

Repressor element-1 silencing transcription factor (REST) is a transcriptional regulator that regulates the expression of various nerve-specific genes. In the central nervous system, the expression of REST increases with aging and it protects nerves against apoptosis and oxidative stress (9,10). The expression

of REST is decreased in neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (10). On the other hand, REST inhibits axon regeneration (11). REST has multiple roles, including neuroprotection and neurotoxicity, and a previous study identified it as a critical regulator in neural survival (12). A previous study by our group reported that axon regenerative capacity is poor in aging mice compared to young mice using a mouse model of peripheral nerve injury (13). Furthermore, we found that the expression of REST increases in peripheral nerves with aging in the peripheral nervous system (14).

Based on these findings, we hypothesized that REST plays a major role in the reduction of peripheral nerve axon regenerative capacity with aging. Furthermore, as the expression of REST increases with aging and given its functions in regulating neuronal gene expression and inhibiting axon regeneration, we hypothesized that identifying and regulating molecules controlled by REST in peripheral nerves could improve the decline in axon regenerative capacity associated with aging.

In this study, animal models and REST-regulated cells were used to investigate the mechanism of REST-mediated axon regeneration in peripheral nerves *in vivo* and *in vitro*.

Materials and methods

Animals. The present study was approved by the Animal Care Committee of Juntendo University, Tokyo, Japan (registration no. 1555; approval no. 2023202, date of approval: December 11, 2023).

Ten male C57BL/6J mice (Young group: 10-week-old mice, n=5; Aged group: 70-week-old mice, n=5) for immunofluorescence staining; 10 male C57BL/6J mice (Young group: 8-week-old mice, n=5; Aged group: 78-week-old mice, n=5) for qPCR and western blotting; and 6 male aged C57BL/6J mice for treatment of GP130 receptor agonist-1 (Ga1, Selleck, Tokyo, Japan) analysis (78-week-old, treated with vehicle, n=3; treated with Ga1, n=3) were purchased from JAPAN SLC, Inc. Mice were housed at five animals/cage in a sterile environment controlled at a temperature of 22± 2˚C, humidity of 40-60%, and 12-h light and dark cycle, and were given water and CRF-1 gamma-ray-irradiated (15 kGy) (Oriental Yeast Co., Ltd.) *ad libitum*. Ten mice for immunofluorescence staining, and 10 mice for qPCR and western blotting were monitored only once when they were carried out, then they were sacrificed immediately. Six mice treated with vehicle and Ga1 were monitored 5 times, when they were carried out, before and after treatment, 1 h after treatment and 24 h after treatment. 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. No mice were reached humane endpoints. All mice were anaesthetized using 5% isoflurane and sacrificed by cervical dislocation and used for each experiment. Death was confirmed when breathing and heart rate had stopped.

There are some reports that low estrogen affects peripheral neuropathy. Because estrogen decreases with age, males with less estrogen fluctuations and susceptibility to estrogen were used in this study.

Cell culture. Mouse embryonic fibroblast cell line NIH3T3 (Cell Line Service) was cultured in a humidified incubator with 5% CO_2 at 37˚C. The culture medium was Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12) (Sigma-Aldrich) supplemented with 10% fetal bovine serum and penicillin (100 U/ml).

Construct REST expression-regulated cells. Using cultured cell lines, REST expression-regulated cells were constructed. The lentiviral vector used to overexpress REST in our study was constructed by VectorBuilder Inc. The vector ID is VB900006-3284rup. Meanwhile, the mock plasmid acted as the negative control. They were propagated in Escherichia coli DH5α. All plasmid DNA used for transfection was isolated using QIAGEN® Plasmid Maxi kit from propagated Escherichia coli. To make REST-overexpressed (REST-OE) cells, cells were transfected with isolated REST plasmid using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. To make REST-low expressed (siREST) cells, cells were transfected with REST-targeting siRNA (Sigma-Aldrich, SASI_Mm01_00196017) and control siRNA (Sigma-Aldrich, SIC001-10NMOL) using Lipofectamine RNAimax (Thermo Fisher Scientific) according to the manufacturer's instructions. REST-targeting siRNA can be purchased by registration and logging in to website of Sigma-Aldrich at https://www.sigmaaldrich.com/JP/ja/login?redirect=%2FJP%2Fja, and entering 'REST' into the gene search box and choosing the mouse at https://www.sigmaaldrich.com/JP/ja/semi-configurators/sirna?activeLink=selectAssays. Control siRNA can be purchased at https://www.sigmaaldrich.com/JP/ja/product/sigma/sic001.

Histochemical assessment. The harvested sciatic nerve (SN) and dorsal root ganglia (DRG) were fixed in 4% paraformaldehyde at room temperature for 72 h and paraffin blocks were prepared. Immunofluorescence staining was performed to assess the expression of REST and growth associated protein 43 (GAP43). Tissue sections were prepared by cutting the harvested SN and DRG at a thickness of 3 μm. Samples were deparaffinized and autoclaved at 121˚C for 10 min for antigen retrieval. After treatment with True View™ (SP-8400, Vector, CA, USA) to suppress autofluorescence, samples were blocked using 2% bovine serum albumin (A2153, Sigma Aldrich, MO, USA) in PBS containing 0.05% Tween 20 for 30 min. Samples were then reacted with antibodies against the target proteins at 4˚C for 15 h. After washing with Tris-buffered saline with Tween 20 (TBST), a goat anti-mouse IgG antibody labeled with Alexa Fluor 488 (A11001, Thermo Fisher Scientific) was used as a secondary antibody, and a rabbit IgG monoclonal antibody as a negative control. The intensity of fluorescence in each section was quantified in the photon counting mode using a fluorescence imaging microscope (Leica, TCSSP5). The antibodies used in the present study were against REST, a transcription factor that regulates the expression of nerve-specific proteins, and GAP43, a neuronal protein known for its important role in axonal outgrowth. Primary antibodies were as follows: rabbit polyclonal anti-REST (1:100, 22242-1-AP, ProteinTech, IL, USA), and rabbit polyclonal anti-GAP43 (1:100, 16971-1-AP, ProteinTech).

Table I. Primer sequences used for RT-qPCR in the present study.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (3'-5')	Seq ID
Rest	ACCTGCAGCAAGTGCAACTA	CCGCATGTGTCGCGTTAGA	XM_036164926.1
Gap43	AAGGCAGGGGAAGATACCAC	TTGTTCAATCTTTTGGTCCTCAT	NM_008083.2
Il-6	CCACTTCACAAGTCGGAGGCTTA	TGCAAGTGCATCATCGTTGTTC	NM_001314054.1
Il-6 receptor	GCCGGATCCACCTGCCAACCTT	GGGCCACCGGGAGCAGCAACAC	X53802.1
Gp130	TCCCATGGGCAGGAATATAG	CCATTGGCTTCAGAAAGAGG	NM_010560.3
Jak1	CATGGTGGAAGAGTTTGTGGA	CAGCTGTTTGGCAACCTTGAA	NM_146145.2
Stat3	AGGAGTCTAACAACGGCAGC	ACAGGATTGATGCCCAAGCA	AY299489.1
Gapdh	TGTGTCCGTCGTGGATCTG	TTGCTGTTGAAGTCGCAGG	GU214026.1

RT-qPCR, reverse transcription-quantitative PCR; Rest, repressor element-1 silencing transcriptional factor; Gap43, growth associated protein 43; Il-6, interleukin 6; Il-6 receptor, interleukin 6 receptor; Gp130, glycoprotein 130; Jak1, Janus kinase 1; Stat3, signal transducer activator of transcription 3; Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

In the photon counting mode, fluorescence intensity (gray value) was measured at 10 randomly selected sites from the perikaryon in a region of interest set in a fluorescence-emitting area, and mean fluorescence intensity was calculated. Fluorescence intensity measured using each antibody was compared between the Young and Aged groups.

Western blotting. For western blotting, protein was extracted by 1xradio immunoprecipitation assay buffer. Equal amounts of proteins from the SN and DRG in animal models, and REST expression-regulated cells were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a poly vinylidene di-fluoride (PVDF) membrane by Trans-Blot Turbo (BIORAD). Non-specific sites were blocked with PVDF Blocking Reagent (Toyobo Co., Ltd.) for 1 h at room temperature following which the membrane was washed with TBST three times, for 10 min each. The blot was then incubated overnight at 4˚C with appropriate primary antibody in solution 1 (Toyobo Co., Ltd.) according to the supplier's specific instructions. Primary antibodies were as follows: 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-glycoprotein 130 (GP130, 1:1,000, #3732; Cell Signaling), anti-signal transducer and activator of transcription 3 (STAT3, 1:1,000, #9132; Cell Signaling), anti-phosphorylated STAT3 (pSTAT3, 1:1,000, #9131; Cell Signaling), and mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:2,000, sc-32233; Santa Cruz). The blots were washed with TBST and incubated with appropriate secondary antibody for 2 h at room temperature. After washing, Amersham Imager 680 (GE Healthcare Life SciencesA) was applied, and blot images were captured using a gel documentation system. Relative optical density of protein bands was analyzed using gel software image lab 3.0. The membranes were stripped and reprobed with GAPDH as a loading control.

Quantitative PCR. Total RNA was isolated from the SN, DRG in animal models and REST expression-regulated cells by using RNeasy Microkit (Qiagen) in accordance with the manufacturer's instructions. Complementary DNA was synthesized using PrimeScript™ RT reagent Kit (Takara). Next, qPCR was performed with SYBR Green real-time PCR assay (Thermo Fisher Scientific) according to the ΔΔCq method. The expression levels of targets were normalized to GAPDH. Used primers are listed in Table I.

Analysis of REST and GAP43 expression in young and aged mice. Young mice (n=5) and aged mice (n=5) were sacrificed by cervical dislocation on the day that SN and DRG were harvested. The expression of REST and GAP43 in SN and DRG was compared between the young and aged mice by immunofluorescence staining, qPCR, and western blotting.

Analysis of molecules expression of JAK1/STAT3 pathway in REST expression-regulated cells. The expression of interleukin 6 (IL6), IL6 receptor, GP130, janus kinase 1 (JAK1), and STAT3, which are components of the JAK1/STAT3 pathway involved in regulating GAP43 expression (15), was evaluated by qPCR in REST-regulated cells. The expression of GP130 was evaluated by western blot additionally. As the expression of GAP43 is promoted when STAT3 is phosphorylated by JAK1, we evaluated STAT3 and pSTAT3 by western blotting (16).

Analysis of GAP43 expression in REST-OE treated with Ga1. Since expression of JAK1/STAT3 pathway was investigated and GP130 was found to be important, we treated REST-OE cells with Ga1 to investigate the effect on axonal regeneration markers. The stock solution of Ga1 was prepared by transferring 5 mg to dimethyl sulfoxide (DMSO) at a concentration of 10 mM. Further dilutions were made fresh in cell culture medium. The final concentration of DMSO was 0.1% and Ga1 was 10 μM. After 48 h culture, REST-OE cells were incubated with 10 μM Ga1 for an additional 30 min at 37˚C. After being treated by Ga1, the expression of REST, GP130, and GAP43 in REST-OE was evaluated by western blotting and qPCR. DMSO was used as the vehicle control.

Analysis of GAP43 expression in SN of Aged mice treated with Ga1. Six 78-week-old mice were used and divided into a DMSO group (n=3) and Ga1 group (n=3). DMSO was

used as the vehicle control. The Ga1 group mice received an intraperitoneal injection of Ga1 dissolved by DMSO at a dose of 10 mg/kg. Since 10 mg/kg Ga1 was used to mice to evaluate the nervous system in the previous study reported by Alam *et al* (17), a dose of 10 mg/kg was administered to mice in this study. Only one dose was given during the entire experimental period. The mice were sacrificed at 24 h after treatment and SN was harvested to confirm the effect of Ga1 on the expression of axon regeneration marker GAP43. The expression of REST and GAP43 in SN was evaluated by western blotting and qPCR.

Date analysis and statistics. Data are presented as the mean ± standard deviation and were analyzed for significant differences using unpaired Student's t-test, significance was defined as P<0.05 (Prism 7; GraphPad Software).

Results

Expression of REST and GAP43 in the sciatic nerve and dorsal root ganglia. To investigate the difference in the expression of REST and GAP43 in young (Young group) and aged (Aged group) mice, the expression of REST and GAP43 in the SN and DRG were quantified by immunofluorescence staining, western blotting, and qPCR. Immunofluorescence staining revealed a significant increase in the fluorescence intensity of REST in the Aged group compared to the Young group in the SN (Young group 132.3±14.5; Aged group 189.4±12.1, P=0.0003) (Fig. 1A) and DRG (Young group 115.9±25.5; Aged group 174.7±41.1, P=0.026) (Fig. 1B). Furthermore, a significant decrease in the fluorescence intensity of GAP43 in the Aged group compared to the Young group was found in the SN (Young group 192.3±16.3; Aged group 119.2±9.0, P<0.0001) (Fig. 1C) and DRG (Young group 168.5±16.3; Aged group 89.0±45.0, P=0.005) (Fig. 1D).

Western blotting revealed a significant increase in the expression of REST in the Aged group compared to Young group in the SN (1.29±0.04-fold, P=0.002) (Fig. 1E) and DRG (3.57±0.52-fold, P=0.009) (Fig. 1F). On the other hand, a significant decrease was observed in the expression of GAP43 in the Aged group compared to the Young group in the SN (0.68±0.07-fold, P=0.026) (Fig. 1E) and DRG (0.74±0.09-fold, P=0.012) (Fig. 1F).

qPCR revealed a significant increase in the expression of REST in the Aged group compared to the Young group in the SN (2.12±0.46-fold, P=0.006) (Fig. 1G) and DRG (1.75±0.29-fold, P=0.009) (Fig. 1H). A significant decrease was found in the expression of GAP43 in the Aged group compared to the Young group in the SN (0.75±0.21-fold, P=0.031) (Fig. 1I). No significant difference between the expression of GAP43 in the Aged group and the Young group was observed in the DRG (0.99±0.21-fold, P=0.935) (Fig. 1J). GAP43 is known to be strictly regulated in terms of its expression at the mRNA level and protein level, by regulating mRNA stability post-transcriptionally and by post-translational modification including phosphorylation and palmitoylation (18). Future study may clarify whether GAP43 is degraded by post-translational modification. In addition, GAP43 mRNA and protein may be regulated by different mechanisms that are tissue-dependent, such as in the SN and DRG.

The expression of REST and GAP43 in REST-regulated cells. To determine whether REST plays a role in GAP43 expression, REST plasmid and siRNA were used to construct REST-OE cells and siREST cells using NIH3T3. Western blotting and qPCR revealed a significant increase in the expression of REST in REST-OE compared to the Control (protein: 18.8±5.5-fold, P=0.038; mRNA: 662.6±53.6-fold, P<0.0001) (Fig. 2A and B) and a significant decrease in the expression of REST in siREST compared to the Control (protein: 0.63±0.25-fold, P=0.026; mRNA: 0.33±0.18-fold, P<0.0001) (Fig. 2C and D). Therefore, REST-OE cells and siREST cells were used for the following studies. Interestingly, qPCR revealed a significant decrease in the expression of GAP43 in REST-OE and a significant increase in the expression of GAP43 in siREST compared to the Control (REST-OE: 0.58±0.31-fold, P=0.016; siREST: 2.60±0.50-fold, P<0.0001) (Fig. 2E and F). This relationship between REST and GAP43 supports the findings in the animal models.

The expression of molecules of JAK1/STAT3 pathway in REST-regulated cells. To determine the role of REST in GAP43 expression, the involvement of molecules in the JAK1/STAT3 pathway was investigated. The expression levels of IL-6, IL-6 receptor, GP130, JAK1, and STAT3 were investigated using qPCR. qPCR revealed a significant increase in IL-6 expression in REST-OE and siREST compared to the Control (REST-OE: 1.98±0.18-fold, P=0.017; siREST: 2.81±0.26-fold, P=0.010) (Fig. 3A and B). There was no significant difference in IL-6 receptor expression between REST-OE and the Control (0.96±0.02-fold, P=0.184) (Fig. 3C), or between siREST and Control (1.20±0.29-fold, P=0.442) (Fig. 3D). A significant decrease in GP130 expression was observed in REST-OE, whereas a significant increase was observed in siREST compared to the Control (REST-OE: 0.52±0.01-fold, P=0.004; siREST: 2.26±0.11-fold, P=0.004) (Fig. 3E and F). JAK1 expression significantly decreased in REST-OE and significantly increased in siREST compared to the Control (REST-OE: 0.68±0.08-fold, P=0.003; siREST: 1.66±0.20-fold, P=0.005) (Fig. 3G and H). No significant difference in STAT3 expression was observed between REST-OE and Control (1.14±0.02-fold, P=0.095) (Fig. 3I), whereas a significant increase was noted in siREST compared to the Control (1.51±0.04-fold, P=0.012) (Fig. 3J). Since GP130 was considered to be an important molecule, GP130 expression was also evaluated by western blotting. As a result, a significant decrease in GP130 expression was observed in REST-OE, whereas a significant increase was observed in siREST compared to the Control (REST-OE: 0.69±0.04-fold, P=0.003; siREST: 1.48±0.17-fold, P=0.013) (Fig. 3K and L).

The expression of GAP43 is promoted when STAT3 is phosphorylated by JAK1 (16). Therefore, the expression of STAT3 and pSTAT3 protein was evaluated by western blotting. Western blotting revealed no significant difference between the expression of STAT3 in REST-OE and Control, however a significant decrease in the expression of pSTAT3 in REST-OE compared to the Control (STAT3: 1.10 ± 0.15-fold, P=0.459, pSTAT3: 0.76±0.04-fold, P=0.015) (Fig. 4A). Moreover, a significant increase in the expression of STAT3 and pSTAT3 was observed in siREST compared to the Control (STAT3: 1.28±0.08-fold; P=0.043, pSTAT3:

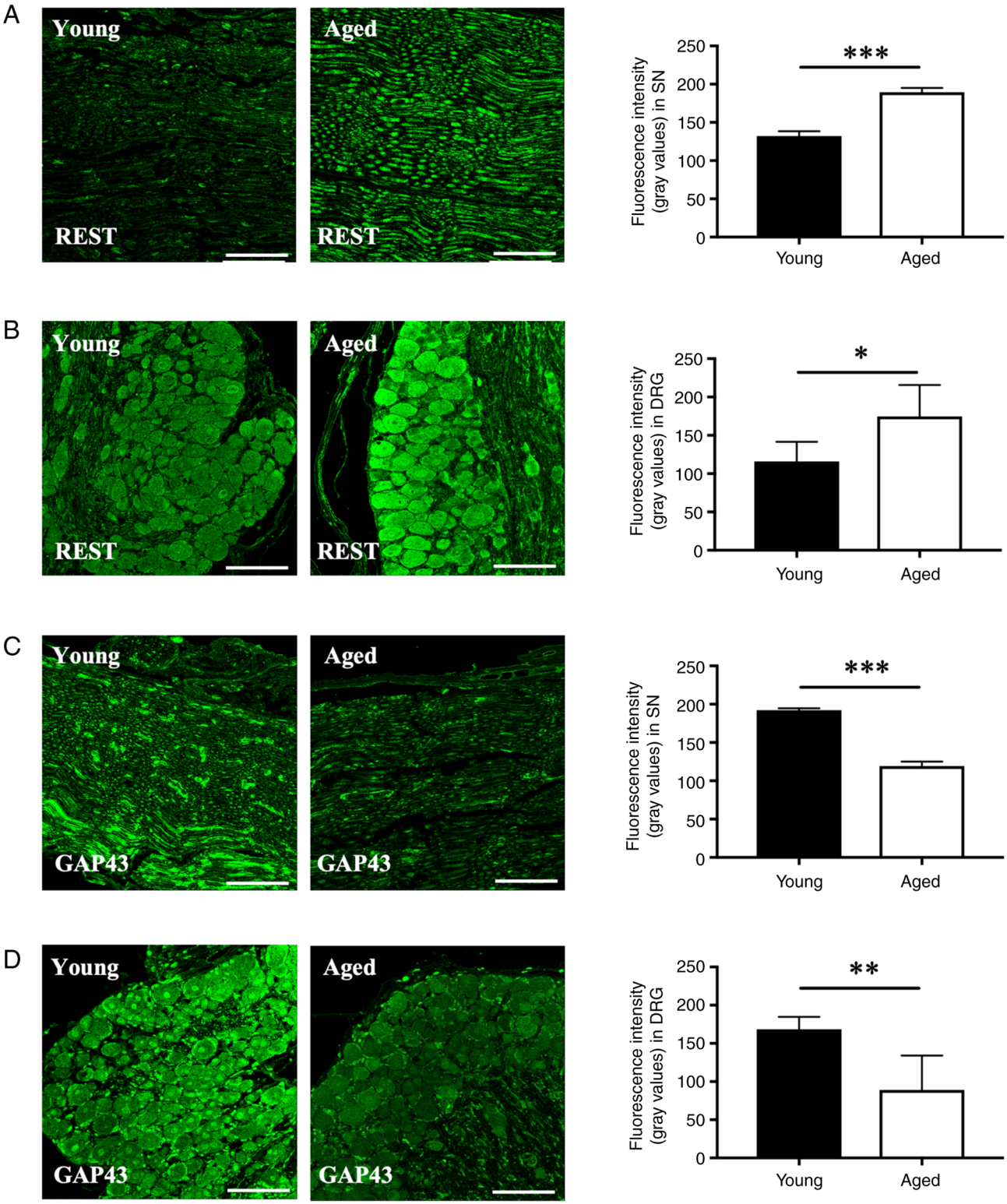

Figure 1. Continued.

1.33±0.07-fold, P=0.033) (Fig. 4B). Furthermore, the ratio of phosphorylation/total STAT3 protein was significantly decreased in REST-OE compared to the Control (0.69±0.09-fold; P=0.005) (Fig. 4C), whereas the ratio of that was significantly increased in siREST compared to the Control (1.21±0.07-fold; P=0.005) (Fig. 4D). These findings suggest that the expression of STAT3 does not change, but the activation of STAT3 is low in REST-OE.

In summary, despite similar changes of IL6 and IL6 receptor in REST-OE and siREST, the expression of GP130, JAK1, and phosphorylation of STAT3 were decreased in REST-OE, whereas the expression of GP130, JAK1, and phosphorylation of STAT3 were increased in siREST. These findings suggest that REST may regulate the expression of GAP43 by the JAK1/STAT3 pathway via the expression of GP130 (Fig. 5).

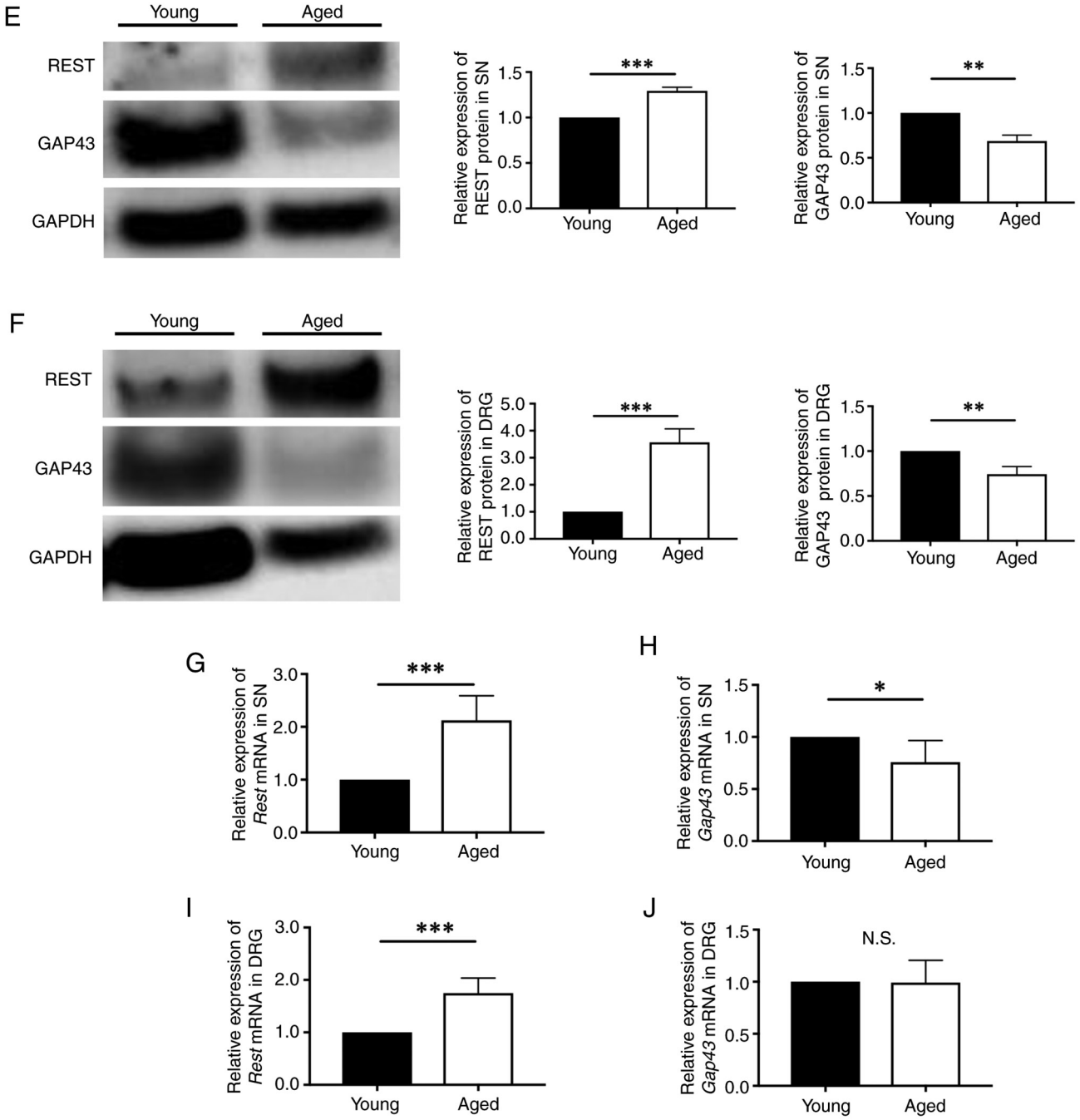

Figure 1. Expression of REST and GAP43 in the SN and DRG of young and aged mice are evaluated by immunofluorescence staining, western blotting and RT-qPCR. Aged mice (70- or 78-week-old, white bars) compared with young mice (10- or 8-week-old, black bars). Histochemical assessment of the expression of REST in the (A) SN and (B) DRG of young and aged mice by immunofluorescence staining (scale bar, 100 μm). Histochemical assessment of the expression of GAP43 in the (C) SN and (D) DRG of young and aged mice by immunofluorescence staining (scale bar, 100 μm). Western blotting analysis of the expression of REST and GAP43 in the (E) SN and (F) DRG. RT-qPCR analysis of the expression of (G) REST and (H) GAP43 in the SN. RT-qPCR analysis of the expression of (I) REST and (J) GAP43 in the DRG. Data are expressed as mean ± standard deviation (n=5 mice per group). *P<0.05, **P<0.01 and ***P<0.001. REST, repressor element-1 silencing transcription factor; GAP43, growth-associated protein 43; SN, sciatic nerve; DRG, dorsal root ganglia; RT-qPCR, reverse transcription-quantitative PCR.

The expression of GAP43 in REST-OE cells cultured with GP130 agonist. As REST possibly regulates the expression of GAP43 by the JAK1/STAT3 pathway via the expression of GP130, we predicted that regulation of GP130-related molecular expression may change the expression of GAP43 in REST-OE cells. To investigate this hypothesis, we used Ga1, a GP130 agonist. The expression of REST, GP130, and GAP43 was investigated by western blotting and qPCR. Western blotting and qPCR revealed no significant difference between the expression of REST in Ga1 and DMSO control (protein: 1.01±0.03-fold, P=0.54; mRNA: 1.05±0.33-fold, P=0.85) (Fig. 6A and B), no significant difference between the expression of GP130 in Ga1 and a DMSO control (protein: 0.95±0.07-fold, P=0.28; mRNA: 0.92±0.13-fold, P=0.34) (Fig. 6A and C). Interestingly, a significant increase was observed in the expression of GAP43 in Ga1 compared to a DMSO control (protein: 1.41±0.10-fold, P=0.018; mRNA: 1.66±0.26-fold,

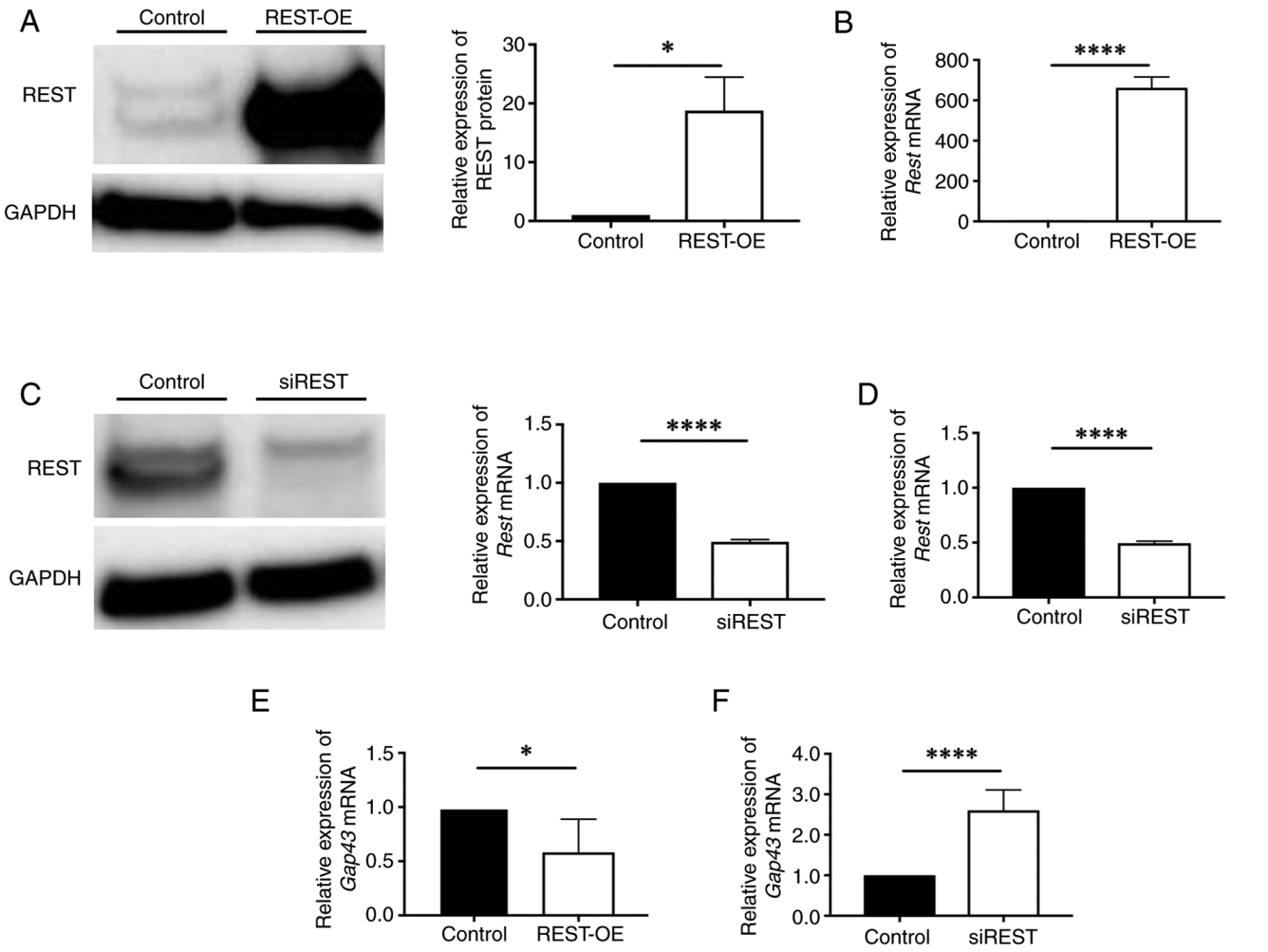

Figure 2. Expression of REST and GAP43 in REST-regulated cells. REST-OE and siREST (white bars) compared with the Control (black bars). Graphs show quantification of relative protein and mRNA abundance. (A) Western blotting analysis and (B) RT-qPCR analysis of the expression of REST in REST-OE. (C) Western blotting analysis and (D) RT-qPCR analysis of the expression of REST in siREST. RT-qPCR analysis of the expression of GAP43 in (E) REST-OE and (F) siREST. Data are expressed as mean ± standard deviation. *P<0.05 and ****P<0.0001. REST, repressor element-1 silencing transcription factor; GAP43, growth-associated protein 43; RT-qPCR, reverse transcription-quantitative PCR; REST-OE, REST-overexpressed; siREST, REST-low expressed.

P=0.040) (Fig. 6A and D). Thus, Ga1 enhanced GAP43 expression without changes of REST and GP130 expression.

The expression of GAP43 in mice treated with Ga1. Ga1 enhanced GAP43 expression in REST-OE cells; therefore, we dosed Ga1 into aged mice that had high REST expression to investigate its influence *in vivo*. The expression of REST and GAP43 was investigated by western blotting and qPCR. Western blotting and qPCR revealed no significant difference between the expression of REST in Ga1 and a DMSO control (protein: 1.48±0.72-fold, P=0.54; mRNA: 1.07±0.19-fold, P=0.55) (Fig. 7A and B). Interestingly, a significant increase was observed in the expression of GAP43 in Ga1 compared to a DMSO control (protein: 2.25±0.55-fold, P=0.016, mRNA: 1.54±0.22-fold; P=0.013) (Fig. 7A and C). Our findings suggest that Ga1 enhanced GAP43 expression *in vitro* and *in vivo*.

Discussion

Our findings suggest that the JAK1/STAT3 pathway is involved in the pathology of the reduction in peripheral nerve axon regenerative capacity with aging. Then, our *in vitro* findings using REST-OE cells and siREST cells suggest that regulating GP130 expression is important in addressing the reduction of peripheral nerve axon regenerative capacity that occurs with aging. Furthermore, our finding that treatment with the GP130 agonist Ga1 enhanced the expression of the axon regeneration marker GAP43 suggests that GP130 is an important molecule for peripheral nerve axon regeneration.

Previous studies reported that the JAK1/STAT3 pathway is activated by cytokines binding to GP130 (19,20). Quarta *et al* (21) reported that suppression of JAK1/STAT3 pathway activity in GP130 knockout mice resulted in decreased axon regeneration and delayed functional recovery after nerve injury. Furthermore, inhibition of leukocyte migration inhibitory factor, which is a ligand for GP130, suppressed axon regeneration after nerve injury, whereas administration of ciliary neurotrophic factor, which is another ligand for GP130, promoted axon regeneration (22,23). In the present study, expression of GP130 and JAK1, and phosphorylation of STAT3 in the JAK1/STAT3 pathway *in vitro* were decreased in REST-OE cells and increased in siREST cells. Furthermore, REST-OE cells in aged mice treated with Ga1 promoted axon regeneration. Our findings support the findings of previous

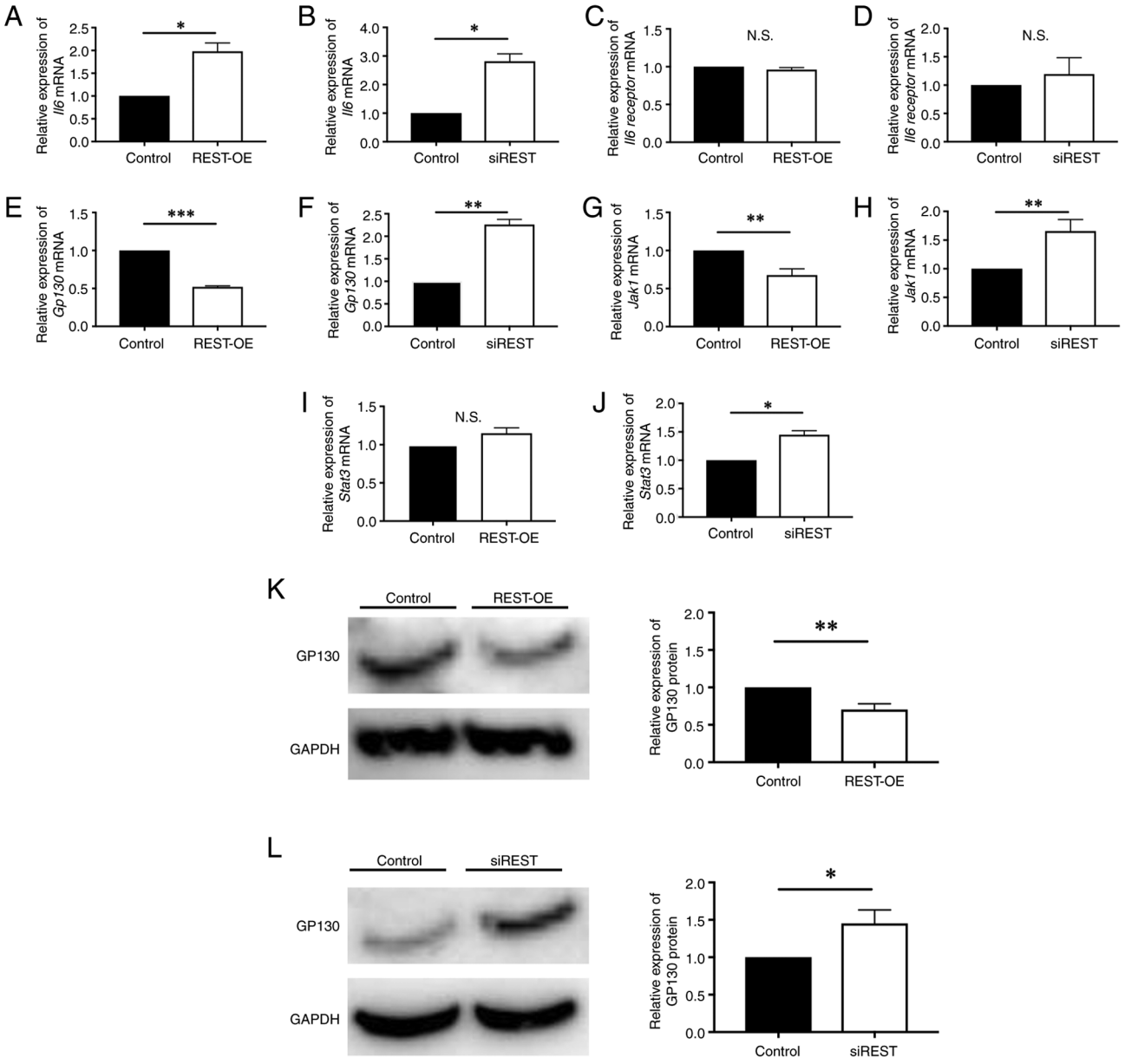

Figure 3. Expression of molecules of the JAK1/STAT3 pathway in REST-regulated cells evaluated by RT-qPCR and western blotting. REST-OE and siREST (white bars) compared with the control (black bars). RT-qPCR analysis of: IL6 in (A) REST-OE and (B) siREST; IL6 receptor in (C) REST-OE and (D) siREST; GP130 in (E) REST-OE and (F) siREST; JAK1 in (G) REST-OE and (H) siREST; and STAT3 in (I) REST-OE and (J) siREST. Western blotting analysis of GP130 in (K) REST-OE and (L) siREST. Data are expressed as mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001. JAK1, janus kinase 1; STAT3, signal transducer and activator of transcription 3; REST, repressor element-1 silencing transcription factor; RT-qPCR, reverse transcription-quantitative PCR; REST-OE, REST-overexpressed; siREST, REST-low expressed; GP130, glycoprotein 130.

studies that reported that GP130 is a key protein and potential therapeutic target for treating poor axon regenerative capacity with aging.

Known axon regeneration markers include superior cervical ganglion (SCG10), small proline-rich protein 1A (SPRR1A), and GAP43 (24-26). SCG10 is a marker that expresses in microtubules of regenerating axons (24,27). SPRR1A is an axon regenerative marker which is increased in neurons after nerve injury (25). The protein GAP43 is a marker that can assess axon regeneration in the distal axon terminals of motor and sensory nerves (28-30). Therefore, in the present study, to assess the axon regenerative capacity with aging, we analyzed the expression of GAP43, which we hypothesized would be able to assess axon regeneration at the distal end of axons. There are some reports on intracellular signaling pathways involved in peripheral nerve axon regeneration (15,31-34). Among these pathways, the PI3K/AKT pathway and JAK1/STAT3 pathway are involved in GAP43 expression (35). Furthermore, it has been reported that PI3K inhibitors do not inhibit axon regeneration, whereas JAK inhibitors inhibit axon regeneration (35). Based on these findings, GAP43, as a marker of axon regeneration, and the JAK1/STAT3 pathway, as an intracellular signaling pathway, were analyzed to assess the pathology of the reduction in axon regenerative capacity associated with aging in the present study.

REST inhibits axon regeneration by suppressing the expression of L1 cell adhesion molecule, which is an adhesion

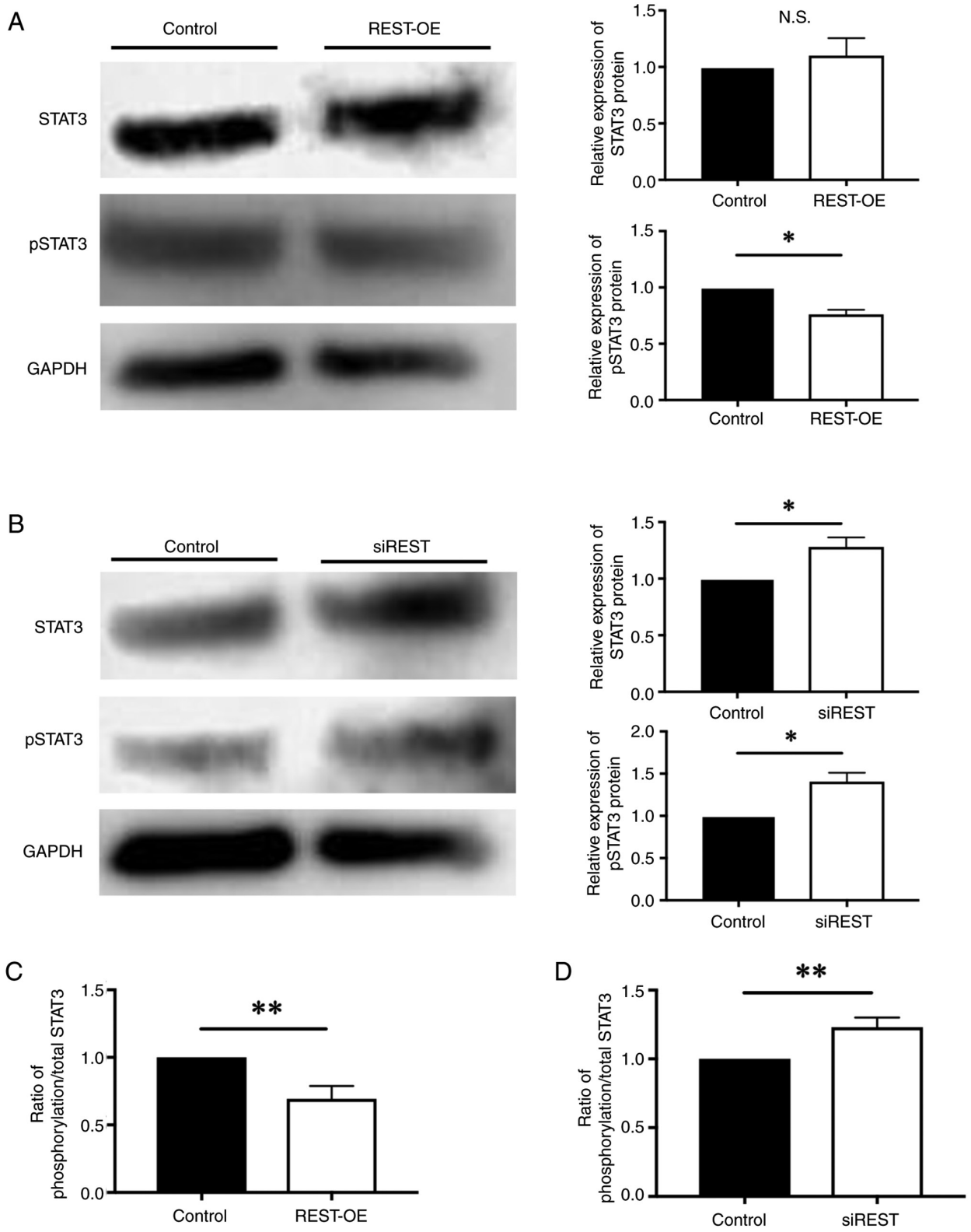

Figure 4. Expression of STAT3 and phosphorylation of STAT3 in REST-regulated cells evaluated by western blotting. REST-OE and siREST (white bars) compared with the control (black bars). Graphs show quantification of relative protein abundance. Western blotting analysis of STAT3 and pSTAT3 in (A) REST-OE and (B) in siREST. Ratio of phosphorylation STAT3 level for total STAT3 protein levels in (C) REST-OE and (D) in siREST. Data are presented as mean ± standard deviation. *P<0.05 and **P<0.01. STAT3, signal transducer and activator of transcription 3; REST, repressor element-1 silencing transcription factor; REST-OE, REST-overexpressed; siREST, REST-low expressed; p, phosphorylated.

factor that promotes axon regeneration, and suppressing Elk-1, which is a transcription factor that promotes axon regeneration, by inhibiting its phosphorylation (11,36,37). Gervasi *et al* (38) reported that inhibiting carboxy-terminal domain small phosphatase 1, which stabilizes REST by dephosphorylation, increases brain-derived neurotrophic factor expression

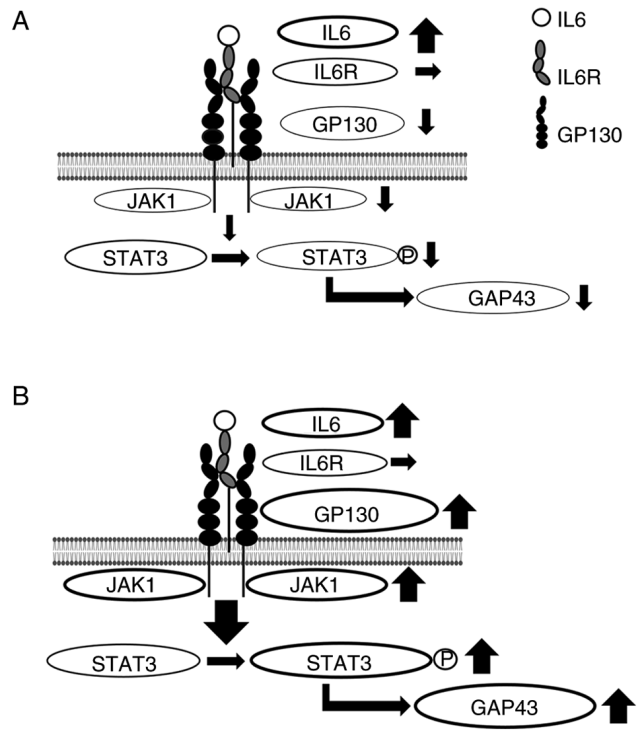

Figure 5. Summary of the expression of molecules of the JAK1/STAT3 pathway in REST-regulated cells. Despite similar changes of IL6 and IL6 receptor in REST-OE and siREST, the expression of GP130, JAK1 and pSTAT3 was increased in REST-OE and the expression of GP130, JAK1 and pSTAT3 was decreased in siREST. These findings suggest that REST regulates the expression of GAP43 by the JAK1/STAT3 pathway via the expression of GP130. (A) Summary of the expression of molecules in REST-OE. In REST-OE, the expression of IL6 was significantly increased, there was no significant difference in the expression of IL6 receptor and the expression of GP130, JAK1 and pSTAT3 were significantly decreased compared with the control. (B) Summary of the expression of molecules in siREST. In siREST, the expression of IL6 was significantly increased, there was no significant difference in the expression of IL6 receptor, and the expression of GP130, JAK1 and pSTAT3 was significantly increased compared with the control. STAT3, signal transducer and activator of transcription 3; REST, repressor element-1 silencing transcription factor; REST-OE, REST-overexpressed; siREST, REST-low expressed; p, phosphorylated; GP130, glycoprotein 130; p, phosphorylated; IL, interleukin; IL6R, IL6 receptor; GAP43, growth-associated protein 43.

and promotes axon regeneration. Thus, several studies have reported a mechanism of axon regeneration inhibition by REST. However, the effects of REST on the expression of molecules involved in the JAK1/STAT3 pathway remain to be elucidated.

The present study investigated the effects of REST on the intracellular signaling pathways associated with axon regeneration in peripheral nerves. We found that REST inhibits axon regeneration by suppressing the activity of the JAK1/STAT3 pathway via GP130. Our findings suggest the importance of GP130 for understanding the pathology of the reduction in axon regenerative capacity associated with aging. It is known that the function of motor and sensory nerves is declined in elderly (39). Furthermore, the improvement of the function of motor and sensory nerves can be led by the enhancement of axon regeneration (40). The results of this study suggested that GP130 could be a potential therapeutic target with problems of peripheral nerve systems in elderly.

Our study has some major limitations that must be taken into account when interpreting our results. Firstly, the REST-regulated cells were constructed by fibroblasts, which are non-neuronal cells. Gene expression patterns vary between cell lines (41). However, there have been no reports of differences in gene expression patterns between nervous system cells and fibroblasts, although basic research of nervous systems using fibroblasts has been reported (42,43). This suggests that NIH3T3 is suitable for experiments analyzing the expression of nervous system genes; thus, this cell line was used in the present study. Secondly, REST is a transcriptional regulator that protects neural homeostasis by regulating the expression of various nervous system genes and has multiple functions (10). In this study we focused on only axon regeneration; however, further studies are needed to comprehensively assess axon regeneration *in vivo*. The function of transcription factors is being investigated for the treatment of various diseases (44,45). Cao *et al* (44) reported that PTEN, which is a multifunctional cancer transcriptional repressor, had a cell survival function and that increased PTEN expression promoted apoptosis and suppressed cancer. Thirdly, the experiments of GP130 knockdown were not conducted in this study. It has been reported that axonal regeneration after nerve injury is reduced in GP130 knockout mice (20). Moreover, it has also been reported that axonal regeneration is reduced when the GP130 ligand is knocked out (21,22). Based on these reports, it is well known that GP130 is necessary for axonal regeneration. In this study, REST-OE cells were used to investigate the molecules expression of JAK1/STAT3 pathway involved in regulating GAP43 expression. Then, it was revealed that GP130 expression was 48% decreased in REST-OE compared to Control in this study. In previous reports of gene knockdown experiments, experiments were conducted with 30 to 50% reduction in expression of target gene using siRNA, and with 46% reduction in expression of target gene using shRNA (46-48). In other words, the 48% reduction in GP130 in REST-OE in this study is considered to equivalent to the gene knockdown state. Therefore, it is considered that the experiments using REST-OE mimic the experiments of GP130 knockdown and can evaluate the effect on axonal regeneration marker GAP43. According to above reason, the experiments of GP130 knockdown were not conducted in this study. Fourthly, mice treated with Ga1 in this study did not evaluate using DRG. However, several previous studies have been reported that have evaluated axonal regeneration without using DRG, but only using SN (49,50). Therefore, the evaluation of the SN would be sufficient for evaluating axonal regeneration. Furthermore, we evaluated the expression of GAP43 in the SN rather than the DRG since GAP43 is a protein that is expressed in the distal axon terminals (28).

In conclusion, we found that reduced JAK1/STAT3 pathway activity, caused by decreased GP130 expression due to REST, is a key factor in the reduction of axon regenerative capacity with aging and represents a potential therapeutic target. This study may improve axon regenerative capacity by aging.

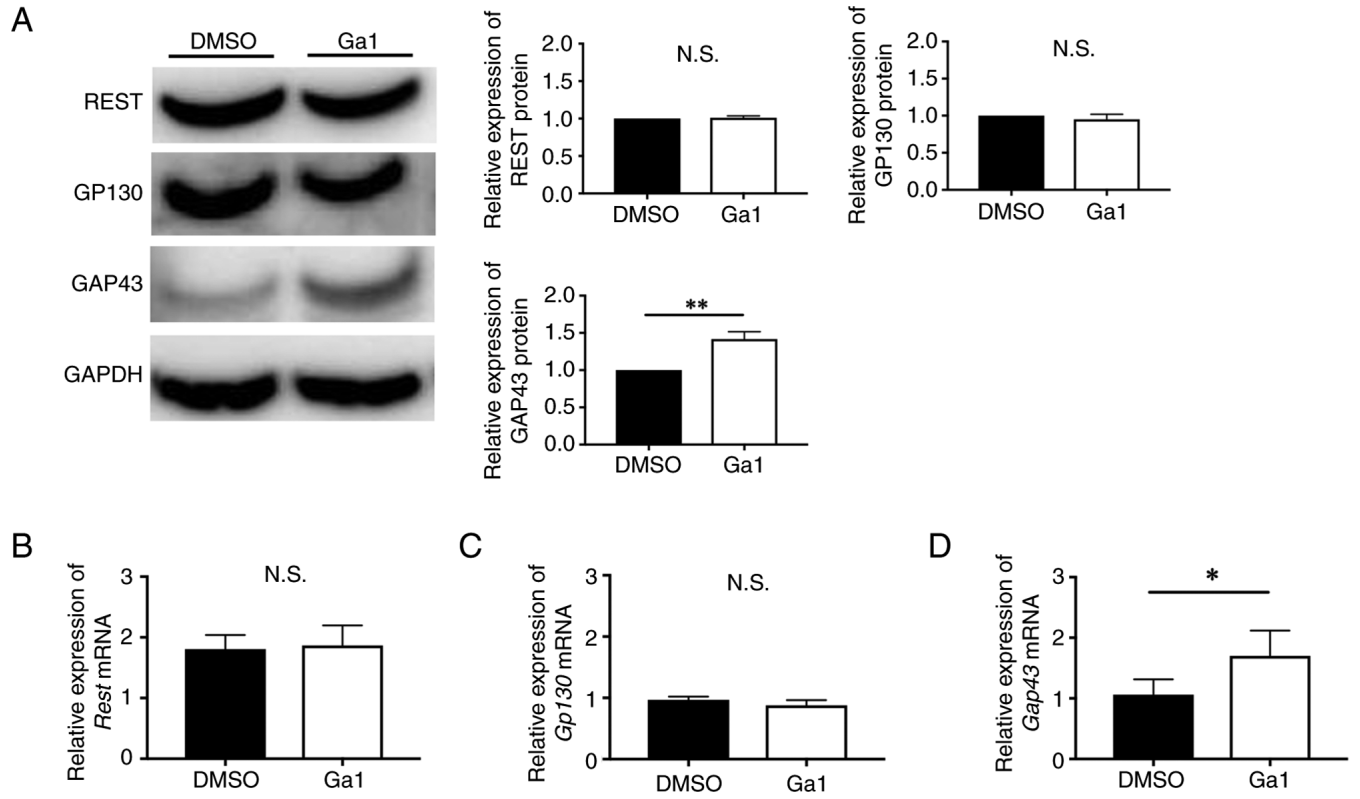

Figure 6. Expression of REST, GP130 and GAP43 in REST-OE cultured with Ga1. REST-OE cultured with Ga1 (white bars) compared with REST-OE cultured with DMSO (black bars). Graphs show quantification of relative protein and protein and mRNA abundance. (A) Western blotting analysis of REST, GP130 and GAP43. RT-qPCR analysis of (B) REST, (C) GP130 and (D) GAP43. Data are presented as mean ± standard deviation. *P<0.05 and **P<0.01. REST, repressor element-1 silencing transcription factor; REST-OE, REST-overexpressed; Ga1, GP130 receptor agonist-1; GP130, glycoprotein 130; DMSO, dimethyl sulfoxide; GAP43, growth-associated protein 43; RT-qPCR, reverse transcription-quantitative PCR; N.S., not significant.

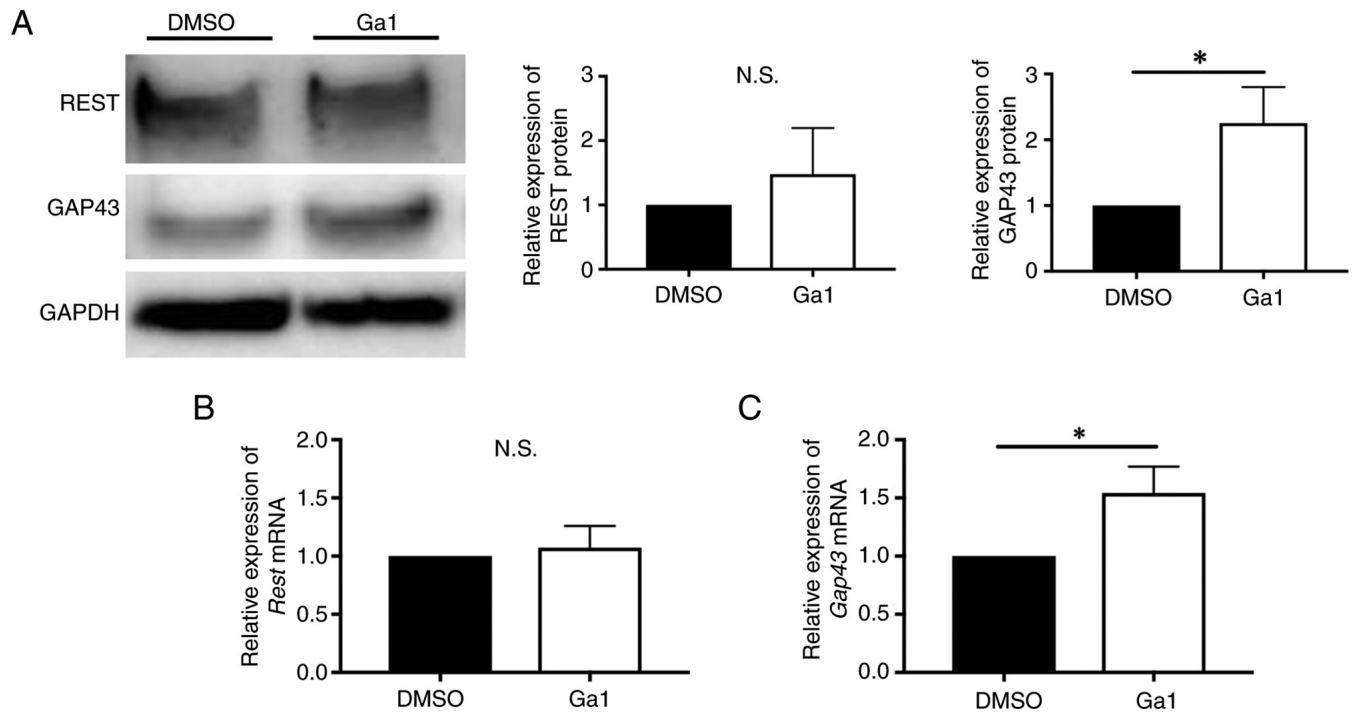

Figure 7. Expression of REST and GAP43 in aged mice treated with Ga1. Aged mice treated with Ga1 (white bars) compared to aged mice treated with DMSO (black bars). Graphs show quantification of relative protein and protein and mRNA abundance. (A) Western blotting analysis of REST and GAP43. RT-qPCR analysis of (B) REST and (C) GAP43. Data are presented as mean ± Standard deviation (n=3 mice per group). *P<0.05. REST, repressor element-1 silencing transcription factor; GAP43, growth-associated protein 43; RT-qPCR, reverse transcription-quantitative PCR; N.S., not significant; DMSO, dimethyl sulfoxide; Ga1, GP130 receptor agonist-1.

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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. SK, TNK and YY designed the methodology. SK, TS, NI and KK conducted the investigation. SK, KN, NH and MI analyzed and interpreted the data. SK and KN prepared the original draft, while SK, KN and MI reviewed and edited the manuscript. KN secured funding. SK, KN, DK and NH confirm the authenticity of all the raw data. All authors have read and approved the final 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. 2023202).

Patient consent for publications

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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