Age-related differences for expression of the nerve-specific proteins after peripheral nerve injury

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Received June 29, 2022; Accepted September 6, 2022

DOI: 10.3892/etm.2022.11618

Abstract. The effects of aging on axon regeneration currently remain unclear. In addition, the up-regulated expression of neurotrophic factors that occurs within one week of peripheral nerve injury has been shown to play an important role in the axon regeneration. To investigate the effects of aging on axon regeneration, the expression of nerve-specific proteins immediately after peripheral nerve injury were compared between young and aged mice. A mouse peripheral nerve injury model was prepared using the sciatic nerve compression method. In each group, Luxol fast blue staining and immunofluorescence staining were performed to assess the degree of Wallerian degeneration in the sciatic nerve, and to evaluate the expression of repressor element 1-silencing transcription factor (REST)/neuron-restrictive silencer factor (NRSF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), nerve growth factor (NGF), and semaphorin 3A (Sema3A) in the dorsal root ganglion, respectively. Wallerian degeneration was observed in both young and aged mice after peripheral nerve injury. Significant increases were observed in the expression of REST/NRSF ($P<0.0001$), NT3 ($P=0.0279$), and Sema3A ($P=0.0175$) following peripheral nerve injury in young mice, while that of BDNF ($P=0.5583$) and NGF ($P=0.9769$) remained unchanged. On the other hand, no significant differences were noted in the expression of these nerve-specific proteins in aged mice. Based on the results of the present study, compensatory changes induced by peripheral nerve injury were initiated by the up-regulated expression of REST/NRSF in young mice, but not in aged mice.

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Key words: aging, nerve-specific proteins, neurotrophic factor, peripheral nerve injury, repressor element 1-silencing transcription factor/neuron-restrictive silencer factor, Wallerian degeneration

Introduction

In the central nervous system, repressor element-1 silencing transcription/neuron-restrictive silencer factor (REST/NRSF) maintains homeostasis by suppressing the apoptosis of neurons (1-3). Its expression increases with age and protects nerves against aging stress (3). We previously reported the up-regulated expression of REST/NRSF in neurons in the peripheral and central nervous systems with aging (4). However, the effects of REST/NRSF on axon regeneration following peripheral nerve injury currently remain unclear.

The ability to regenerate axons damaged by peripheral nerve injury decreases with age (5,6). Our previous findings, obtained using a mouse peripheral nerve injury model, suggested that axon regeneration was significantly slower in aged mice than in young mice (7). Moreover, 'angiogenesis' and 'Schwann cell migration', the processes required for axon regeneration immediately after peripheral nerve injury, were impaired in aged mice (7).

The up-regulated expression of neurotrophic factors that occurs within one week of peripheral nerve injury has been shown to play an important role in Schwann cell migration and myelination during axon regeneration (8,9). Based on these findings, the expression of nerve-specific proteins immediately after peripheral nerve injury needs to be examined in order to obtain more detailed insights into axon regeneration.

A peripheral nerve injury model in young and aged mice was used to investigate the effects of aging on axon regeneration in the present study. The degree of Wallerian degeneration and the expression of nerve-specific proteins immediately after peripheral nerve injury were compared between these groups. Furthermore, the effects of REST/NRSF on axon regeneration,

which currently remain unclear, were discussed based on the expression of nerve-specific proteins.

Materials and methods

Animal Model. The present study was approved by the Animal Care Committee of Juntendo University, Tokyo, Japan (registration no. 1555; approval no. 2021312).

Forty male C57BL/6 mice (Young group: 10-week-old mice, n=20; Aged group: 70-week-old mice, n=20) purchased from Japan SLC, Inc. (Shizuoka, Japan) were used. Mice were housed at 5 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 that was CRF-1 gamma-ray irradiated at 15 kGy (Oriental Yeast Co., Ltd.) *ad libitum*. There are some reports that low estrogen affects peripheral neuropathy (10,11). Because estrogen decrease with age (12,13), males with less estrogen fluctuations and susceptible to estrogen were used in this study.

Peripheral nerve injury model. The Young group (n=20) and Aged group (n=20) were divided into Control and Crush groups to create four groups (A: Young control (n=10), B: Young crush (n=10), C: Aged control (n=10), and D: Aged crush (n=10)). Chronic constriction injury (CCI) is a partial nerve injury that is mostly used in rodents and is performed using a hemostatic forceps (14). It induces incomplete nerve injury. In the present study, this CCI model was used and defined as the peripheral nerve injury group (Crush group).

Under general anesthesia with isoflurane inhalation anesthetic solution (4% isoflurane for induction and 2% for maintenance) (7), a skin incision was made on the lateral side of the right hindlimb. A light microscope (Zeiss, Axioskop2, magnification, x40) was used to manipulate the sciatic nerve. The sciatic nerve was dissected from the surrounding tissues (Fig. 1A) and crushed for 30 sec using the hemostatic forceps with sufficient strength to flatten the sciatic nerve to caused Wallerian degeneration (Fig. 1B and C), according to a previously reported method (14). The postoperative activity of mice was not limited, and they were maintained in the same environment as that before the procedure. Under general anesthesia, the right sciatic nerve and L3-5 dorsal root ganglion (DRG) of each group were harvested (15). Control group were only harvested the samples, and samples from the Crush group were harvested one week after surgery. Mice were sacrificed by cervical dislocation on the day the sciatic nerves and DRG were harvesting. The harvested sciatic nerve and DRG were fixed in 4% paraformaldehyde at room temperature for 72 h and paraffin blocks were prepared.

Histochemical assessment of the degree of Wallerian degeneration in peripheral nerves. Luxol fast blue (LFB) staining was performed to assess the degree of Wallerian degeneration after peripheral nerve injury (16). Tissue sections were prepared by cutting the sciatic nerve in the longitudinal axis at a thickness of 3 μm.

Tissue sections of the sciatic nerve in the longitudinal axis were divided into three areas (the crushed site, proximal to the crushed site, and distal to the crushed site) in the Crush groups. Similarly, tissue sections of the sciatic nerve were divided into three areas in the Control group without peripheral nerve injury. The area distal to the crushed site was used in the present study because this was the site at which Wallerian degeneration occurred. Using a light microscope (Carl Zeiss, KS400), the percentage of the area stained by LFB to the total nerve fiber area was calculated in both groups (16,17).

Histochemical assessment of the expression of nerve-specific proteins after peripheral nerve injury. Immunofluorescence staining was performed to assess the expression of nerve-specific proteins after peripheral nerve injury, as described previous by Goto *et al* (4). Tissue sections were prepared by cutting the DRG at a thickness of 3 μm. Samples were deparaffinized and autoclaved at 121˚C for 10 min for antigen retrieval. After a treatment with True View™ (SP-8400, Vector) to suppress autofluorescence, samples were blocked using 2% bovine serum albumin (A2153; Sigma-Aldrich; Merck KGaA) in PBS containing 0.05% Tween-20 (PBS-Tween) for 30 min. Samples were then reacted with antibodies against the target proteins at 4˚C for 15 h. After washing with PBS-Tween, a goat anti-mouse IgG antibody labeled with Alexa Fluor 488 (A11001; Thermo Fisher Scientific, Inc.) 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/NRSF, a transcription factor that regulates the expression of nerve-specific proteins, neurotrophin 3 (NT3), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF), which are neurotrophic factors, and semaphorin 3A (Sema3A), an axon guidance factor. The following antibodies were obtained from commercial sources: rabbit polyclonal anti-REST/NRSF (1:200, 22242-1-AP; ProteinTech), rabbit polyclonal anti-NT3 (1:50, 12369-1-AP; ProteinTech), rabbit polyclonal anti-BDNF (1:1,000, GTX132621; GNT), rabbit polyclonal anti-NGF (1:50, ab6199; Abcam), and rabbit polyclonal anti-Sema3A (1:50, ab23393; Abcam).

In the photon counting mode, fluorescence intensity was measured at 20 randomly selected sites from the perikaryon in a region of interest (ROI) set in a fluorescence-emitting area, and mean fluorescence intensity was calculated. Fluorescence intensity measured using each antibody was compared between the four groups.

Statistical analysis. Data are presented as the mean ± standard deviation (SD) and were analyzed for significant differences using a two-way ANOVA with age and nerve injury set as two independent variables (Prism 7; GraphPad Software). After the two-way ANOVA, Turkey's multiple comparisons test was used as a post hoc test. Differences were considered to be significant at $P<0.05$.

Results

In the sciatic nerve, the percentage of the area of the myelin sheath to the total nerve fiber area has been used to assess Wallerian degeneration in peripheral nerve injury (16). LFB stains the myelin sheath blue and is used to assess the degree of its degeneration (Fig. 2A-D) (17). The percentages of the area

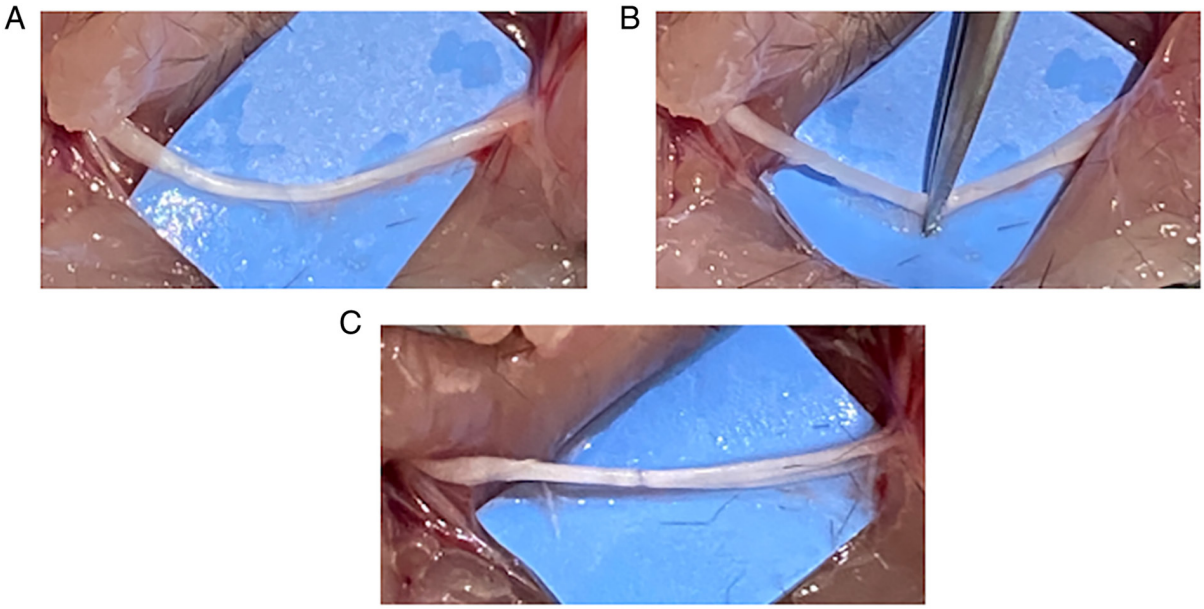

Figure 1. Surgical procedure to create the peripheral nerve injury model. (A) Mouse right sciatic nerve was dissected from the surrounding tissue. (B) The sciatic nerve was crushed for 30 sec using a hemostatic forceps. (C) The chronic constriction injury was made by a hemostatic forceps.

of the myelin sheath to the total nerve fiber area were 85.7±2.6 and 74.0±4.5% in the Young control and Aged control groups, respectively, and did not significantly differ (P=0.1891). Following peripheral nerve injury, sciatic nerve fiber swelling, decreased staining by LFB, and vacuolation were observed in both the Young and Aged crush groups (Fig. 2B and D). The percentages of the area of the myelin sheath to the total nerve fiber area were 39.9±22.1 and 41.2±7.8% in the Young and Aged crush groups, respectively, and were significantly lower in the Crush groups than in the Control groups in the Young and Aged groups (Young group: P<0.0001, Aged group: P<0.0001) (Fig. 2B, D and E).

The expression of nerve-specific proteins in DRG was quantified by immunofluorescence staining. The fluorescence intensity of REST/NRSF was significantly higher in the Aged control group (147.8±15.8) than in the Young control group (110.2±9.5) (P<0.0001) (Table I, Fig. 3A and C). Following peripheral nerve injury, the fluorescence intensity of REST/NRSF significantly increased in the Young group (Young crush group: 175.7±11.5, P<0.0001), but not in the Aged group (Aged crush group: 157.7±16.7, P=0.5739) (Fig. 3B, D and E). We then investigated the expression of the neurotrophic factors NT3, BDNF, and NGF. The fluorescence intensity of NT3 was 116.3±24.8 in the Young control group and 91.7±30.9 in the Aged control group (Fig. 4A and C), with no significant difference (P=0.2618) (Table I). Following peripheral nerve injury, the fluorescence intensity of NT3 significantly increased in the Young group (Young crush group: 155.0±27.1, P=0.0279), but not in the Aged group (Aged crush group: 104.3±28.3, P=0.7740) (Fig. 4B, D and E). The fluorescence intensity of BDNF was 123.3±39.6 in the Young control group and 116.4±38.5 in the Aged control group (Fig. 5A and C), with no significant difference (P=0.9836) (Table I). Following peripheral nerve injury, the fluorescence intensity of BDNF did not significantly differ in the Young group (Young crush group: 148.6±48.0, P=0.5583) or Aged group (Aged crush group: 123.8±35.9, P=0.9802) (Fig. 5B, D and E). The fluorescence intensity of NGF was significantly higher in the Young control group (135.1±17.2) than in the Aged control group (68.1±13.7) (P<0.0001) (Table I, Fig. 6A and C). On the other hand, following peripheral nerve injury, the fluorescence intensity of NGF did not significantly differ in the Young group (Young crush group: 132.0±19.5, P=0.9769) or Aged group (Aged crush group: 73.3±15.1, P=0.9094) (Fig. 6B, D and E). Furthermore, the fluorescence intensity of Sema3A, an axon guidance factor, was significantly higher in the Young control group (147.0±12.0) than in the Aged control group (126.5±16.1) (P=0.0266) (Table I, Fig. 7A and C). Following peripheral nerve injury, the fluorescence intensity of Sema3A significantly increased in the Young group (Young crush group: 168.5±11.3, P=0.0175), but remained unchanged in the Aged group (Aged crush group: 135.1±16.8, P=0.7631) (Fig. 7B, D and E).

Discussion

In the present study, we investigated whether the degree of Wallerian degeneration after peripheral nerve injury was affected by aging using a mouse peripheral nerve injury model. In peripheral nerves, the number and density of axons decrease with age (6,18,19). However, LFB staining in the present study showed that the percentage of the area of the myelin sheath to the total nerve fiber area did not significantly differ between the Young and Aged control groups, and aging did not markedly affect the myelination of peripheral nerves. Furthermore, following peripheral nerve injury, sciatic nerve fiber swelling, decreased staining by LFB, and vacuolation were observed in both the Young and Aged groups, and the percentage of the area of the myelin sheath was significantly lower in the Crush group than in the Control group in both the Young and Aged groups. In other words, Wallerian degeneration after peripheral nerve injury was detected in both the

Table I. Comparison of the expression of the nerve-specific proteins between Young and Aged group.

Nerve-specific proteins	Young	Aged	P-value
REST/NRSF (gray values)	110.2±9.5	147.8±15.8	P<0.0001
NT3 (gray values)	116.3±24.8	91.7±30.9	P=0.2618
BDNF (gray values)	123.3±39.6	116.4±38.5	P=0.9836
NGF (gray values)	135.1±17.2	68.1±13.7	P<0.0001
Sema3A (gray values)	147.0±12.0	126.5±16.1	P=0.0266

REST/NRSF, repressor element 1-silencing transcription/neuron-restrictive silencer factor; NT3, neurotrophin-3; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; Sema3A, semaphorin 3A.

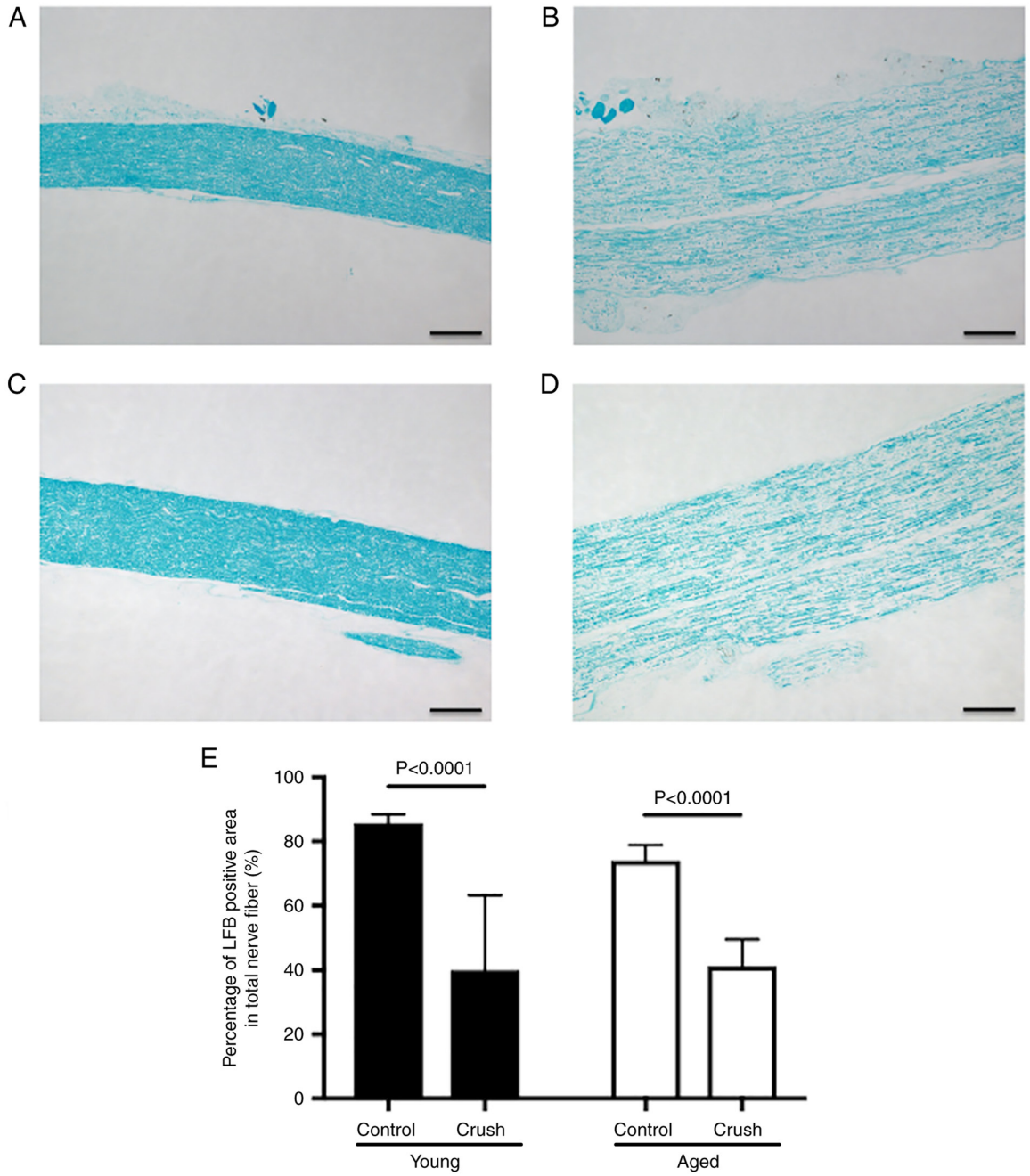

Figure 2. Histochemical assessment of the degree of Wallerian degeneration in the peripheral nerve by LFB staining. LFB assesses the degree of degeneration of the myelin sheath. The longitudinal axis of the sciatic nerves in the Young group (10 weeks) and Aged group (70 weeks) were examined. (A) Young control group; (B) Young crush group; (C) Aged control group; (D) Aged crush group; (E) Comparison of the degree of Wallerian degeneration after peripheral nerve injury in the Young and Aged groups. Scale bar, 200 μm. LFB, Luxol fast blue.

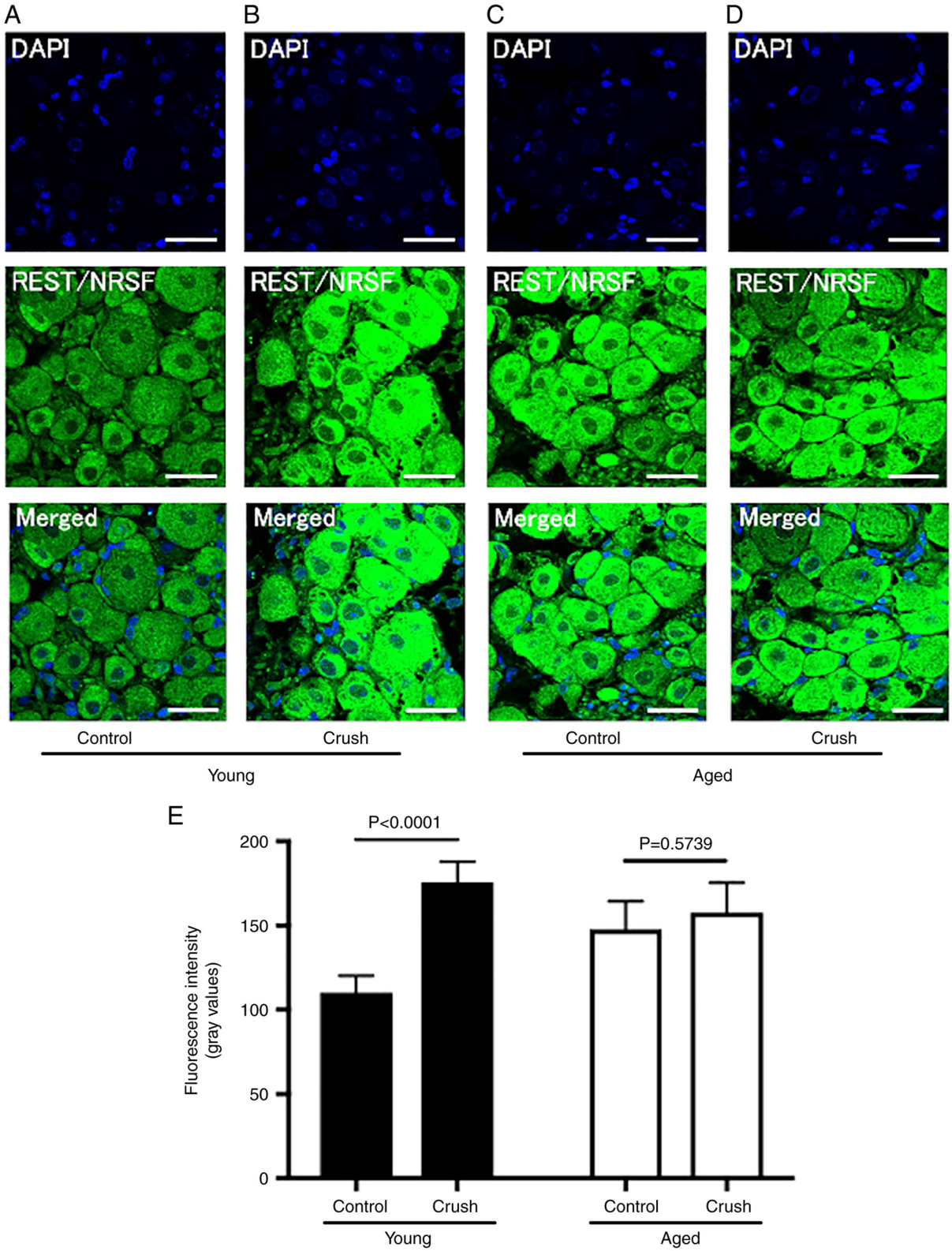

Figure 3. Histochemical assessment of the expression of REST/NRSF in the DRG by immunofluorescence staining. The expression of nerve-specific proteins was quantified by immunofluorescence staining using a REST/NRSF antibody. DRG in the Young group (10 weeks old) and Aged group (70 weeks old) were used and fluorescence intensity was compared. REST/NRSF stained green; DAPI stained blue; scale bar, 20.0 μm. (A) Young control group. (B) Young crush group. (C) Aged control group. (D) Aged crush group. (E) The fluorescence intensity of REST/NRSF. DRG, dorsal root ganglion; REST, repressor element 1-silencing transcription factor; NRSF, neuron-restrictive silencer factor.

Young and Aged groups. However, the effects of aging on axon regeneration after peripheral nerve injury currently remain unknown (20-22).

Peripheral nerve injury always induces inflammation. It is a complex series of molecular and cellular events through the recruitment of circulating proteins and leukocytes to the injury

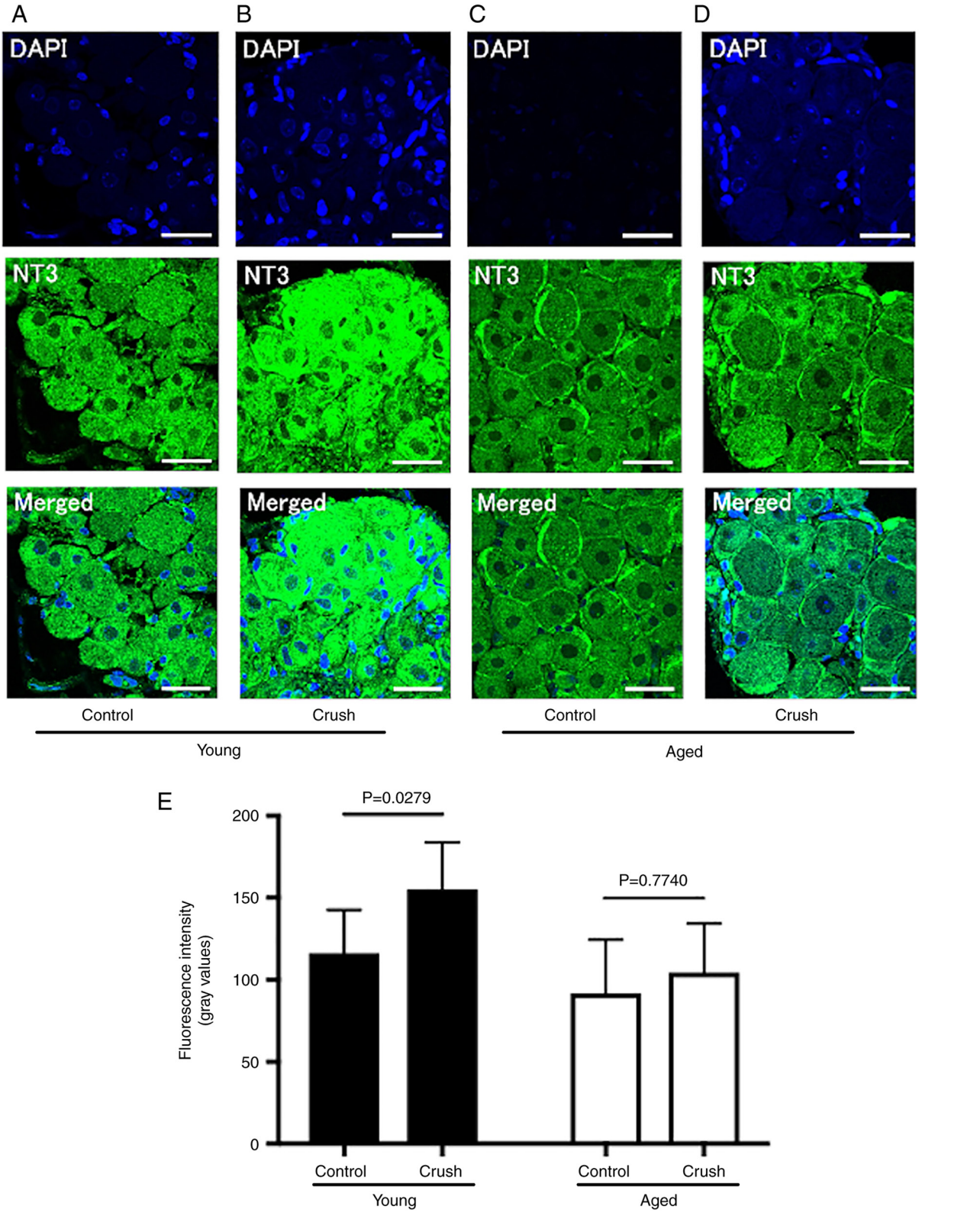

Figure 4. Histochemical assessment of the expression of NT3 in DRG by immunofluorescence staining. The expression of nerve-specific proteins was quantified by immunofluorescence staining using a NT3 antibody. In the present study, DRG in the Young group (10 weeks old) and Aged group (70 weeks old) were used and fluorescence intensity was compared (NT3 stained green; DAPI stained blue; scale bar, 20.0 μm). (A) Young control group. (B) Young crush group. (C) Aged control group. (D) Aged crush group. (E) The fluorescence intensity of NT3. NT3, neurotrophin 3; DRG, dorsal root ganglion.

site within hours to days after peripheral nerve injury (23). These reactions associated with inflammation are thought to have a significant effect on axon regeneration (24,25). To investigate the effects of aging on axon regeneration after peripheral nerve injury, we quantified the expression of nerve-specific proteins in young and aged mice with similarly degenerated peripheral nerves. In peripheral nerve injury, the expression of most of the genes required for axon regeneration and synaptogenesis is

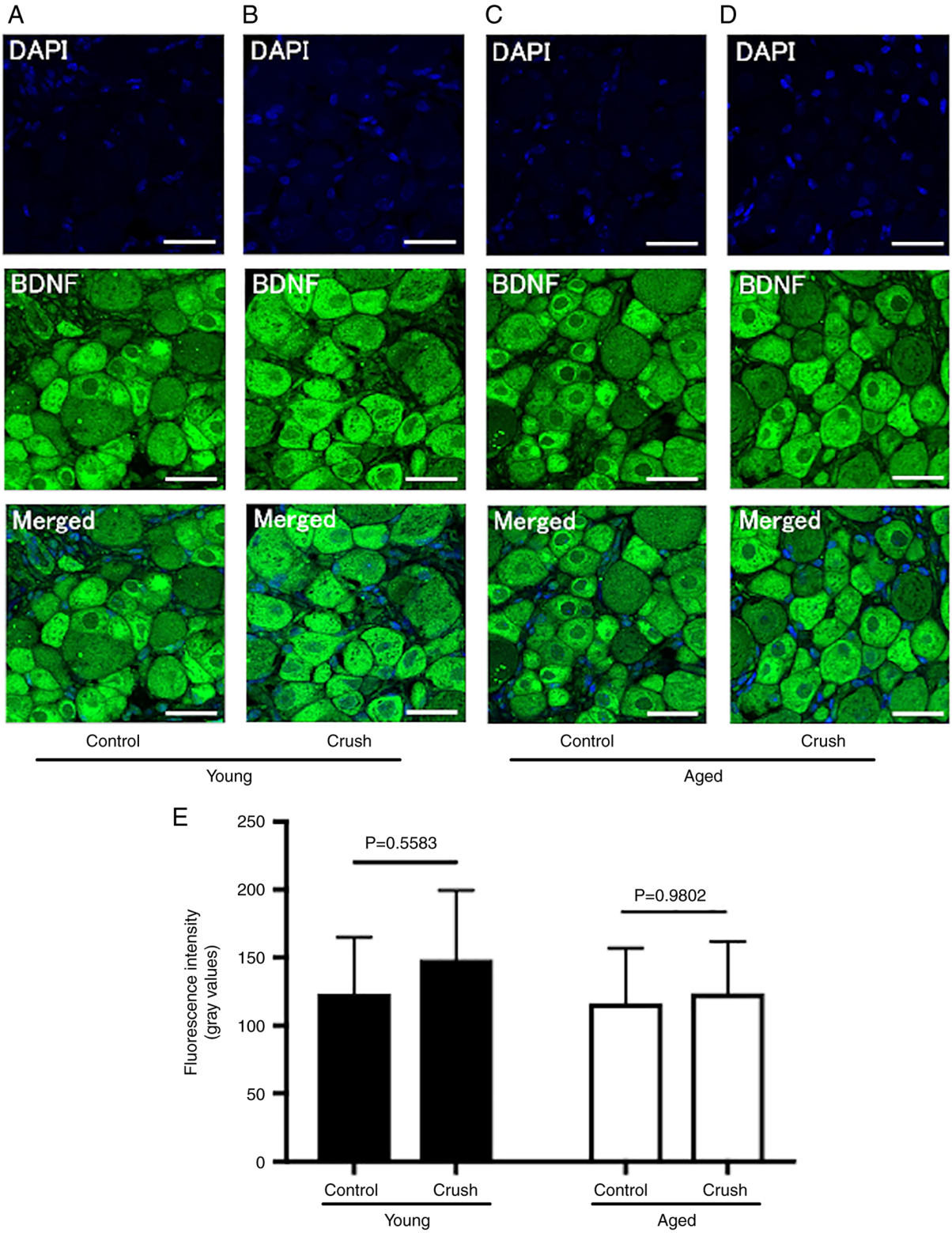

Figure 5. Histochemical assessment of BDNF expression in DRG by immunofluorescence staining. The expression of nerve-specific proteins was quantified by immunofluorescence staining using a BDNF antibody. In the present study, DRG in the Young group (10 weeks) and Aged group (70 weeks) were used and fluorescence intensity was compared (BDNF stained green; DAPI stained blue; scale bar, 20.0 μm). (A) Young control group. (B) Young crush group. (C) Aged control group. (D) Aged crush group. (E) The fluorescence intensity of BDNF. BDNF, brain-derived neurotrophic factor; DRG, dorsal root ganglion.

regulated by REST/NRSF (26-28), and REST/NRSF possesses a wide number of functions through its regulation of more than 1000 target genes (3,29-31). In the central nervous system, the expression of REST/NRSF was shown to increase with age, and maintained homeostasis by suppressing the apoptosis of neurons (1-3). We also previously demonstrated that the expression of REST/NRSF increased with age in peripheral nerves (4). In the present study, the expression of REST/NRSF was significantly higher in the Aged control group than in the Young control group, consistent with the previous studies.

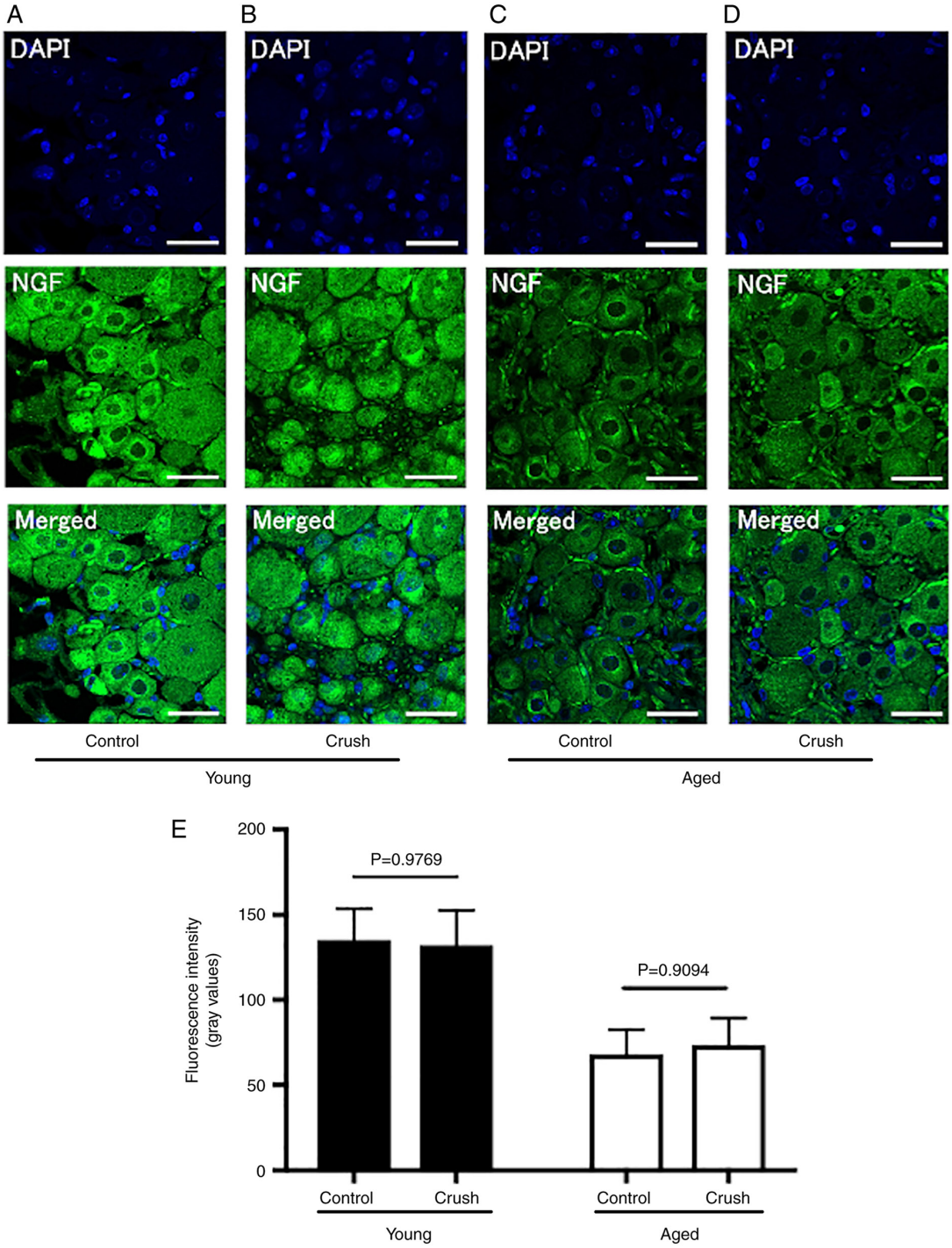

Figure 6. Histochemical assessment of the expression of NGF in DRG by immunofluorescence staining. The expression of nerve-specific proteins was quantified by immunofluorescence staining using a NGF antibody. In the present study, DRG in the Young group (10 weeks) and Aged group (70 weeks) were used and fluorescence intensity was compared (NGF stained green; DAPI stained blue; scale bar, 20.0 μm. (A) Young control group. (B) Young crush group. (C) Aged control group. (D) Aged crush group. (E) The fluorescence intensity of NGF. NGF, nerve growth factor; DRG, dorsal root ganglion.

The up-regulated expression of REST/NRSF after peripheral nerve injury has also been demonstrated (32-35), and indicates the initiation of axon regeneration in the injured peripheral nerve (32). In the peripheral nerve injury model used in the present study, the expression of REST/NRSF was significantly increased in the Young group, but not in the Aged group, suggesting that axon regeneration was initiated immediately after peripheral nerve injury in the Young group, but not in the Aged group.

The expression of neurotrophic factors has been shown to play an important role in axon regeneration after peripheral nerve injury (8,9). NT3 is a necessary factor for Schwann cell survival and differentiation in the absence of axons (9,36), BDNF maintains peripheral nerve homeostasis by regulating neuron survival maintenance, neurite outgrowth promotion, and synaptogenesis (9,37-40), and NGF promotes axon elongation in peripheral nerves (41-44). Therefore, the neurotrophic factors investigated in the present study are markers for

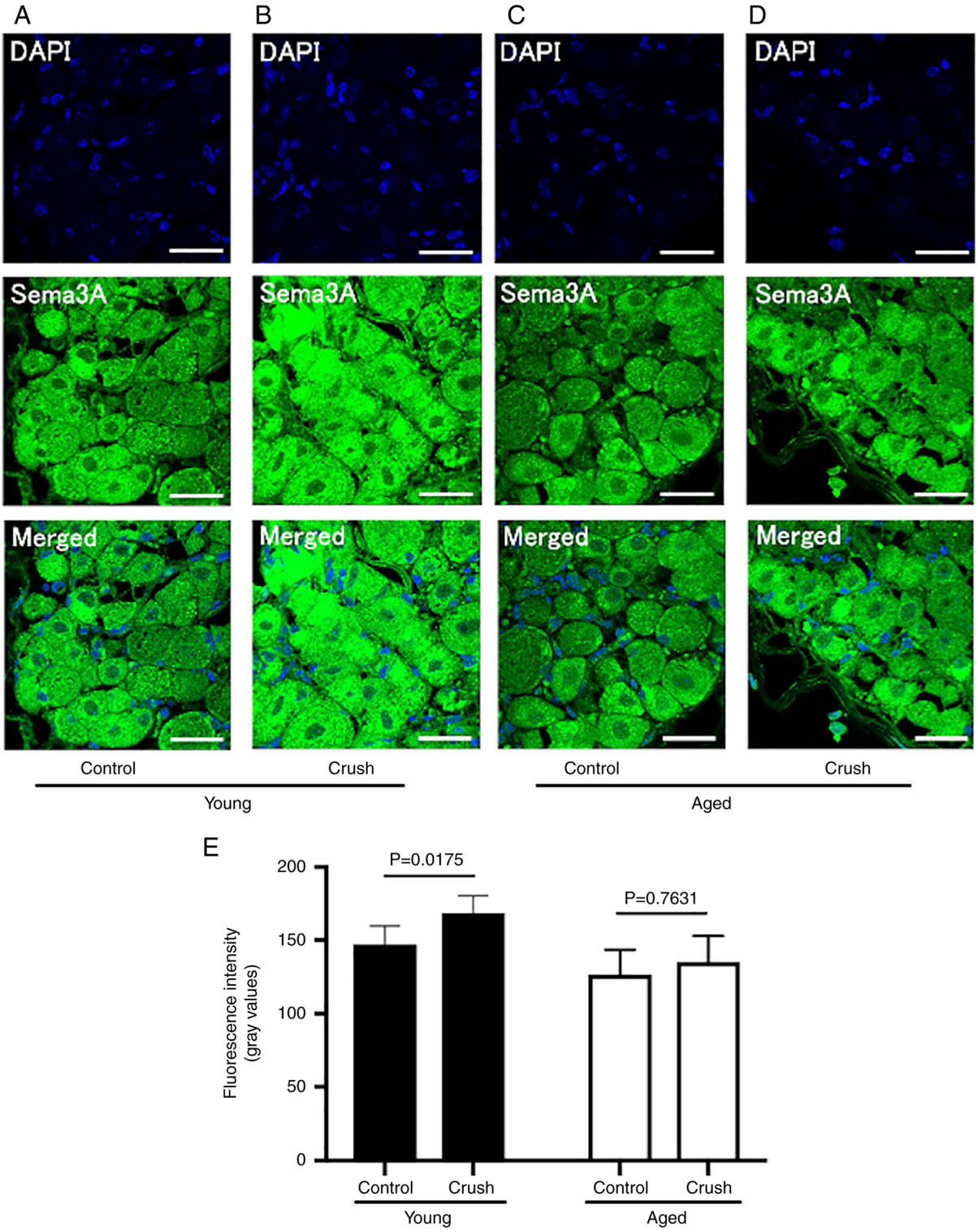

Figure 7. Histochemical assessment of the expression of Sema3A in DRG by immunofluorescence staining. The expression of nerve-specific proteins was quantified by immunofluorescence staining using a Sema3A antibody. In the present study, DRG in the Young group (10 weeks) and Aged group (70 weeks) were used and fluorescence intensity was compared (Sema3A stained green; DAPI stained blue; scale bar, 20.0 μm. (A) Young control group. (B) Young crush group. (C) Aged control group. (D) Aged crush group. (E) The fluorescence intensity of Sema3A. Sema3A, semaphorin 3A; DRG, dorsal root ganglion.

Schwann cell migration (NT3), myelination (BDNF), and axon elongation (NGF). To investigate the effects of aging on the expression of these neurotrophic factors, their expression was compared between the Young and Aged control groups. The results obtained showed no significant differences in the expression of NT3 or BDNF between these groups, while the expression of NGF was significantly lower in the Aged control group than in the Young control group, suggesting that Schwann cell migration may not be affected by aging in the Control group in the presence of axons. Furthermore, the results on the expression of BDNF suggest that myelination was not affected by aging. However, the results on the

expression of NGF indicate that axon elongation decreased with aging. These results are consistent with our previous findings showing that the ability to regenerate axons in peripheral nerve is decreased with age (7). Moreover, we investigated changes in the expression of neurotrophic factors characterized by age-related changes in the Young and Aged groups following peripheral nerve injury. In comparisons with the respective Control groups, the expression of BDNF and NGF did not significantly differ between the Young and Aged crush groups, while the expression of NT3 significantly increased in the Young group, but not in the Aged group. In other words, Wallerian degeneration occurred and axons were absent in injured peripheral nerves, axon regeneration was initiated by the increased expression of REST/NRSF, and the migration of Schwann cells, which is the initial stage of axon regeneration, was induced by the upregulation of NT3 in the Young group. However, based on the results for the expression of REST/NRSF and NT3, axon regeneration was not initiated in the Aged group following peripheral nerve injury. The expression of BDNF and NGF, which have been suggested to play a role in myelination and axon elongation after Schwann cell migration for axon regeneration, was not increased in the Young or Aged group one week after peripheral nerve injury in the present study.

There are several limitations in this study. First, in creating the CCI model for peripheral nerve injury, the compression method with a hemostatic forceps was used, so the degree of nerve injury may be different with each mice. It has been reported that the expression of neurotrophic factor varies depending on the degree of nerve injury (Neurapraxia, Axonotmesis, Neurotmesis) (9). However, there were no complication (death, disability, etc). Next, in this study, we performed evaluation the expression of nerve-specific proteins such as REST/NRSF and discussed how they affect axon regeneration. On the other hand, although inflammatory cytokines are up-regulated with aging or nerve injury (45,46), age-related differences in inflammatory cytokine levels after peripheral nerve injury are unclear (47). However, the degree of inflammation after nerve injury was not investigated in this sturdy. In a future study, to investigate the degree of inflammation and age-related differences in inflammatory cytokine levels after peripheral nerve injury may provide a more detailed understanding of the effects of aging on axon regeneration. Moreover, we also investigated the expression of nerve-specific proteins in the cytoplasm and the nucleus in the perikaryon of DRG, but there was no significant difference among them. Therefore, the fluorescence intensity of the whole cell was measured by the method of this study. Therefore, since all DRG samples were used by histochemical assessment in this study, the quantification of nerve-specific proteins by western blot were not able to be performed. This is the limitation of this study and a future issue. Last, since this study is a fixed-point observation one week after peripheral nerve injury, it only evaluates the effect of peripheral nerve injury and aging in a limited manner. It has been reported that the expression of nerve-specific proteins varies greatly depending on the time after nerve injury (9,48-51). We believe that it may be possible to evaluate the effect of peripheral nerve injury and aging in more detail by evaluating with time course. However, in assessment of the axon regeneration process, it is highly meaningful to investigate the expression of these nerve-specific proteins at one week after peripheral nerve injury. Because the up-regulated expression of neurotrophic factors and Schwann cell migration, an early stage of axon regeneration, occur within one week after nerve injury (52). Therefore, in this study, we performed evaluation at one week after peripheral nerve injury and discussed it.

Based on the results of present study, while compensatory changes for peripheral nerve injury were initiated by the upregulation of the REST/NRSF, followed by Schwann cell migration in the Young group, these compensatory changes did not occur in the Aged group. The regulation of REST/NRSF expression appears to be essential for axon regeneration when peripheral nerves are exposed to stress. In peripheral nerves, aging-associated functional and electrophysiological disorders have been reported in clinical study (6). This study and our previous study showed the REST/NRSF expression is increased with age (4), and the expression is also increased by nerve injury according to the results of this study. Therefore, focusing on the expression of REST/NRSF is expected to elucidate the pathology of nerve injury, and is also expected to contribute significantly to the treatment of age-related peripheral nerve injury.

In conclusion, the present results suggested that Wallerian degeneration occurred after peripheral nerve injury in the Young and Aged groups. On the other hand, compensatory changes for peripheral nerve injury and Schwann cell migration were initiated in the Young group, but not in the Aged group.

Acknowledgements

Not applicable.

Funding

No funding was recieved.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HO mainly wrote the manuscript and acquired, analyzed and interpretated the data. KN wrote the manuscript and made substantial contributions to conception and design of the study, and interpretation of data. SN and KS contributed to acquisition, analysis and interpretation of data. SK, TS KG, AK, NN and YS contributed to acquisition of data. SK and TS confirm the authenticity of all the raw data. IN made substantial contributions to conception and design. MI contributed to the analysis and interpretation of data. All authors 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. 2021312).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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