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Changes in Type II NRG-1 Expression during Regeneration Following Autologous Nerve Transplantation in Rats

Ya-Kang WANG[1], Wei-Xia YANG[2], Rui LIU[1], Jian-Bin GUO[1], Yu-Min ZHANG[1], Kun LI[1], Yao LU[1], Junning WANG[3]

[1]Xi'an Jiaotong University, Honghui Hospital, Department of Orthopaedics, Xi'an, PR China
[2]Worker's Hospital of Aecc Xi'an Aero-Engine LTD, Department of Pathology, Xi'an, PR China
[3]Xi'an Jiaotong University, Honghui Hospital, Department of Respiratory and Gastroenterology, Xi'an, PR China

Corresponding author: Junning WANG, Yao LU ✉ wangjunningde@163.com, drluyao@163.com

ABSTRACT

AIM: To investigate the changes in type II neuregulin-1 (NRG-1) during the regeneration process following autologous sciatic nerve transplantation in rats.

MATERIAL and METHODS: In total, 40 healthy male Sprague-Dawley (SD) rats of clean grade with body weights between 250 g and 300 g were randomly divided into an experimental and control group, with 20 rats per group. Five time points were set, including the 3rd, 7th, 14th, 21st and 28th days after surgery. In the experimental group, reversed autologous transplantation of the sciatic nerve was performed, while in the control group, the sciatic nerve was simply exposed without autologous transplantation. At the different time points, changes in the rat footprints were observed, the sciatic functional index (SFI) was calculated, changes in the regeneration of the myelin sheath at the nerve end after transplantation were observed by transmission electron microscopy, changes in type II NRG-1 protein expression were detected by a western blot analysis, and changes in type II NRG-1 mRNA expression were detected by real-time PCR.

RESULTS: The SFI in the experimental group was lower than that in the control group at all time points after surgery, and the SFI in the experimental group gradually increased; these differences were statistically significant ($p<0.05$). The expression of type II NRG-1 protein in the experimental group was significantly increased on the 3rd day after nerve transplantation and peaked on the 7th day, which continued until the 28th day after surgery, indicating a significant difference from the control group ($p<0.01$). NRG-1 mRNA expression was markedly increased on the 7th day after nerve transplantation, further increased, and peaked on the 14th day ($p<0.01$). The area of medullated nerve fibers (μm^2) in the experimental group significantly differed from that in the control group on the 7th, 14th, 21st and 28th days ($p<0.01$), and the diameters of the axons in the experimental group notably differed from those in the control group on the 7th, 14th and 21st days ($p<0.01$).

CONCLUSION: Type II NRG-1 expression peaked between the 3rd day and 14th day after autologous nerve transplantation and is likely involved in the regulation of myelin sheath regeneration during this period.

KEYWORDS: Neuregulin-1 (NRG-1), Myelin sheath, Regeneration

INTRODUCTION

Neuregulin-1 (NRG-1) is a nutrient factor that contains an epidermal growth factor-like domain and mainly exists in nerve tissue, especially neurons, where it is concentrated in axons, presynaptic membranes and growth cones; NRG-1 is also found in neuroglial cells and is closely related to synaptic plasticity, neural development and regeneration (20). According to the different structures of its N-terminus, NRG-1 can be divided into type I, II, III, IV, V, and VI and other subtypes. Type II NRG-1 is also known as glial

Ya-Kang WANG : 0000-0002-1403-9032
Wei-Xia YANG : 0000-0001-6729-913X
Rui LIU : 0000-0002-2422-8232
Jian-Bin GUO : 0000-0002-9956-4097
Yu-Min ZHANG : 0000-0003-2878-1024
Kun LI : 0000-0002-6165-6105
Yao LU : 0000-0002-5345-5231
Junning WANG : 0000-0002-7920-1010

growth factor (GGF); its N-terminus has an immunoglobulin-like domain that can be turned into soluble proteins by enzyme digestion and then separated and released from the surface of nerve cells (7,8,14). Type II NRG-1 can activate various enzymes inside and outside of the cell by binding to ErbB2 and ErbB3 receptors and causing further cascades, amplifying and activating multiple types of signaling pathways, such as the Ras and calmodulin signaling pathways, ultimately regulating cell proliferation, apoptosis, migration, regeneration and differentiation (9,23,30).

Recent studies have shown that type II NRG-1 plays an important role in the growth of myelin sheaths during peripheral nerve development and has a significant effect on the regeneration and repair of myelin sheaths after nerve injury (20,28,32). In a model of spinal nerve ligation, it was found that the mRNA expression of type II NRG-1 was significantly higher than that in the contralateral normal nerve, but there was no significant difference over time, suggesting that type II NRG-1 may play an initial role in the early repair of nerve injury (16). In vitro experiments have revealed that soluble type II NRG-1 has dual functions; it can promote the process of myelination at low doses but inhibit the process of myelination at high doses (28). Through in vivo studies using a nerve injury model, the authors found that local injections of synthetic type II NRG-1 could promote the recovery of injured nerve function, as represented by increases in the quantity, diameter, and thickness of the myelin sheath and an increase in the sciatic nerve index (5). In addition, type II NRG-1 biomaterials synthesized by tissue engineering could promote recovery from nerve injury (13,22,24).

However, there have been no relevant reports exploring the effect and role of type II NRG-1 after autologous nerve transplantation. In this study, autologous sciatic nerve transplantation was applied as a model to observe the changes in the expression of type II NRG-1 and the morphology of the sciatic nerve myelin sheath during regeneration of the transplanted nerve. Moreover, the functional recovery of injured limbs in rats was evaluated, and the possible effects of type II NRG-1 on the regeneration of the myelin sheath in autologous nerve transplantation were explored.

MATERIAL and METHODS

Preparation of an Animal Model of Autologous Nerve Transplantation

In total, 40 healthy male Sprague-Dawley (SD) rats of clean grade weighing 250-300 g were provided by the animal experimental center of Xi'an Jiaotong University and randomly divided into an experimental group and a control group, with 20 rats per group. Five time points were set, namely, the 3rd, 7th, 14th, 21st and 28th days after surgery, and 4 rats per group were tested at each time point. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Honghui Hospital affiliated with Xi'an Jiao Tong University School of Medicine (approval no. 201901004).

First, the 20 SD rats in the experimental group underwent autologous nerve transplantation, and the sciatic nerve was exposed in the 20 SD rats in the control group. An intraperitoneal injection of 1% pentobarbital sodium at 40 mg/kg was administered for anesthesia in the experimental group. Then, skin preparation and routine disinfection were performed. A sideling incision of approximately 1.5 cm was created at the lower margin of the left ischial tuberosity, and then, the sciatic nerve could be visualized after muscle separation. Blunt dissection was performed, and 1 cm of the sciatic nerve was incised on the lower margin of the piriformis. After 180° of inversion, 11-0 epineurial sutures were placed for nerve anastomosis under a microscope at tenfold magnification (Figure 1A). After surgery, the sarcolemma and skin were sutured successively, and a local muscle injection of 800,000 units of penicillin was administered to diminish inflammation. The rats were housed in separate cages. In the control group, the sciatic nerve was exposed without any injury, and the rest of the procedure was the same as that in the experimental group.

Footprint Collection and Calculation of the Sciatic Functional Index (SFI) After Transplantation

On the 3rd, 7th, 14th, 21st, and 28th days after surgery, four rats per group were selected, and the soles of their hind feet were coated with blue ink. Then, the rats were placed on a track with white paper in a blind lane with a length of 75 cm, width of 10 cm, and height of 15 cm, and the bilateral footprints of the rats were recorded. At least 5 complete sets of footprints were recorded per experimental animal, and the SFI was measured and calculated by electronic scanning and PRISM 6.0 software. The formula was SFI=(-38.3×(EPL-NPL)/NPL)+(109.5×(ETS+NTS)/NTS)+(13.3×(EIT-NIT)/NIT)-8.8, where EPL and NPL represent the distance between the heel and the tip of the third toe, ETS and NTS represent the distance between the first toe and the fifth toe, and EIT and NIT represent the distance between the second toe and the fourth toe. E (experimental limb) represents the footprint on the operated side (left hind foot), and N (nonoperated limb) represents the contralateral nonoperated footprint (right hind foot) (Figure 1B) (1).

After the footprints were measured, intraperitoneal anesthesia was administered to four rats per group on the 3rd, 7th, 14th, 21st and 28th days after surgery. Then, the intermediate segment of the operated transplanted sciatic nerve was cut into three sections, each 0.3 cm long. One section was placed in 0.1 M alkaline phosphate buffer for observation by electron microscopy; the other two sections were placed at -80° and prepared for analysis by western blot and RT-PCR.

Regeneration of the Myelin Sheath Observed by Transmission Electron Microscopy

Sciatic nerve samples were prepared, and 1 mm^3 of tissue was cut from each group, fixed for 2-4 hours with 3% glutaraldehyde and 0.1 M phosphate buffer, rinsed with 0.1 M phosphate washout fluid and fixed with 1% osmium tetroxide. Then, the samples were dehydrated with 50%, 70%, 90% and 100% acetone successively. After dehydration, the samples were soaked at room temperature in 100% acetone, embedded in solution (1:1) and solidified in an oven. Then,

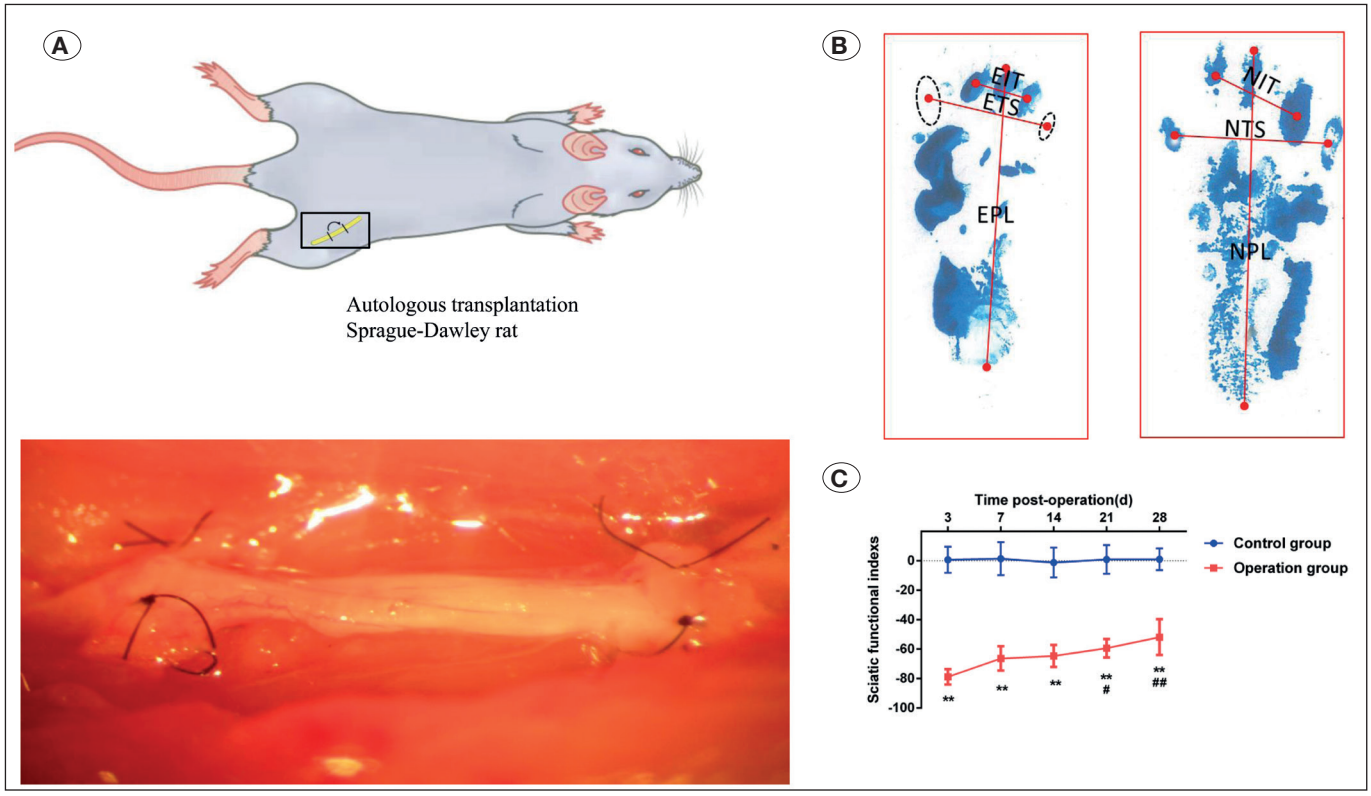

Figure 1: A) Surgical method used for autologous sciatic nerve transplantation in the experimental group. The rat hind limb was dissected, and the sciatic nerve was exposed, reversed 180° and repaired. Two 11-0 epineurial sutures were placed at the bottom under a microscope at tenfold magnification. **B)** SFI acquisition and measurement method. Footprint on the operative side (left hind limb); the dotted ellipse is the footprint of the rat that is not fully stepped on the center of the ellipse and is taken as the starting point of the measurement. Footprint on the contralateral side (right hind limb). **C)** Change in the SFI between the 3rd and 28th days after surgery. ** $p<0.01$ for the comparison between each time point in the experimental group and the control group; # represents the different days versus the 3rd day in the groups; # $p<0.05$, ## $p<0.01$.

sectioning at 50 nm was performed with an ultrathin slicer, followed by double staining with 3% uranyl acetate-lead citrate. Finally, the following indicators were observed and recorded during transmission electron microscopy: 1) area of regenerated nerve; 2) number of nerve fibers per unit area (number/339.75 μm^2); 3) average diameter of medullated nerve fibers; and 4) degree of myelination of medullated nerve fibers (G-ratio, the ratio of the diameter of the axon to the diameter of the nerve fibers).

Variable Expression of type II NRG-1 Detected by Western Blot Analysis

The prepared sciatic nerve was minced, ground, and lysed, and then, tissue protein was extracted. The protein content was determined by a bicinchoninic acid assay. Membrane transfer was carried out by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a polyvinylidene fluoride filter membrane. Then, primary and secondary antibodies were added to conduct a chemiluminescence reaction. Finally, in a darkroom, development and fixation were performed, and the film was scanned or photographed. Then, the molecular weight and net optical density of the object band were analyzed by PRISM 6.

Variable Expression of type II NRG-1 Detected by PCR

The tissue was ground, weighed and loaded into a centrifuge tube. TRIzol reagent was added, and successively, 0.2 ml of chloroform and isopropanol were applied to extract the total RNA. Diethylpyrocarbonate (DEPC)-treated water was added for dilution, and the sample concentration was determined by UV spectrophotometry. In addition, the optical density at 260 and 280 nm (OD260 and OD280) were determined, and cDNA was synthesized by reverse transcription.

The primer sequences are as follows:

5'-3': GCAACCTCAAGAAGGAGGTC;

3'-5': CGTTCCCATTCTTGAACCAT (16).

Real-time fluorescence quantitative PCR was conducted by thermal cycling. The data were subsequently exported, and the amount of target cDNA was determined by the number of cycle times (Ct) of the threshold amplification calculated by the ABI Sequence Detection System software. The difference between the Ct value of the target gene and that of the internal reference gene, i.e., GAPDH, was the relative Ct value, and the relative DNA content was calculated by the following formula: average relative content=$2^{-\text{average } \Delta\Delta CT}$.

Statistical Methods

IBM SPSS Statistics 20 and Prism 16 software packages were used for the statistical analysis. The data are expressed as the mean±standard deviation. Comparisons between groups at different time points were performed by one-way analysis of variance (ANOVA), and two-way ANOVA was applied to compare data considering the group as factor A and time as factor B (A1: autologous nerve transplantation, A2: exposed sciatic nerve).

RESULTS

Analysis of Footprints

The footprints of the rats were recorded at 5 time points, namely, the 3rd, 7th, 14th, 21st and 28th days after nerve transplantation, and the SFI of the rats was calculated to evaluate the recovery of motor function after nerve injury. The SFI at each time point in the experimental group was lower than that in the control group, with a statistically significant difference ($p<0.01$, two-way ANOVA; the time main effect was 7.382, the group main effect was 1.624, and their interaction effect was 6.698, $p<0.01$). The null hypothesis that the error variance in the dependent variable is equal across groups was tested by Levene's test of equality of error variances, and the SFI was found to gradually increase over time and was markedly increased on the 21st and 28th days after nerve transplantation ($p<0.05$, one-way ANOVA), indicating that function on the operated side recovered gradually after autologous nerve transplantation (Figure 1C).

Western Blot Analysis

The western blot analysis results demonstrated that after autologous nerve transplantation, the protein expression of type II NRG-1 in the experimental group was significantly increased compared to that in the control group at each time point ($p<0.01$, two-way ANOVA; the time main effect was 624.8, the group main effect was 17.92, and their interaction effect was 15.71, $p<0.01$). Its expression increased on the 3rd day after nerve transplantation and further increased from the 7th day until the 28th day, showing significant differences from that on the 3rd day ($p<0.01$, one-way ANOVA). However, its expression gradually decreased on the 14th, 21st and 28th days compared to that on the 7th day ($p<0.05$, one-way ANOVA, Figure 2A, B).

Real-Time PCR Analysis

Analysis of the real-time PCR results revealed that the mRNA expression of type II NRG-1 on the 7th, 14th, 21st and 28th days in the experimental group was higher than that in the control group, and a statistically significant difference was observed ($p<0.01$, two-way ANOVA; the time main effect was 206.3, the group main effect was 15.31, and their interaction effect was

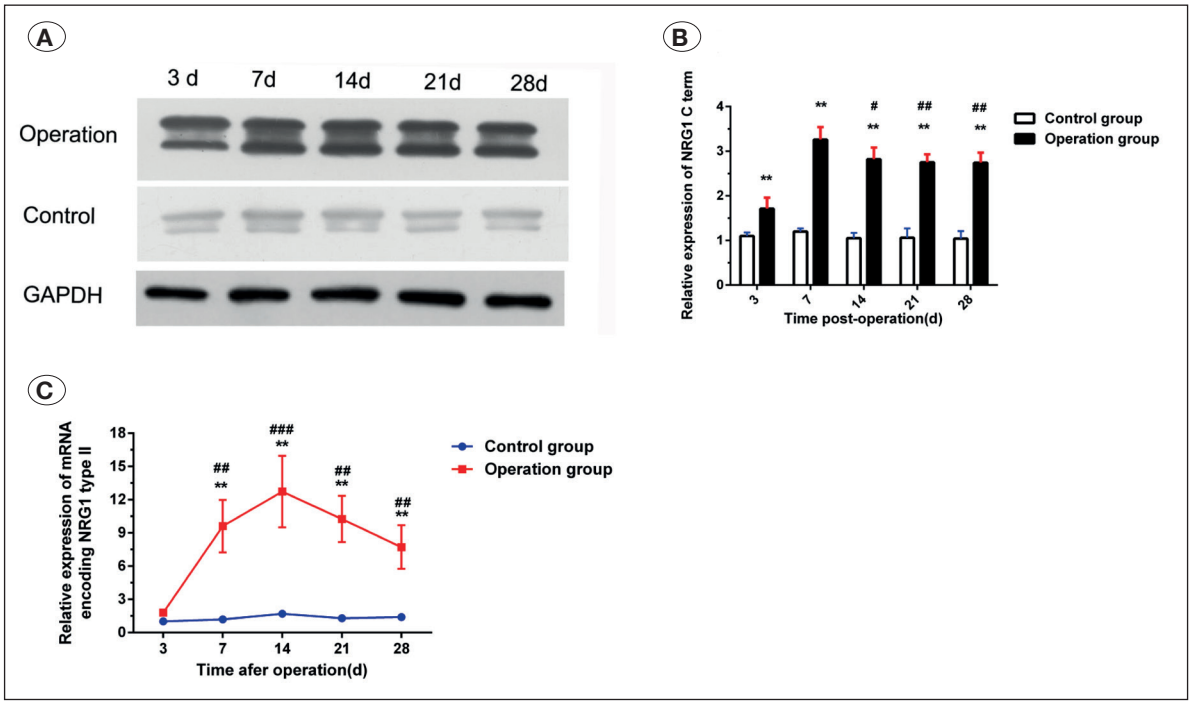

Figure 2: A) Changes in type II NRG-1 protein expression were detected by western blot analysis between the 3rd and 28th days after surgery. **B)** Semiquantitative analysis of the sciatic myelin protein levels was performed by western blot analysis and quantification by Phosphor Imager analysis. The myelin protein levels are normalized to actin, as indicated. The data represent the mean (±SEM) of two different experiments. ** $p<0.01$ for the comparison between each time point in the experimental group and the control group. # $p<0.05$, ## $p<0.01$ for the 14th, 21st and 28th days compared to the 7th day. **C)** Changes in type II NRG-1 mRNA expression were detected by real-time PCR between the 3rd and 28th days after surgery. ** $p<0.01$ for the comparison between each time point in the experimental group and the control group. ## $p<0.01$, ### $p<0.001$ for the 7th, 14th, 21st and 28th days compared to the 3rd day.

12.48, p<0.01). The mRNA expression level was also markedly increased on the 3rd day after nerve transplantation and then further increased, peaking on the 14th day. The observations on the 14th, 21st and 28th days after surgery showed that its expression was significantly increased compared with that on the 3rd day (p<0.01, one-way ANOVA, Figure 2C).

Electron Microscopy Analysis

Regarding the morphological observations by electron microscopy, on the 3rd day after transplantation, the myelin sheath encased by medullated nerve fibers began to show distortion and deformation, with macrophage infiltration, but it remained in its original form, and no disintegration occurred (Figure 3K, F). On the 7th day after surgery, extensive axon disintegration and collapse were observed; mitochondria in the axoplasm were swollen, the structure was blurred, and the formation of new myelin sheaths could be observed (Figure 3L, G). On the 14th and 21st days after nerve transplantation, the diameter of the nerve fibers became larger, the thickness of the myelin sheath was significantly increased, and surrounding Schwann cells were observed (Figure 3M, N, H, I). The diameter of the myelin sheath was increased on the 28th day, but this increase in thickness was not significant Figure 3O, G).

The area of medullated nerve fibers (μm^2) in the experimental group significantly differed from that in the control group on the 7th, 14th, 21st and 28th days (p<0.01, two-way ANOVA; the time main effect was 4.58, the group main effect was 64.83 and their interaction effect was 4.18, p<0.01), and this area in the experimental group significantly differed from that on the 3rd day (20.78 ± 7.82) at each time point (p<0.01, one-way ANOVA), indicating that the myelin sheath area gradually increased after transplantation. There were statistically significant differences in the number of medullated nerve fibers per unit area between the experimental group and the control group on the 3rd, 14th, 21st and 28th days (p<0.01, two-way ANOVA; the time main effect was 4.58, the group main effect was 10.09 and their interaction effect was 19.46, p<0.01). However, in the experimental group, only the comparison between the number obtained on the 21st day (7.00 ± 1.93) and that on the 3rd day (2.00 ± 1.00) showed a significant difference (p<0.0001, one-way ANOVA), suggesting that the number of nerve fibers did not significantly increase within 0-14 days but began to obviously increase on the 21st day. Significant differences were found in the axon diameter between the experimental group and the control group on the 7th, 14th and 21st days (two-way ANOVA; the group main effect was 65.53, p<0.01, the time main effect was 2.35, p=0.07, and their interaction effect was 3.68, p=0.015, with no significant difference). In the experimental group, only the comparison between the 7th day (0.18 ± 0.09) and the 3rd day (1.74 ± 0.69) showed a significant difference (p<0.01, one-way ANOVA), indicating that the diameter of the nerve fibers did not increase 1 week after transplantation. There were no significant differences in the G-ratio among the time points or within the experimental group (p>0.01, two-way ANOVA; the group main effect was 3.27, p=0.07, the time main effect was 1.03, p=0.4, and their interaction effect was 1.13, p=0.35, with no significant differences). Because this study was an observational experiment, significance could not be assessed. The above index changes are shown in Figure 3.

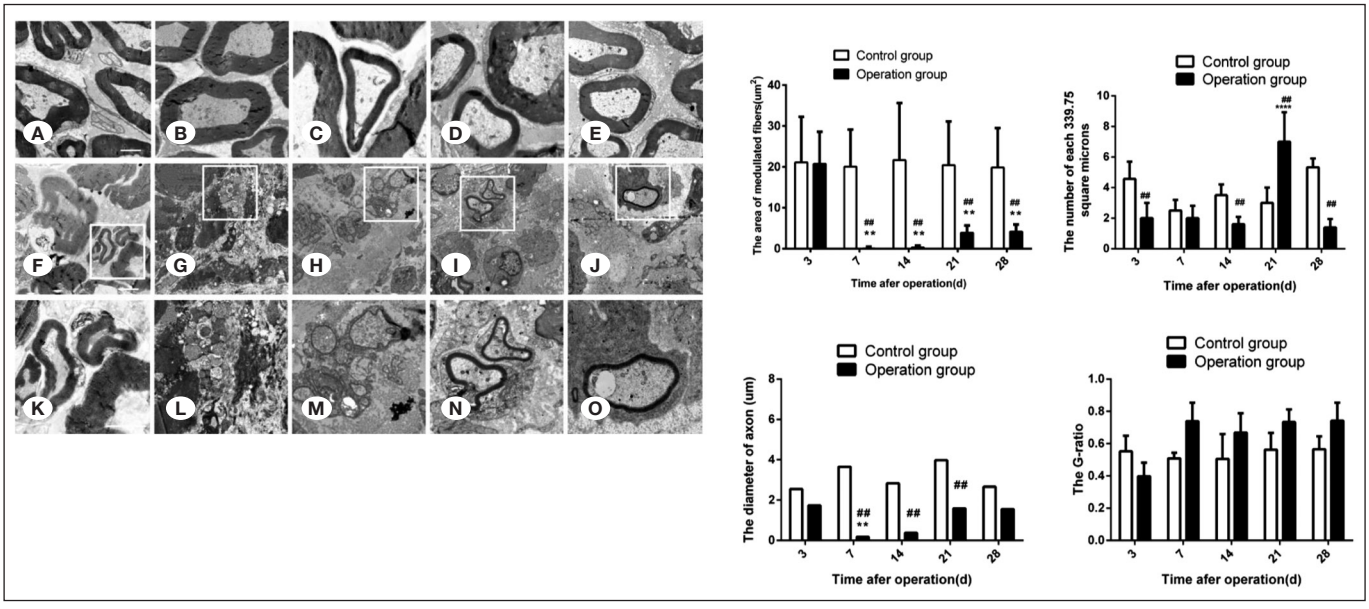

Figure 3: Transmission electron microscopy results. All scale bars are 2 microns. **K-O** are magnified 15,000 times, **A-J** are magnified 8,000 times. **Experimental group: K, F** (3rd day); **L, G** (7th day); **M, H** (14th day); **N, I** (21st day); **O, J** (28th day). **Control group: A** (3rd day), **B** (7th day), **C** (14th day), **D** (21st day), **E** (28th day). Bar chart of the electron microscopy results: * represents the comparison of the index between different time points and that on the 3rd day in the experimental group; # represents the comparison between the experimental group and the control group; **, ## p<0.01, **** p<0.0001.

DISCUSSION

Studies conducted in recent years have shown that in the rat sciatic nerve extrusion model, type II NRG-1 shows high expression and performs a positive regulatory function (3,10,11,25), which can cause an increase in the myelin sheath thickness, number and diameter and an increase in the SFI (5), and inhibit Schwann cell apoptosis (31). However, the expression and function of type II NRG-1 in autologous nerve transplantation remain unknown. Therefore, in this study, the authors established a rat model of autologous nerve transplantation and performed western blot, real-time PCR, footprint ichnology and electron microscopy morphology analyses to observe the changes in the expression of type II NRG-1 protein and mRNA in autologous transplantation, evaluate the SFI, observe and analyze myelin sheath regeneration, and explore the effect of type II NRG-1 on regeneration after autologous nerve transplantation.

In the process of myelin sheath regeneration, Schwann cells undergo two stages of differentiation and eventually regenerate into the myelin sheath. The first stage consists of the degeneration and disappearance of the axon, which peaks three days after nerve injury (4,15,26,27). The second stage consists of the axon entering the stump of denervation. NRG-1 promotes the growth and differentiation of Schwann cells when the cells regenerate into the myelin sheath (2,12). NRG-1 works mainly during the period from the 3rd to 30th day after injury (4,6,18). Hence, the experimental observation period in this study was from the 3rd day to the 28th day after transplantation. In this experiment, degeneration of the primary axon, phagocytosis and clearing of macrophages, and initial myelin formation in Schwann cells were observed on the 7th day after transplantation. Coincidentally, the mRNA and protein expression of type II NRG-1 also began to peak from the 7th to 14th days. Therefore, it was preliminarily speculated that type II NRG-1 becomes active on the 7th day after neural transplantation and plays a critical regulatory role.

In the footprint analysis, the SFI began to increase after 1 week, showing that the hind limb function gradually recovered, while the corresponding western blot analysis indicated that the protein content of type II NRG-1 began to increase on the 3rd day, reached the peak value after 1 week and then remained at a certain level. No other changes occurred, but this effect gradually weakened in the later stages. Furthermore, the mRNA expression of type II NRG-1 peaked and then gradually decreased in the later stages, indicating that type II NRG-1 may play a role in the initial stage of recovery from neural transplantation, similar to the process of recovery after nerve injury (10). In addition, type II NRG-1 was released through the cell surface as a soluble protein and bound the ErbB receptor to elicit its effects, which may be closely related to its structure and function. Type II NRG-1 is gradually degraded by various secretory enzymes (TACE, BACE, ADAM10 and ADAM19) in the later stages (19,29).

A knockout mouse experiment demonstrated that type I and type III NRG-1 regulate the process of myelin regeneration and positively regulate the thickness and diameter of the myelin sheath (21,25), although the specific regulatory effect of type II NRG-1 is still unclear. In this experiment, the changes in the thickness of the myelin sheath were the most obvious between the 7th day and 21st day through observation by electron microscopy, indicating that the changes in the regeneration of myelin mainly occurred between the 14th and 21st days, which is similar to the results of the research conducted by Kataria et al. (17), which showed that the peak of type II NRG-1 protein expression also occurred during this stage. Moreover, according to the measurement and analysis, the diameter and area of the myelin sheath in the neural transplantation model significantly differed from those in the control group on the 7th, 14th and 21st days, but the changes in the myelin sheath were not obvious from the 21st to 28th day. Additionally, the number of myelin sheaths mainly increased from the 14th to 21st day, which might also be related to the high expression of type II NRG-1 at this stage, or type II NRG-1 may have a specific regulatory effect on one specific indicator; however, this speculation has yet to be proven by gene knockout or other experimental techniques.

CONCLUSION

The number of myelin sheaths mainly increased from the 14th to 21st days.Type II NRG-1 peaked between the 3rd day and 14th day after autologous nerve transplantation and was probably involved in the regulation of myelin sheath regeneration during this period.

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AUTHORSHIP CONTRIBUTION

Study conception and design: YKW, WXY, YL, JW
Data collection: YKW
Analysis and interpretation of results: RL, JBG
Draft manuscript preparation: YKW
Critical revision of the article: WXY
Other (study supervision, fundings, materials, etc...): KL
All authors (YKW, WXY, RL, JBG, YMZ, KL, YL, JW) reviewed the results and approved the final version of the manuscript.

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