Ulnar nerve palsy associated with closed midshaft forearm fractures

Ulnar Nerve Palsy Associated With Closed Midshaft Forearm Fractures

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Abstract

Background: We examined whether or not peripheral nerves can be regenerated using uncultured adipose-derived regenerative cells (ADRCs). We also searched humoral factors which might promote proliferation or migration of Schwann cells.

Methods: Thirty rats were randomly assigned to 3 groups. A 10-mm sciatic nerve defect was bridged using a silicon tube filled with physiological saline (control group), type I collagen gel (collagen group) and a mixture of ADRCs and type I collagen gel (ADRC group). The regenerated tissues were studied 2 weeks after surgery.

Results: Continuity of regenerated tissue was observed in all rats in the control group and the ADRC group. In the collagen group, only two rats had a bridge of thin tissue which was barely visible macroscopically. Protein gene product 9.5 staining confirmed significantly faster regeneration in the ADRC group. The distributions of the PKH-26 positive areas and the S-100 protein positive areas were different, which suggests that the transplanted cells had not differentiated into Schwann cells. In real-time RT-PCR, neuregulin-1 (Neu-1) and vascular endothelial growth factor A (VEGFA) expression were detected in uncultured ADRCs before transplantation. The regenerated tissue in the ADRC group had higher levels of Neu-1 and VEGFA expression compared to the control group.

Conclusions: ADRCs promote peripheral nerve regeneration. The mechanism does not involve differentiation of transplanted cells into Schwann cells, but probably involves secretion of some type of humoral factor such as Neu-1 or VEGFA, promoting proliferation or migration of Schwann cells.

Introduction

Microsurgery began to be used to repair nerve injuries in the 1960s. Thereafter the treatment has markedly advanced. In 1972, Millesi et al. used autologous sural nerve grafts for median and ulnar nerve repair [1]. Subsequently, autologous nerve grafts began to be used that can bridge large peripheral nerve defects without tension after trauma or malignant tumor resection. However, autologous nerve grafting has had problems such as limitation in the defect size and complaints about the donor site. Therefore, artificial nerve conduits have been developed which apply the principles of tubulization [2]. Development has been advanced in two aspects: materials filling the tubes and materials making up the tubes. Many reports have described the effectiveness of filling the tubes with materials such as nerve growth factors [3-6] and various cellular factors [7-9]. The nerve growth factors disappear if they are not continuously infused. Cellular factors have not been widely applied clinically due to ethical and technical reasons. In this study, we examined whether or not peripheral nerves can be regenerated using adipose-derived regenerative cells (ADRCs) [10] which can be easily isolated in large amounts from autologous adipose tissue. ADRCs were used as much as possible without culturing or induced differentiation to enable easier future clinical application of these cells. Ikeda et al. examined regenerated axons in rats over time [7]. They observed that the regenerative ability of axons was high. In addition, they reported that the number of regenerated axons did not differ significantly at 8 weeks after transplantation between a group having tubes filled with physiological saline alone and a group having tubes filled with Schwann cells. Thus, we evaluated regenerated axons which were excised 2 weeks after transplantation to examine early axonal outgrowth.

Materials and Methods

Study Design

The experiment was conducted with the approval of the Kanazawa University Advanced Science Research Center. Thirty 10-week-old Wistar rats were randomly assigned to 3 groups. A 10-mm sciatic nerve defect was created in each rat. The following were used to fill a silicon tube (inner diameter: 2 mm, wall thickness: 0.5 mm): physiological saline alone for the control group, type I collagen gel alone (Cellmatrix Type I-A, Nitta Gelatin, Osaka) for the collagen group, and a mixture of uncultured ADRCs and type I collagen gel for the ADRC group. The defect was bridged with the tube. We evaluated regenerated tissues which were excised 2 weeks after transplantation.

ADRCs Isolation

Isolation of ADRCs was performed by modifying a previously reported method [11]. Adipose tissue (1.5 g) was harvested from the left inguinal region of a rat and washed with phosphate-buffered saline (PBS, Wako, Osaka). The tissue was cut into strips over a period of 5 minutes. Collagenase (Collagenase, Wako) was dissolved in PBS so that its concentration would be 0.12% in 20 ml, and it was used to digest adipose tissue at 37°C for 45 minutes in a water bath. The mixture was shaken every 15 minutes during the digestion period. Immediately after the reaction was completed, 20 ml of Dulbecco's modified Eagle's medium (D-MEM, Wako) was added and collagenase activity was neutralized. The resulting solution was filtered. The filtrate was centrifuged at 1300 rpm for 6 minutes at 25°C, and the supernatant was removed. The pellet of ADRCs was subsequently used to fill the tubes. Approximately 1×10^6 cells are included in this pellet.

Surgical Method

Surgery was performed in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institute of Health (NIH Publication No. 85-23, Revised 1996) and using aseptic techniques. Anesthesia was achieved with 6.5 mg of sodium pentobarbital (Somnopentyl®, Intervet, Tokyo) administered intraperitoneally. The right sciatic nerve was exposed and transected at the mid-thigh level. The aforementioned silicone tube was used so that it bridged a nerve gap of 10 mm. That is, a 12-mm-long tube was filled with one of the previously mentioned materials. The distal and proximal stumps were inserted 1 mm into the distal and proximal ends of the tube, respectively. Four stitches of 10-0 nylon sutures were used to fixate each stump to the tube. In the ADRC group, the pellet of ADRCs was disrupted and homogenized in type I collagen gel using a syringe, and surgery was performed on each rat using ADRCs from its own adipose tissue. Finally, the gluteus muscles and skin were closed with 6-0 nylon sutures, and the surgery was completed.

Macroscopic Evaluation

Macroscopic evaluation was performed on the continuity of regenerated tissue in the tubes.

Immunohistochemical Evaluation

Paraffin sections were prepared containing cross-sections of the central portion of the regenerated tissues. The presence of Schwann cells was evaluated by staining for S-100 protein (1:300, Abcam, UK).

Paraffin sections were also prepared containing longitudinal sections of the regenerated tissues. They were stained for protein gene product (PGP) 9.5 (1:200, Abcam), and the length of regenerated axons was measured from the beginning of the tube to the last visible positive areas.

PKH-26 Labeling

ADRCs were labeled with PKH-26 dye (PKH Linker Kit, SIGMA-ALDRICH, St. Louis) and transplanted. PKH-26 binds to the cell membrane and has stability for more than 100 days which enables the tracing of PKH-26 labeled transplanted cells in host tissue. Two weeks later, a frozen section was prepared containing a cross-section of the central portion of the regenerated tissue, and the survival of the transplanted cells was examined. The same section was stained for S-100 protein. Comparison was made between the PKH-26 positive areas and the S-100 protein positive areas, and examination was performed to determine whether or not the transplanted cells differentiated into Schwann cells.

Analysis of mRNA Expression

Real-time RT-PCR was performed on pre-transplant ADRCs and regenerated tissues two weeks after transplantation using the following methods. The expression of neuregulin-1 (Neu-1) and vascular endothelial growth factor A (VEGFA) were analyzed.

1. Total mRNA Isolation

RNA was extracted using a NucleoSpin® RNA II kit (TAKARA BIO, Otsu, Japan). Each sample was disrupted and homogenized using a syringe. Thereafter, treatment was performed according to the manufacturer's protocol. The absorbance of the resulting total RNA was measured at 260 nm, and the concentration was calculated.

2. cDNA Synthesis

cDNA was synthesized using the High Capacity RNA-to-cDNA Master Mix® (Applied Biosystems, CA). Treatment was performed according to the manufacturer's protocol, and adjustments were made to yield a total volume of 20 µl. Then the following conditions were used on a GeneAmp® PCR System 9700 (Applied Biosystems) to synthesize cDNA: 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes and a 4°C hold.

3. Real-Time RT-PCR

To 1µl of the resulting cDNA sample, 5µl of TaqMan® Universal Master Mix II (Applied

Biosystems), 0.5 µl of primer, and 3.5 µl of RNAse free water (Applied Biosystems) were added. The total volume was 10 µl. Primers of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Neu-1, and VEGFA were purchased from Applied Biosystems. Reactions were performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with cycles 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 1 minute, and amplification was performed 40 times. The expression ratios of Neu-1 to GAPDH and VEGFA to GAPDH were calculated for each sample.

Statistical Analysis

Experimental results were expressed as means±SD and were analyzed using a t-test. The differences were considered significant when $p<0.05$.

Results

Macroscopic Evaluation

Continuity of regenerated tissue was observed in all rats in the control group and the ADRC group. The gap was bridged by blood clot-like tissue in all rats in the control group (Fig. 1A). Most areas were occupied by white tissue in the ADRC group (Fig. 1B). In the collagen group, only two rats had a bridge of thin tissue which was barely visible macroscopically. Ikeda et al. have reported that regenerated axons could not invade into type I collagen gel alone at 2 weeks owing to lack of migration of Schwann cells [7], and our results are compatible with this report. Therefore, we excluded the collagen group from the following assessment.

Immunohistochemical Evaluation

1. Staining for S-100 Protein

There were no areas positive for S-100 protein in the control group (Fig. 2A), and Schwann cells had not migrated to the central portion of the regenerated tissue. Schwann cells were observed in only the ADRC group (Fig. 2B).

2. Staining for PGP 9.5 Protein

The lengths of the regenerated tissues were compared between the control group (Fig. 3A) and the ADRC group (Fig. 3B). The ADRC group had significantly more axonal regeneration compared to the control group (3.8 ± 0.9 vs. 6.2 ± 0.4 mm, $p=0.0043$) (Fig. 4).

PKH-26 Labeling

The PKH-26 positive areas (red) are shown in Fig. 5. It reveals that transplanted cells had survived two weeks after transplantation. The same section was used to stain for S-100 protein (green). The distributions of the PKH-26 positive areas and the S-100 protein positive areas were different. This finding suggests that the transplanted cells had not differentiated into Schwann cells 2 weeks after transplantation.

Real-time RT-PCR

Neu-1 and VEGFA expression were detected in the pre-transplant ADRCs. Neu-1 and VEGFA expression were detected in the regenerated tissue in the control group and the ADRC group. The regenerated tissue in the ADRC group had significantly higher levels of Neu-1 and VEGFA expression compared to uncultured ADRCs before transplantation (Fig. 6A). The ADRC group had significantly higher levels of Neu-1 and VEGFA expression compared to the control group (Fig. 6B).

Discussion

Regenerative medicine using stem cells is a field that has gained great interest. Stem cells can be divided broadly into pluripotent stem cells and somatic stem cells. Pluripotent stem cells have the ability to differentiate into all types of cells and include embryonic stem (ES) cells [12] and induced pluripotent stem (iPS) cells [13]. Somatic stem cells have the ability to differentiate into only certain types of cells. Pluripotent stem cells have a high ability to differentiate. However, they have risks of neoplastic transformation and their use raises ethical and technical issues. Somatic stem cells have a lower ability to differentiate compared with pluripotent stem cells. However, somatic stem cells have a higher safety profile and early clinical application might be possible.

In recent years, it has been shown that somatic stem cells exist in adipose tissue [11], and these cells began to be called “adipose-derived stem cells” (ADSCs). An in vitro study revealed that ADSCs have the ability to differentiate into the following cells, just like bone marrow-derived stem cells (BMSCs): osteoblasts, chondrocytes, neurons, and cells other than germ cells (e.g., stem cells) [14]. Multiple reports have already been published regarding clinical applications of ADSCs including in breast reconstruction and breast augmentation [15, 16]. There are also reports of their use to treat ischemic heart disease, tracheal fistula [17], intestinal fistula associated with Crohn’s disease [18], and GVHD [19, 20]. The reason why ADSCs are used now is that autologous ADSCs can be easily isolated in large amounts from subcutaneous adipose tissue. In addition, ADSCs can be harvested less invasively compared to BMSCs, and many more

stem cells can be harvested at one time [21, 22]. In clinical applications, autologous ADSCs should be used, if possible, from the perspective of safety, and ADSCs should be minimally manipulated. For the above reasons, we have focused on ADSCs as a new source of tissue stem cells, replacing bone marrow.

Several reports have been published regarding peripheral nerve regeneration using ADSCs, but most reports involved culturing or induced differentiation [23-25]. We proposed that clinical application of peripheral nerve regeneration can become closer to reality with the use of uncultured ADRCs. ADRCs contain ADSCs, vessel-forming cells, such as endothelial and smooth muscle cells and progenitors, and preadipocytes [10]. The result of our present study showed that ADRCs promote peripheral nerve regeneration. The distributions of the PKH-26 positive areas and the S-100 protein positive areas were different. This result suggests that the mechanism does not involve differentiation of ADSCs among ADRCs into Schwann cells but involves secretion of some type of humoral factor by ADRCs, promoting proliferation or migration of Schwann cells. It would be ideal for ADSCs among ADRCs to differentiate into Schwann cells. However, it might be necessary to have a certain amount of mature cells in the target tissue to induce differentiation of stem cells. Then, we examined what types of humoral factors might promote proliferation or migration of Schwann cells. Various extracellular stimulation and conditions promote cell proliferation. In particular, axon-derived Neu-1 very strongly promotes Schwann cell proliferation [26]. The mechanism is known to involve the activation of the intracellular MEK pathway and the phosphatidylinositol-3-kinase pathway [27]. In our study, the results of real-time RT-PCR suggest that ADRCs probably secrete Neu-1, and the relationship between ADRCs and Neu-1 has not been reported yet. VEGFA, on the other hand, has been reported to act directly on not only vascular endothelial cells but also Schwann cells, neurons, and neural stem cells, drawing attention to a close relationship between blood vessels and nerves [28]. In particular, VEGFA has been reported to stimulate Schwann cell proliferation and migration [29, 30]. Therefore, these results suggest that ADRCs promote peripheral nerve regeneration by probably secreting humoral factors such as Neu-1 and VEGFA and by involving in various stages of Schwann cells including proliferation and migration. These findings are consistent with our result that the ADRC group had significantly increased expression of Neu-1 and VEGFA in the regenerated tissue compared to the control group. Perhaps when ADRCs are transplanted near a nerve stump, they change into cells that actively secrete Neu-1 and VEGF even though these cells might not differentiate into Schwann

cells (Fig. 7A). ADRCs possibly regulate nerve stumps to promote secretion of such factors (Fig. 7B), or besement membrane from various cells in ADRCs may be favorable for axonal regeneration. Future studies are needed to examine this speculation. The results of our study suggest that ADRCs might play a very important role as a future tool for peripheral nerve regeneration. It will be necessary to make the longer period model for future clinical applications.

Conclusions

ADRCs promote peripheral nerve regeneration. The mechanism does not involve differentiation of ADSCs among ADRCs into Schwann cells, but probably involves secretion of some type of humoral factor such as Neu-1 or VEGFA, promoting proliferation or migration of Schwann cells.

Conflict of interest

None of the authors have any conflicts of interest or disclosures in relation to this work.

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Figure captions

Figure 1 Macroscopic Findings

The gap was bridged by blood clot-like tissue in all rats in the control group (A), while most areas were occupied by white tissue in the ADRC group (B).

Figure 2 Staining for S-100 Protein (×40)

Paraffin sections were prepared containing cross-sections of the central portion of the regenerated tissues. The presence of Schwann cells was evaluated by staining for S-100 protein. (green: S-100, blue: DAPI)

A. Control group

B. ADRC group

Figure 3 Staining for protein gene product (PGP) 9.5 Protein

Paraffin sections were prepared containing longitudinal sections of the regenerated tissues. They were stained for PGP 9.5 (green), and axonal regeneration was quantitatively evaluated.

A. Control group

B. ADRC group

Figure 4

The lengths of the regenerated tissues were compared between the control group and the ADRC group. The ADRC group had significantly more axonal regeneration compared to the control group ($p=0.0043$).

Figure 5 PKH-26 Labeling (×40)

ADRCs were labeled with PKH-26 dye. Two weeks later, frozen sections were prepared containing cross-sections of the central portion of the regenerated tissues. The PKH-26 positive areas (red) were different from S-100 positive areas (green).

Figure 6. Real-time RT-PCR

A. Comparison between uncultured ADRCs and regenerated tissue (black bar: ADRCs, gray bar:

regenerated tissue)

The regenerated tissue in the ADRC group had higher levels of Neu-1 and VEGFA expression compared to the uncultured ADRCs before transplantation (A).

B. Comparison between control group and ADRC group (black bar: control group, gray bar: ADRC group)

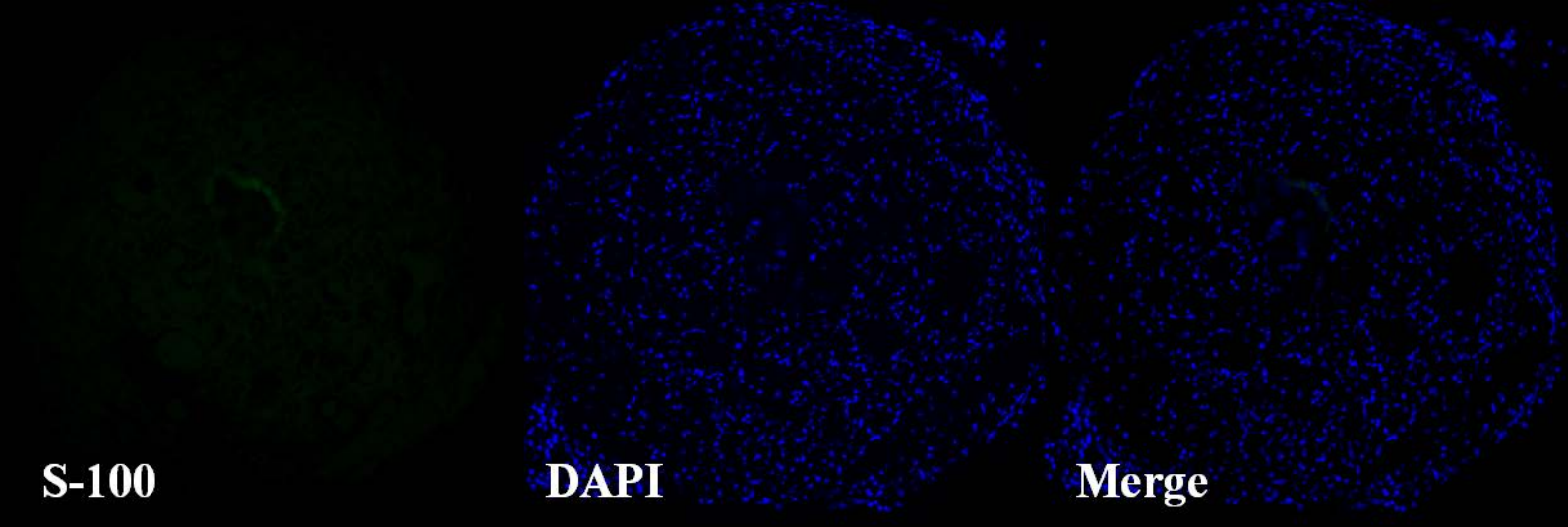
The regenerated tissue in the ADRC group had higher levels of Neu-1 and VEGFA expression compared to the control group (B).

Figure 7 Mechanism of peripheral nerve regeneration using uncultured ADRCs

A. ADRCs can change into cells that actively secrete Neu-1 and VEGF even though these cells might not differentiate into Schwann cells.

B. ADRCs can regulate nerve stumps to promote secretion of humoral factors.


A
10 mm
B
10 mm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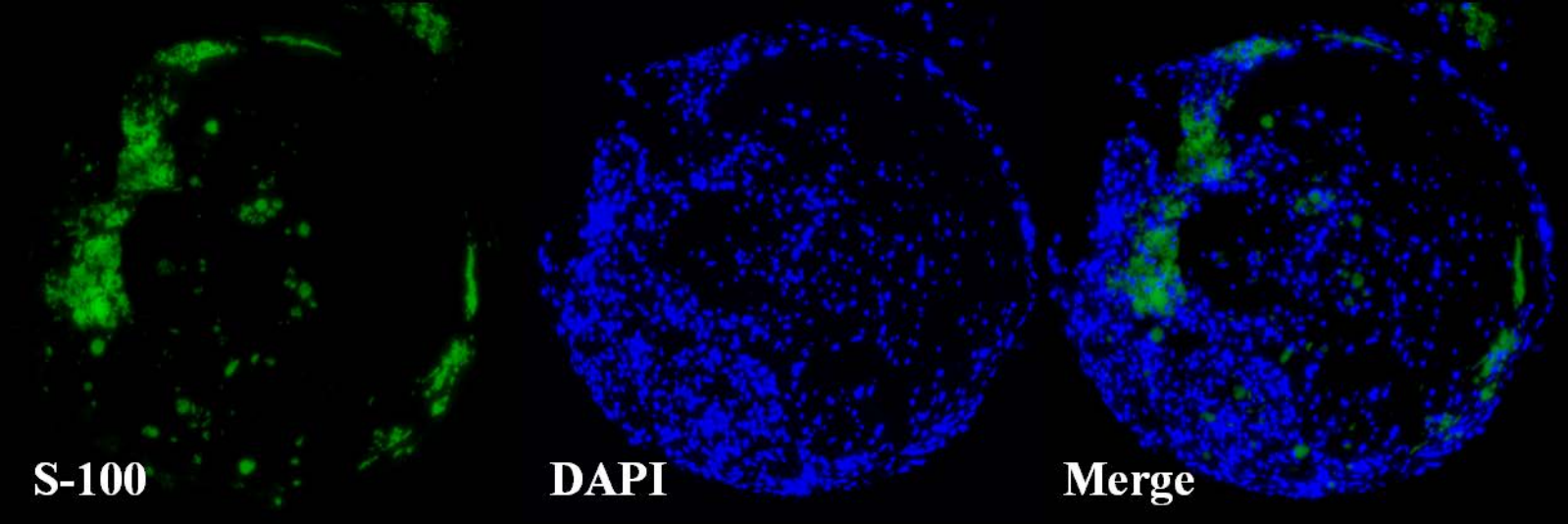
DAPI
Merge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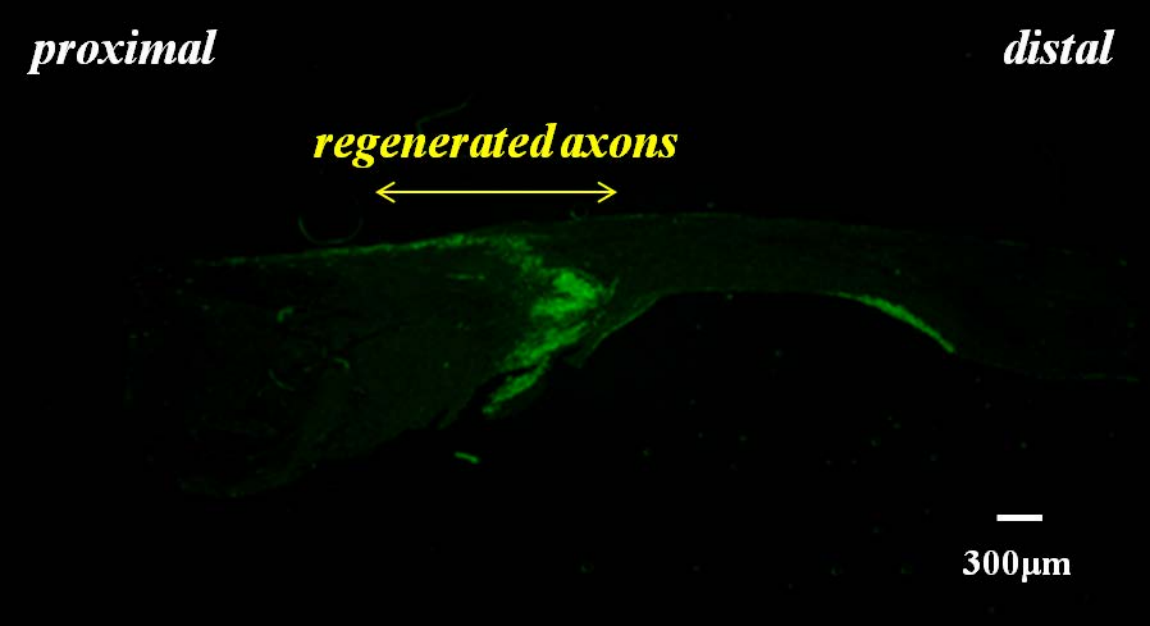
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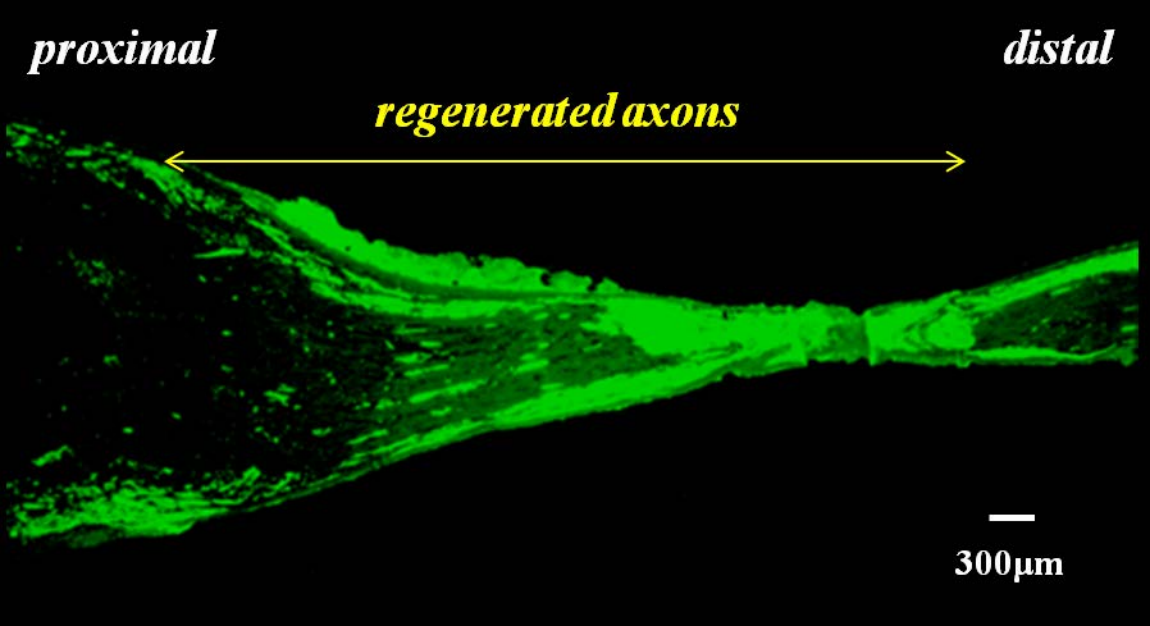
distal
regenerated axons
300μm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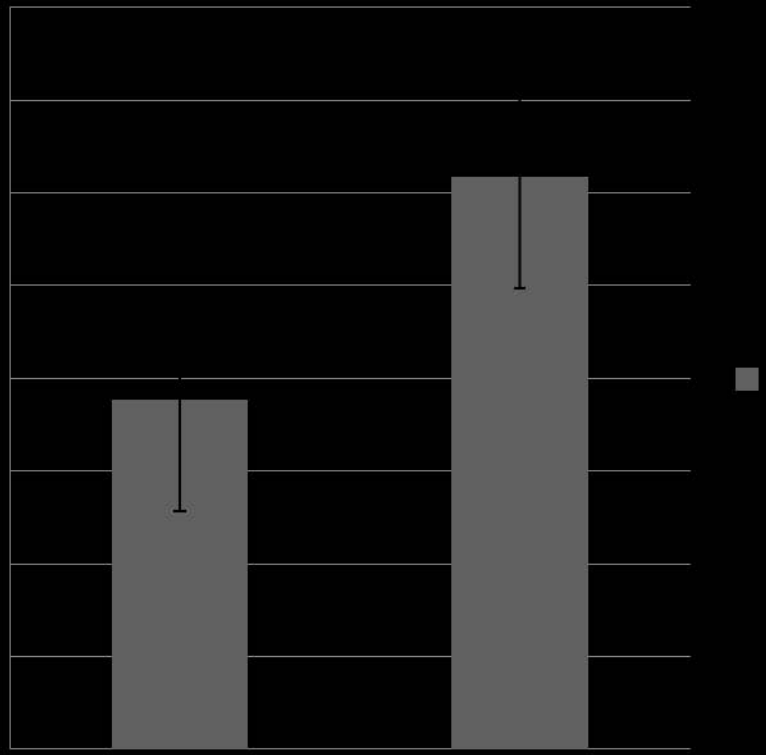

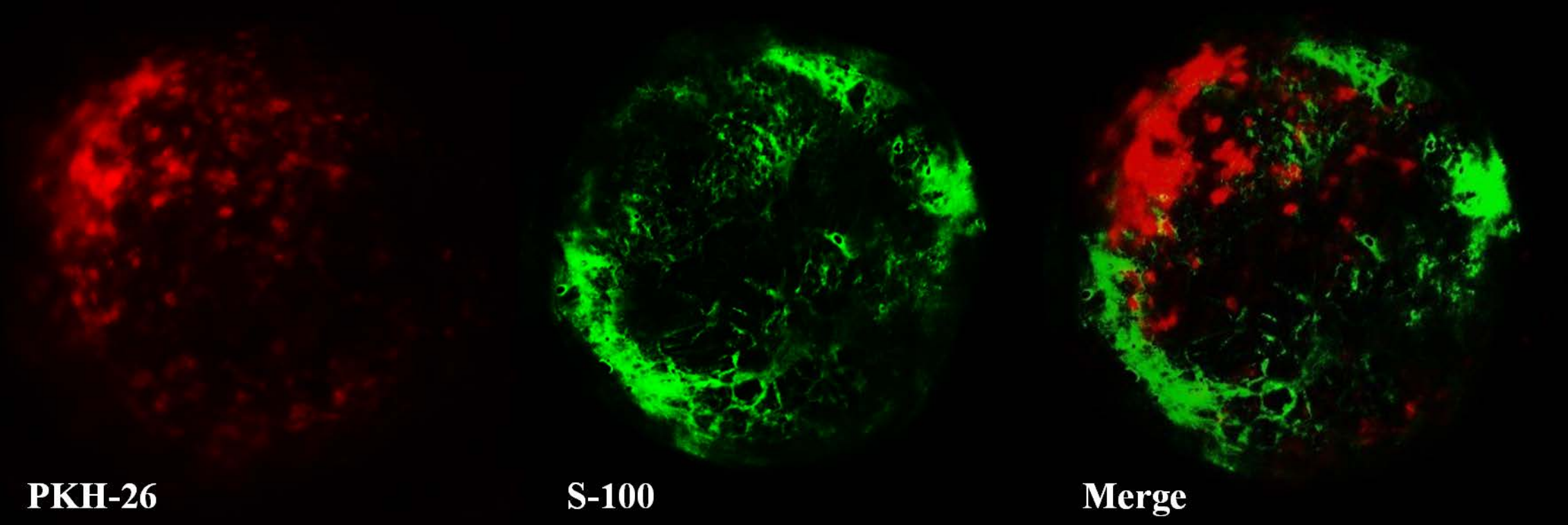
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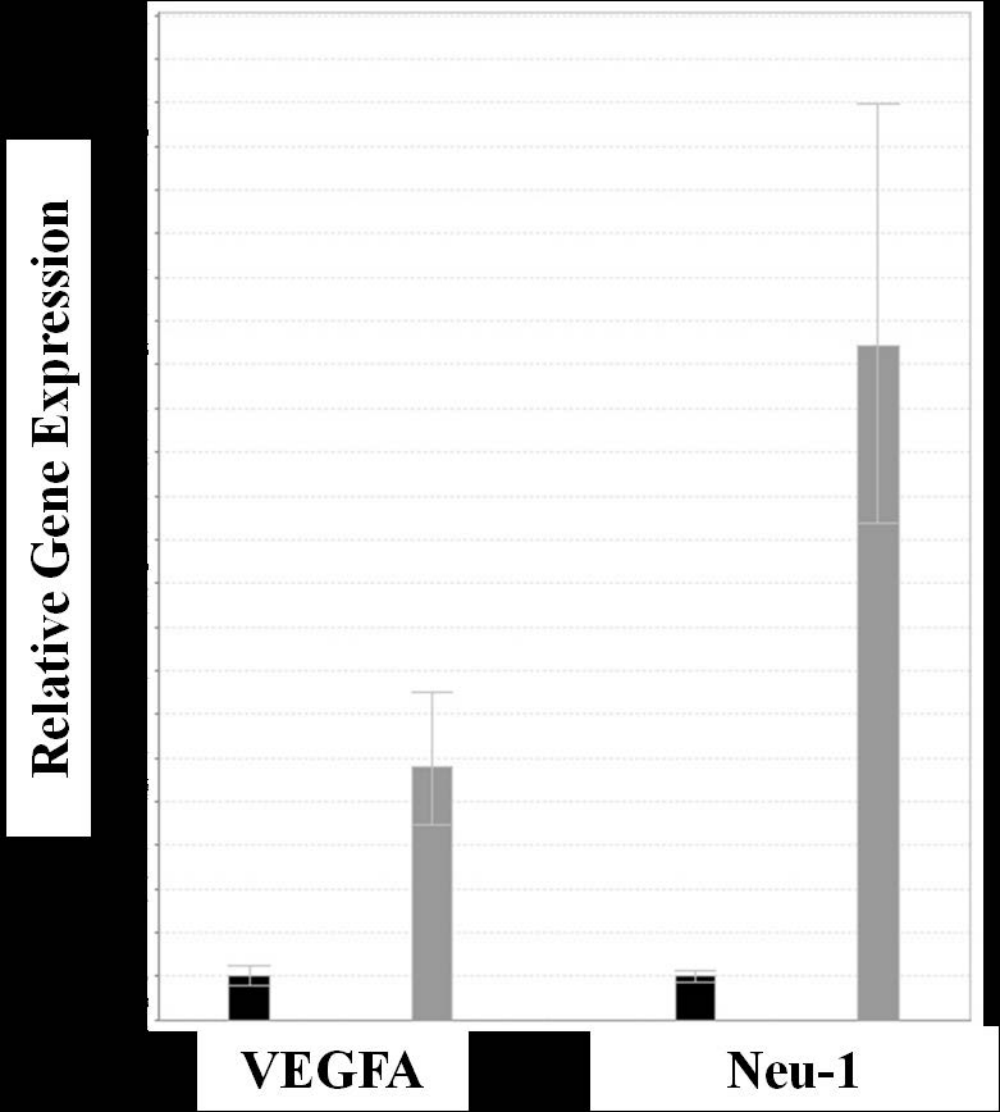
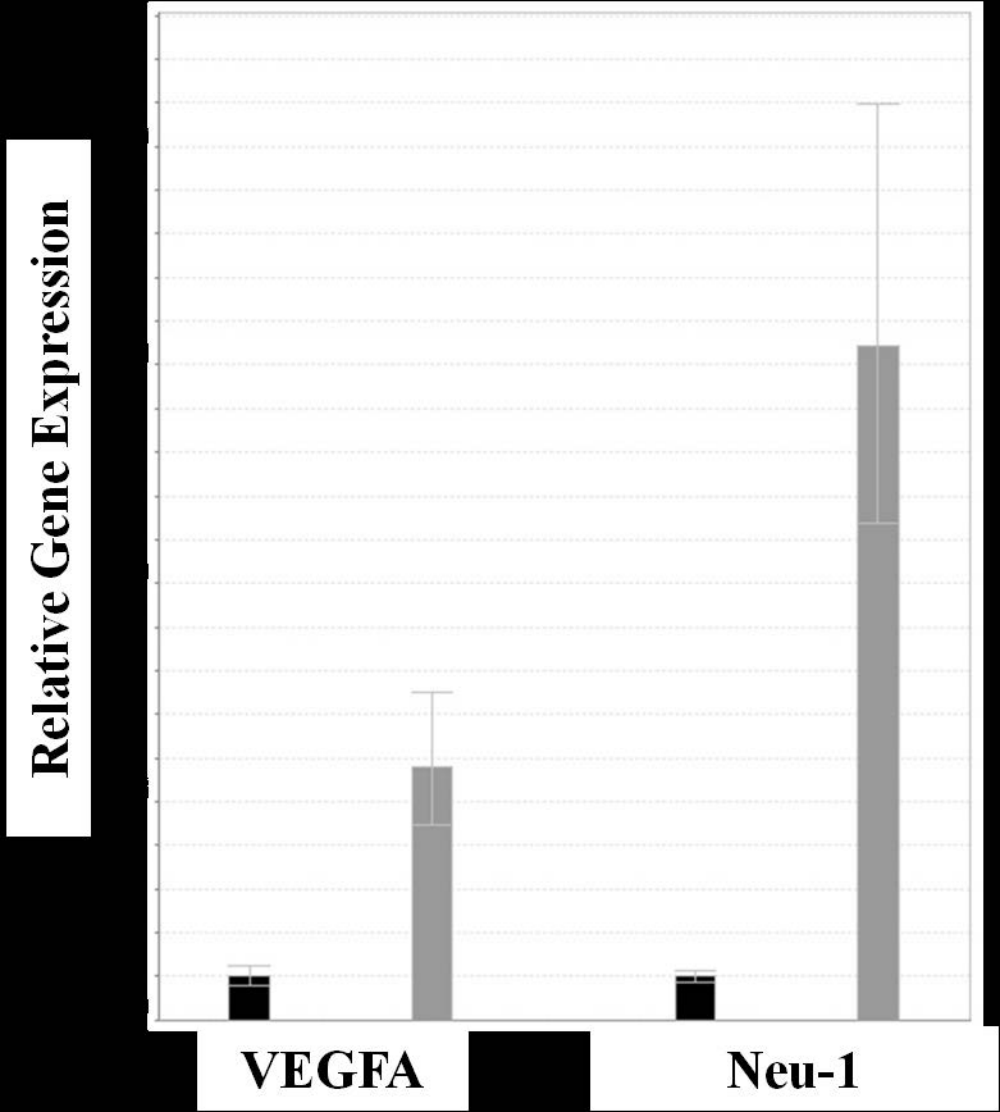
S-100
Merge


Relative Gene Expression
VEGFA
Neu-1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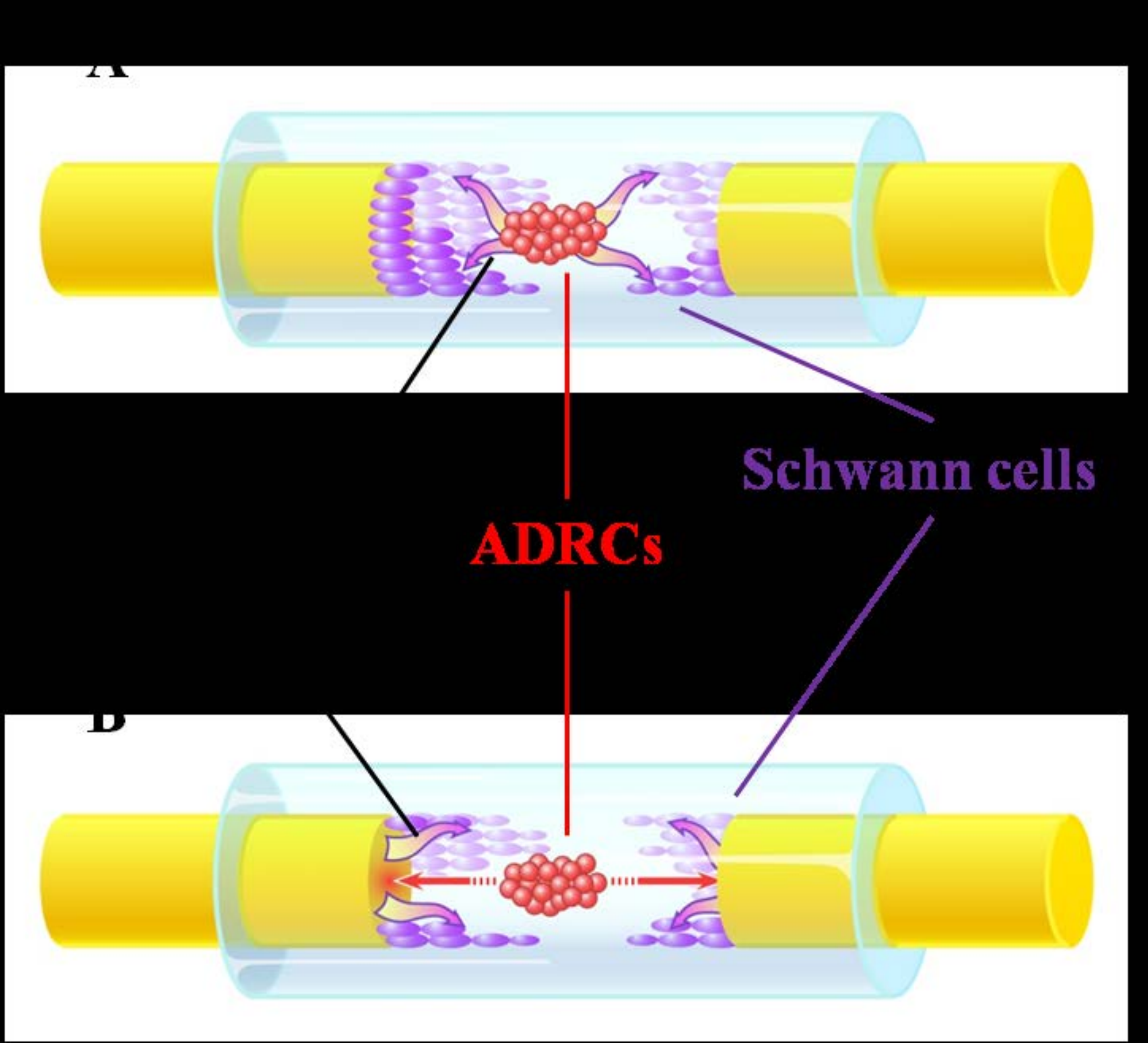
A
Schwann cells
ADRCs
B