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Research paper

Developing a trans-multisynaptic tracer to map the neural circuit of recovered sciatic nerve after treatment with nerve growth factor

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ABSTRACT

Nerve growth factor (NGF) has been shown to support the survival and differentiation of neurons. In this study, we first developed a retrograde trans-multisynaptic tracer PRV580 expressing the mCherry fluorescent protein based on pseudorabies virus Bartha strain to map the neural circuit of sciatic nerve. Secondly, the newly developed PRV580 was used to map the neural circuit of the recovering sciatic nerve upon treatment with NGF. Our results showed that red signals from PRV580 were observed in various brain regions. Among these regions, many areas of the pyramidal system and the extra-pyramidal system had been mapped, accounting for as much as 56.8 % of the total inputs. Furthermore, we found that NGF could significantly increase the ratio of total input (29.05 %) compared to PBS (3.65 %), indicating that NGF indeed can aid in the repair of injured sciatic nerve. These findings indicated that NGF has therapeutic ability for the treatment of peripheral nerve injuries and virus-based tracers can be used to monitor the recovery.

1. Introduction

The sciatic nerve is the largest nerve in the body, which plays important roles in stimulation of leg muscle movement and sending sensory messages from the leg to the spinal cord (Giuffre and Jeanmonod, 2021). Sciatic neuropathy is a common disease, which eventually results in the deficits of motor and sensory functions. There are many causes for sciatic neuropathy, which include i) mechanical injuries, such as sprain of the lumbar spine; ii) non-neurological diseases, such as diabetic neuropathy, ectopic endometriosis, ovarian cysts, pseudomyxoma peritonei (Lin et al., 2009), and infectious diseases (Stafford et al., 2007); iii) physiological causes, such as smoking (Shiri and Falah-Hassani, 2016) and pregnancy (Hall et al., 2016; Sun et al., 2020). Traditional treatments, such as physical therapy (using cold or hot packs and stretching), medication (pain medication, anti-inflammatory, anti-seizure medication, muscle relaxant, narcotic, tricyclic antidepressant) and surgery, play pivotal roles in curing or relieving sciatica.

To repair the injured nerve, nerve growth factor (NGF), a naturally-occurring multifunctional secreted protein with about 27 kDa, has been used to promote neuronal survival, axonal regeneration and morphologic plasticity in both central and peripheral nervous system since its discovery in 1951 by Rita Levi-Montalcini (Onger et al., 2017; Rocco et al., 2018). Various studies showed that NGF can be synthesized and secreted from neuronal cells, immune inflammatory cells, epithelial cells, keratinocytes, smooth muscle cells and fibroblasts (Lambiase et al., 2004; Micera et al., 2004; Micera et al., 2003; Sofroniew et al., 2001). Although NGF can repair the injured nerve, the connectivity level of neural circuit of the recovered nerve has not been described.

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Neurotropic virus-based tracers have been used for mapping neural circuits in the central nervous system (CNS) and the peripheral nervous system (PNS) (Nassi et al., 2015; Xu et al., 2020). Among these tracers, pseudorabies virus (PRV) is a powerful tool, which can retrogradely spread within the neural circuit (Jia et al., 2019) and plays an important role in mapping the neural circuit of the PNS, such as uterus (Chen et al., 2013; Collins et al., 1999; Kirby et al., 2010), lung (Kc et al., 2006), stomach (Li et al., 2015), orbicularis oculi muscle (Gonzalez-Joekes and Schreurs, 2012), eye (Yang et al., 2021), bladder (Yao et al., 2018) and spleen (Zhang et al., 2020). Therefore, development of a PRV-based tracer would be very useful for analyzing the sciatic nerve pathway and the efficiency of the nerve recovery.

2. Materials and methods

2.1. Animals

Eight-week-old male Sprague-Dawley (SD) rats, weighing approximate 200 g, were purchased from Hunan SJA laboratory animal company and housed in cages with free access to food and water under a standard condition 12 h light and 12 h dark cycle. All studies were performed following the National Guides for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committees at Innovation Academy for Precision Measurement Science and Technology, Chinese Academy of Sciences.

2.2. Virus preparation

To prepare the retrograde multi-synaptic tracer, we firstly constructed the plasmid PS580 (pcDNA3.1(+)-left arm-Ubc-mCherry-WPRE-bGHpA-right arm) by inserting the expression cassette of Ubc promoter-mCherry into the AsiSI and *Swa*I treated plasmid PS531 (Jia et al., 2019). The plasmid was verified by DNA sequencing. Secondly, the 2 μg of plasmid PS580 was transfected into BHK21 cells, after 6 h post-transfection, the transfected BHK21 cells was infected by PRV Bartha strain (moi = 1), then the virus sample was collected at 2 days post-infection(dpi). Then, PRV580 was purified by isolating the red-positive plaque for four rounds. Lastly, the purified PRV580 was added into the T75 flask containing BHK21 cells with 90 % confluency. After two days, the supernatant was collected and filtered with 0.22 μm filter. Then the PRV580 was centrifuged to increase the virus titer and remove the culture medium components at 50,000 g for 2 h at 4 °C and repeat again. Then virus pellets were suspended in the cold PBS and stored at −80 °C.

2.3. Plaque assay

Plaque assay was performed to determine the viral titer by using the following protocol (Jia et al., 2019). Briefly, sub-confluent BHK-21 cells were cultured in 6-well plate, then each dilution virus sample was added into individual well and incubated under 5 % CO_2 at 37 °C for 1 h. Then, the cells were overlaid with the first layer and second layer of agar at indicated time point.

2.4. Surgery and virus injection

For labelling the neural circuit of sciatic nerve, the surgical operation was performed in the SD rats under sterile conditions using a method described previously (Chen et al., 2015; di Summa et al., 2010; Xu et al., 2012). The SD rats were anesthetized with 1 % pelltobarbitalum natricum (50 mg/kg) by intraperitoneal injection. After anesthetization, the surgical area between the left knee and the hip was shaved and the skin was cleaned with alcohol and iodine. The skin incision was made to reveal the muscle, which were then dissected to expose the sciatic nerve for surgical operation. Then, a volume of 3 μl of PRV580 (1.5×10^{10} PFU/ml) was injected into the sciatic nerve of rat at anesthetized state using a 10 μl syringe (Hamilton, Nevada, USA) connected to a glass micropipette with 10–15 μm diameter tip under microscopic guidance. After injection, the micropipette was left in the injection site for 3–5 min to help virus infection and then slowly withdrawn. Then, muscle and fascia layer were closed using the resorbable stitches (4/0) and the skin was closed using the continuous running suture (4/0). To minimize tissue necrosis and infection, the surgical area was covered with a layer of erythromycin eye ointment and care was performed to avoid excessive heat. All rats were maintained on the sawdust cages with standard condition 12 h light and 12 h dark cycle and freely received food and water.

For labelling the neural circuit of recovered sciatic nerve, three groups were designed and two steps were performed. The first step is to injury sciatic nerve and repair it with NGF or PBS. In NGF group: the right sciatic nerve was exposed using the surgeical operation as described above. Then the sciatic nerve was completely cut at the midpoint between the pelvic outlet of the sciatic nerve and the bifurcation of the common tibial and peroneal nerves using microscissors. According to the shape of the nerve, under the operating microscope, the epineurium was sutured equidistantly and intermittently with 10–0 non-damaging nylon suture end-to-end anastomosis, and 8–10 stitches were evenly anastomosed. A micro suture was left in the epineurium of the surgical area as a mark, and then the incision was closed layer by layer with sterile 1–0 Pole suture. Next, the 10 μg NGF in PBS was injected into muscle at surgical site every day for three weeks (the NGF was extracted from mouse submandibular gland, which was purchased from Lizhu Group Pharmaceutical Sales Co., Ltd.). In PBS group: the right sciatic nerve was completely cut and sutured like NGF group, while the NGF was replaced with PBS. In wild type group: the sciatic nerve was not cut, and no NGF and PBS treatment. The second step is to label the neural circuit of the recovered sciatic nerve by injecting the PRV580 to the distal end of the injured site (Fig. 2) using the same injection method described as above.

2.5. Tissue preparation, imaging and counting

After 6 days, rats were anaesthetized with 1 % pelltobarbitalum natricum (50 mg/kg) by intraperitoneal injection and were transcardially perfused with 0.9 % saline followed by 4 % paraformaldehyde solution. The brains and spinal cord were removed and post-fixed over night in 4 % paraformaldehyde, and then 30 % sucrose solution for 3 days before being sectioned into 40 μm slices (Leica). The slices were stained with DAPI and imaged (10×) using the Olympus VS 120 slide scanning system. To calculate the percentage of total input and the recovery ratio, one third of all slices (approximately 100 slices) from each rat (each group has three rats) were selected for data analyzing according to the same position relative to bregma. The number of red positive neurons were counted by three technicians. Each technician manually counted the number of red positive neurons of all rats in each group.

3. Results

3.1. Preparation of retrograde trans-multisynaptic tracer PRV580 based on PRV Bartha strain

The mCherry expression cassette was engineered into the gG location of PRV Bartha by homologous recombination (Fig. 1A). The purified recombinant PRV580 can infect BHK21 cells and express red fluorescent protein by using fluorescent microscopy (Fig. 1B). In addition, the virus produced plaque of similar size (Fig. 1C). The growth curve of the virus was tested by determining the viral titer of each sample at the indicated time points. We found that the amount of PRV580 was increased from 12 hpi (hours post-infection) up to the peak value more than 10^7 PFU/ml at 48 hpi (Fig. 1D).

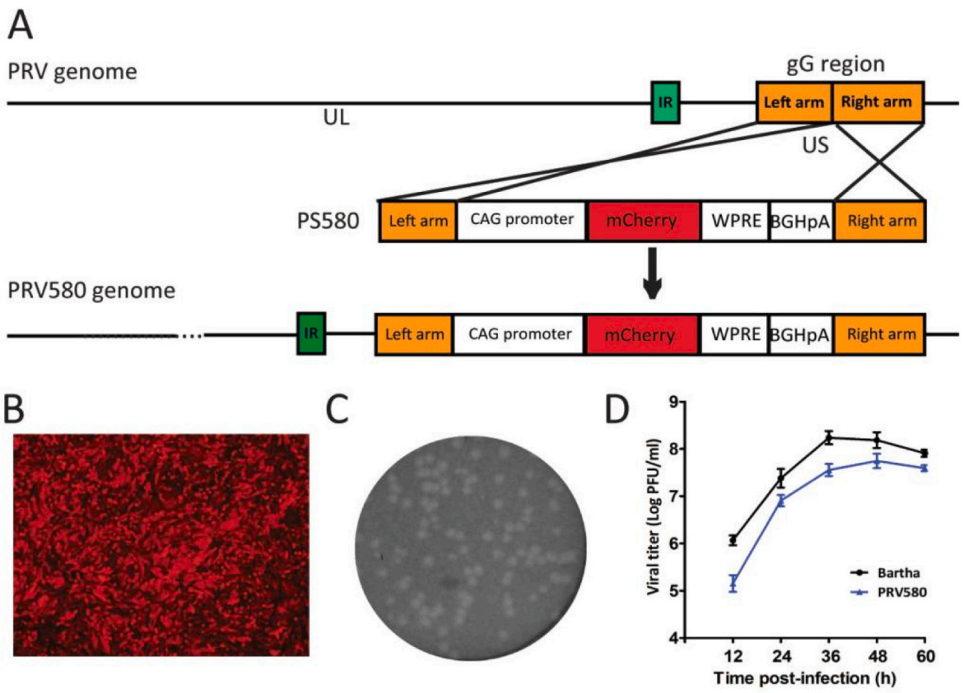

Fig. 1. Preparation of recombinant PRV580. Cloning diagram of recombinant PRV580. The top represents PRV Bartha genome. The middle represents the expression cassette of plasmid PS580, which is flanked by left arm left homologous arm (left arm) and right homologous arm (right arm). The bottom represents the genome of recombinant PRV580. (B) Purification of recombinant PRV580 infects BHK21 cell. (C) The plaque assay was performed on BHK21 cells. (D) The growth curve of PRV580 and its parent virus. Virus infects BHK21 at moi=0.1 and the sample was collected at indicated time points (12, 24, 36, 48 and 60 hpi).

3.2. Brain regions were labelled from the initial injection site of sciatic nerve

One goal of our study is to analyze the labelled brain regions when the retrograde trans-multisynaptic tracer PRV580 was injected into the sciatic nerve. After 6 days post-infection (dpi), the rats showed clear symptoms, such as the exciting, scratching and biting behaviors and then become moribund. Their brains were collected and sectioned into 40-μm slices, and one third of all slices from each animal were selected for data analyzing by same position relative to Bregma. The red signals from tracer PRV580 were observed in many brain regions, such as the primary motor cortex (M1), the secondary motor cortex (M2), the prelimbic cortex (PrL), the medial preoptic nucleus (MPN), the lateral preoptic area (LPO), the periaqueductal gray (PAG), the habenular nucleus (HN), the ventral tegmental area (VTA), the medial hypothalamic nucleus (MHN), the lateral hypothalamic area (LH), the median raphe nucleus (MnR), the paramedian raphe nucleus (PMnR), the pontine reticular nucleus (PRN), the reticulotegmental nucleus of the pons (RtTg), the lateral lemniscus (LL), the medial vestibular nucleus (MVN), the gigantocellular reticular nucleus (GRN), the p1 reticular formation (p1Rt), the mesencephalic reticular formation (mRt), the isthmic reticular formation (isRt), the mammillary nucleus (MN), the prepositus nucleus (Pr) and the reticular nucleus (RN). The number of the red positive neurons is different in different brain regions. The numbers of red positive neurons in MPN, LPO, PAG, VTA, MH, LH, PRN, RtTg, LL, MVN, GRN, mRtm, isRt and RN were relatively much higher than other regions (Fig. 2). To calculate the percentage of total input of different brain regions for each animal, the number of red input neurons of each brain region was divided by the number of all red neurons from all counted brain slices. Among these regions, the percentage of total input is more than 5 % in the PAG, MHN, LH, PRN, MVN and GRN. Interestingly, the red signals in PAG is prominent in all input regions, which is up to 25.02 %, while the percentage of total input in MHN, LH, PRN, MVN and GRN is 13.09 %, 7.06 %, 5.70 %, 6.98 % and 15.29 %, respectively (Fig. 3).

3.3. The neural circuit connection is a direct evidence for NGF role in nerve recovery

The other goal of this work is to analyze the neural circuit connection of injured sciatic nerve after treatment with NGF. The mouse derived NGF was selected for repairing the injured sciatic nerve, since it has been widely used in clinical patient treatment. To analyze the repair efficacy of NGF on injured sciatic nerves, the number of all input neurons in the NGF or PBS groups was divided by the number of input neurons in the wild type group, respectively. The results showed that the total repair ratio of the NGF group was 29.05 %, while the PBS group was only 3.65 % (Fig. 4A). Furthermore, we selected GRN, MVN, and Tg brain regions as the example to analyze the repair ability of NGF. To calculate the recovery ratio of GRN, MVN, and Tg brain regions, the red neurons number in each brain region of NGF or PBS treatment groups was divided by the number of red neurons in the same brain region of wild type group, respectively. In the PBS-treated group, few signals were observed in the GRN, MVN, and Tg regions (Fig. 4B-E). The recovery ratio of GRN, MVN, and Tg is 9.3 %, 5.5 % and 1.7 %, respectively. In the NGF-treated group, many signals were observed in the GRN, MVN and Tg regions (Fig. 4B-E). The recovery ratio of GRN, MVN, and Tg is 62.8 %, 54.5 % and 14.4 %, respectively. Comparison of the data between the NGF or PBS treatment groups, we found that the recovery ratio of GRN, MVN and Tg regions in the NGF group is 6.8-fold, 9.9-fold and 8.5-fold higher than PBS group, respectively. The similar results were also observed in other brain regions, such as PAG, MHN, LH, PRN, RN, isRt, LL, VTA and other regions (Fig. 5). Collectively, these results indicated that NGF has a substantial ability to repair injured sciatic nerve.

4. Discussion

Sciatic nerve as the largest nerve in the body plays important roles in controlling the leg muscle movement and sending sensory messages from the leg to the spine. Determining its neural circuits in the CNS will be very useful in studying the function of the sciatic nerve and providing

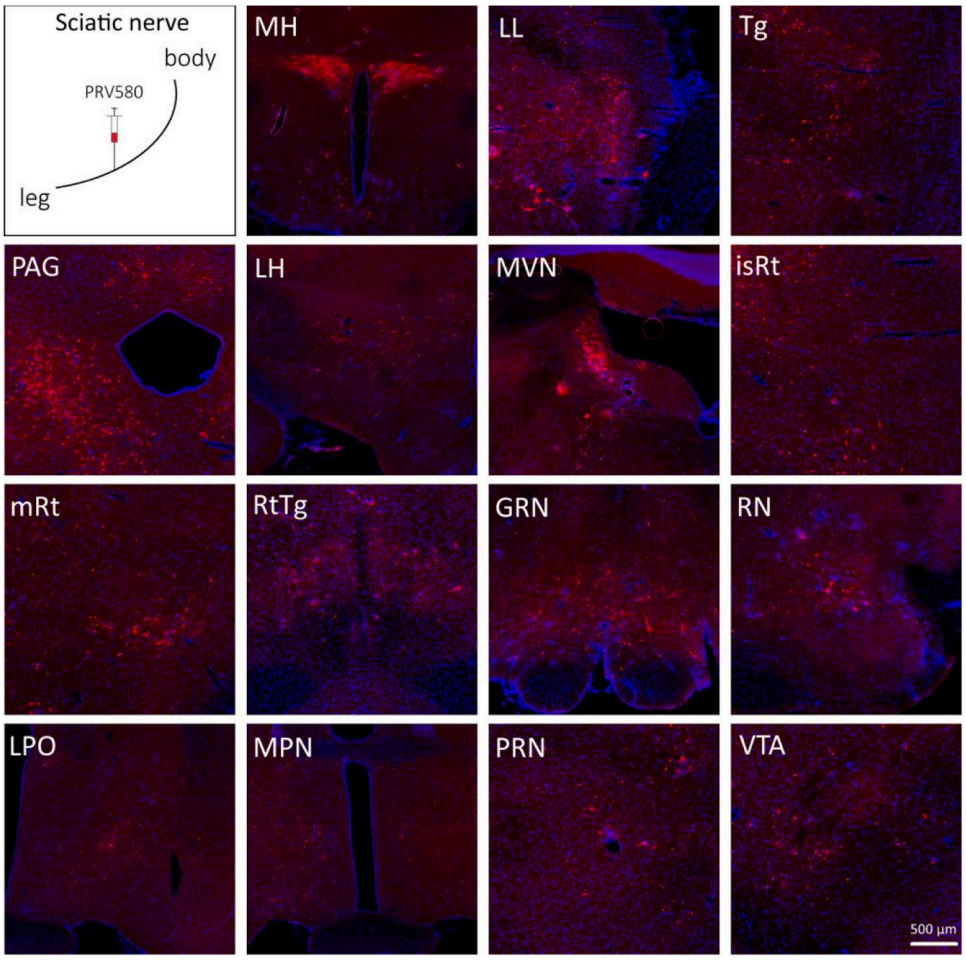

Fig. 2. The sciatic nerve pathway was mapped using reterograde trans-multisynaptic tracer PRV580. The PRV580 (1.5×10^{10} PFU/ml) was injected into the sciatic nerve of rat. The slices were stained with DAPI and imaged. The abbreviations of these labelled brain regions are as follow: medial preoptic nucleus (MPN), lateral preoptic area (LPO), periaqueductal gray (PAG), ventral tegmental area (VTA), medial hypothalamic nucleus (MH), lateral hypothalamic area (LH), pontine reticular nucleus (PRN), reticulotegmental nucleus of the pons (RtTg), lateral lemniscus (LL), medial vestibular nucleus (MVN), gigantocellular reticular nucleus (GRN), mesencephalic reticular formation (mRt), isthmic reticular formation (isRt), reticular nucleus (RN). In addition, the Tg region includes SPTg, PPTg, LDTg, and VTg.

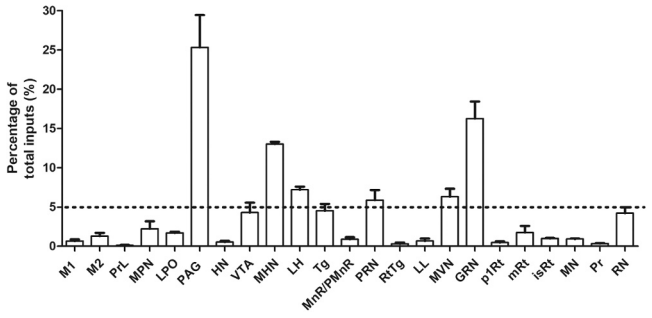

Fig. 3. The percentage of total inputs of the sciatic nerve pathway. The PRV580 (1.5×10^{10} PFU/ml) was separately injected into the sciatic nerve of three rats. To determine the percentage of total inputs of different brain regions for each animal, one third of all slices from each animal were selected for data analyzing. The percentage of total input is calculated as the number of red input neurons of each brain region divided by the number of all red neurons from all counted brain slices.

a new approach for treatment of sciatic nerve-related diseases. Sciatic nerve injury is a common disease in clinical practice. It is widely known that NGF has positive effect on nerve injury. We select the sciatic nerve as a nerve injury model and analyze its neural circuit after treatment with NGF using virus-based tracer.

Neurotropic virus-based tracers have been widely used for depicting the neural circuits of the CNS and the PNS (Nassi et al., 2015; Xu et al., 2020). In our study, we developed a useful tool PRV580 based on PRV Bartha strain to map the neural circuit of the sciatic nerve. Previous studies showed that the pyramidal system helps the body to maintain posture and perform involuntary motor functions by the direct innervation from the cortex to muscle, while the extrapyramidal system is responsible for the regulation of the movement by using the pathway connecting the motor areas (Lee and Muzio, 2021; Stejskalova et al., 2019). We found that many labelled brain regions belong to the pyramidal system and the extrapyramidal system (Fig. 3). Collectively, the percentage of total inputs of the pyramidal system and the extrapyramidal system is up to 56.83 %. These results indicated that the sciatic nerve pathway is involved in the movement, which is consistent with the common opinions (Lee and Muzio, 2021; Stejskalova et al., 2019). However, there are many brain areas that do not belong to the pyramidal system nor the extrapyramidal system. Among these regions, PAG is the most prominent region, which has 25.02 % in all total inputs from the sciatic nerve. As we know, PAG is an essential brain region, which integrates negative emotions with the autonomic, neuroendocrine, and immune systems for the generation of defensive reaction to threat (George et al., 2019). Quick escape behavior is the first response when the animal faces danger, and the process needs many neuronal activity

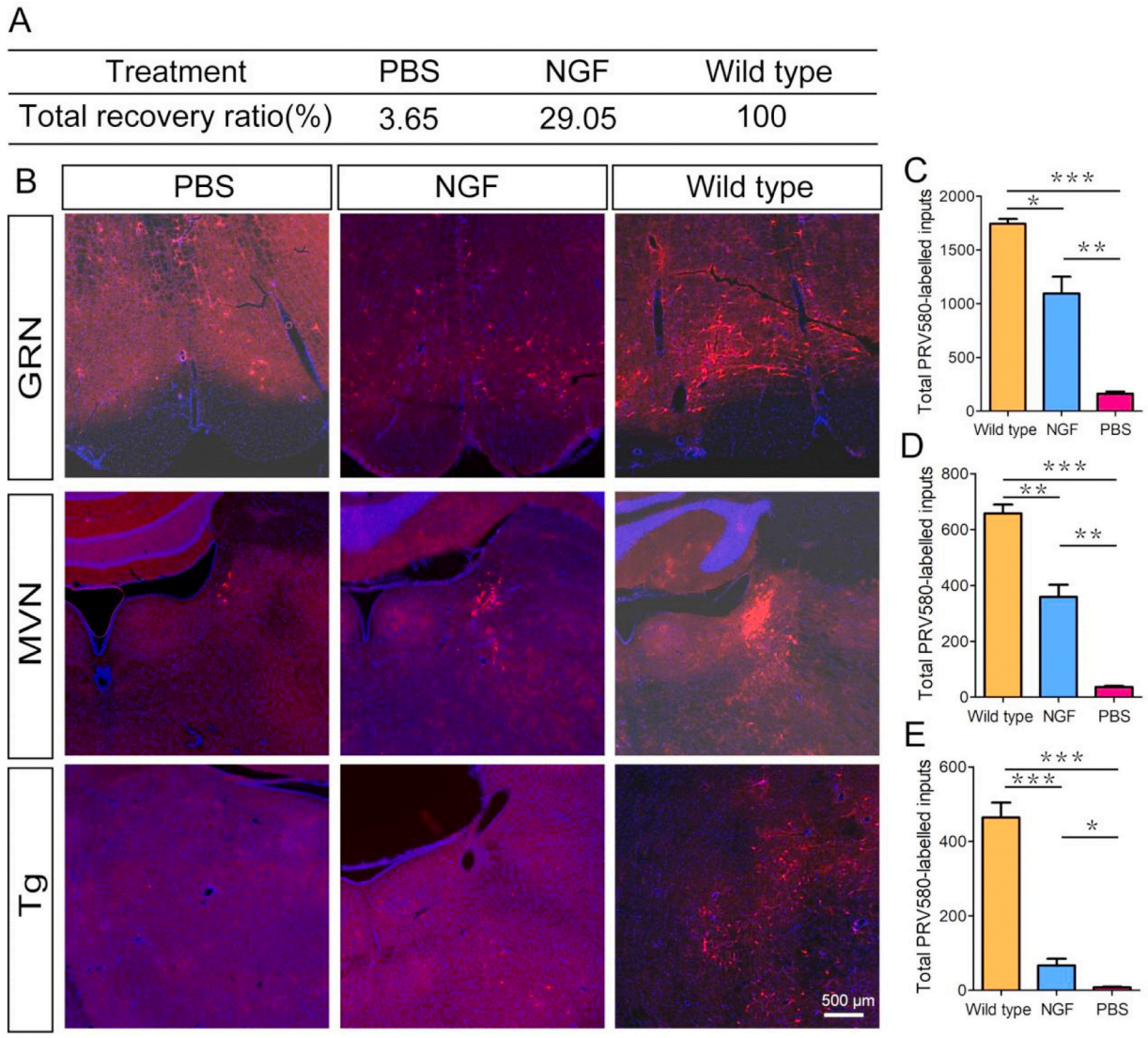

Fig. 4. mNGF can help the injured nerve to repair. (A) The total recovery ratio of the injured sciatic nerve with the help PBS and NGF, respectively. The sciatic nerves of three groups (each group has three rats) were treated with three strategies. PBS-treatment group: the sciatic nerve was completely cut, then sutured, followed with PBS treatment. NGF-treatment group: the sciatic nerve was sutured after transection, and then treated with 10 mg mNGF every day for three weeks. Wild type group: the sciatic nerve was not cut and without any treated. (B) The representative images in GRN, MVN and Tg regions. The labelled input neurons by PRV580 were counted in GRN (C), MVN (D) and Tg (E) in PBS, NGF and wild type groups. The t-test was conducted to compare the difference of recovery ability of the NGF and PBS. Significant differences between pairs are indicated by the p-value. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

from different brain regions to coorporate to achieve safety. Therefore, we hypothesize that PAG is also an important brain region involved in information processing during animal movement.

NGF belongs to a family of neurotrophic factors, which include brain-derived neurotrophic factor and neurotrophin-3. Various studies have shown that NGF can support the recovery of injured nerve (Derby et al., 1993; Machalinski et al., 2012; Onger et al., 2017). Definitely, NGF has the ability of enhancing nerve repair in our study (Fig. 4). However, the injured sciatic nerve is only partially recovered based on the neural circuit connection signals (Fig. 4). This phenomenon might stem from two potential reasons, one is that the two parts of the transection nerve are not fully linked like wild pattern after suturing the nerve, and the other is that NGF might be partially responsible for the axonal growth across the cutting site. Previous study show that NGF-sensitive axon has ability of extending along gradients towards the highest concentration of NGF (Cao and Shoichet, 2001; Ming et al., 2002). In clinical cases, 50 % patients had partial nerve recovery, 22 % patients had complete recovery, while 24 % patient without recovery (Simske et al., 2019). Our work provides a direct evidence to support the opinion of the different repair efficiency results from the recovery degree of the neural circuit connection.

Collectively, our study provides a convenient method for mapping the sciatic nerve pathway and a clear evidence for the injured sciatic nerve recovery after NGF treatment.

Ethical standards

Studies were performed following the National Guides for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committees at Innovation Academy for Precision Measurement Science and Technology, Chinese Academy of Sciences (APM20037A).

CRediT authorship contribution statement

Mei Hongjun : Conceptualization, Methodology, Project administration, Validation, Investigation, Data curation, Formal analysis,

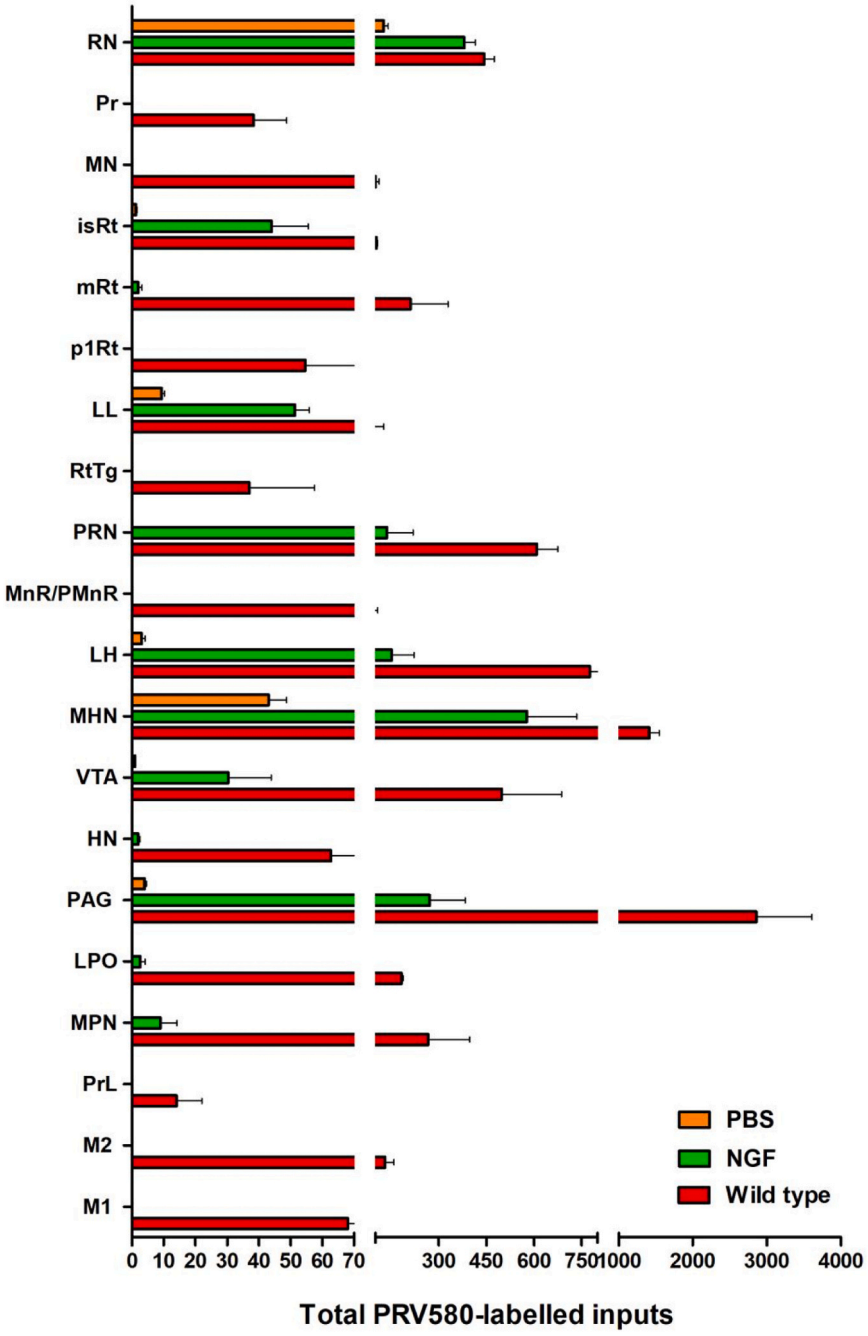

Fig. 5. An overview of the repair ability of NGF on injured sciatic nerve. The sciatic nerves of three groups (each group has three rats) were treated with three strategies. PBS group: the sciatic nerve was completely cut, then sutured, followed with PBS treatment. NGF group: the sciatic nerve was sutured after transection, and then treated with 10 mg mNGF every day for three weeks. Wild type group: the sciatic nerve was not cut and without any treated. The labelled input neurons by PRV580 were counted in different brain regions in PBS, NGF and wild type groups.

Writing – Original draft preparation, Writing – review & editing. **Tan Junfeng:** Methodology, Project administration,Validation. **Hu You:** Project administration,Validation, Data curation. **Shi Xiangwei:** Project administration, Resources, Validation. **Liu Yang:** Formal analysis, Data curation. **Jia Fan:** Conceptualization,Funding acquisition, Supervision, Investigation, Formal analysis, Writing- Original draft preparation, Writing – review & editing. **Xu Fuqiang:** Conceptualization, Funding acquisition, Supervision, Formal analysis.

Conflicts of Interest

The authors declare no competing financial interests.

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