Regenerative Therapy 1 (2015) 86–90

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original article

Neural circuit analysis of axons regenerated by facial–hypoglossal nerve cross-link surgery

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ARTICLE INFO

Article history:
Received 13 August 2014
Received in revised form
7 May 2015
Accepted 16 May 2015

Keywords:
Facial nerve
Hypoglossal nerve
Facial nerve palsy
Cross-link
Neural circuit

ABSTRACT

Introduction: Several methods of nerve reconstruction for facial nerve palsy are known. Although the recently introduced method of "cross-linking" of the facial and hypoglossal nerves with a grafted nerve has proved efficacious, the underlying mechanism is unclear.
Methods: In this study, we created an animal model with Wistar rats and analyzed the newly reconstructed neural circuit by anterograde and retrograde neural tracer methods. The saphenous nerve was harvested as a graft, and its double end-to-side neurorrhaphy with the facial and hypoglossal nerves with epineural windows was carried out under the microscope. After an appropriate interval, small amounts of fluoro-ruby or fluoro-emerald were injected into the animals and analyzed 5 days later by fluorescent microscopy (Anterograde experiment: fluoro-ruby into the hypoglossal nucleus at 5 weeks; retrograde experiment: fluoro-ruby into the distal facial nerve sheath and fluoro-emerald into the distal hypoglossal nerve sheath, both at two months.).
Results: The labeled axons derived from the hypoglossal nucleus were observed passing through the grafted nerve to the facial nerve. On the other hand, retrogradely labeled neurons were observed at both the hypoglossal and facial nuclei with some double-labeled neurons, suggesting that collateral sprouting had occurred.
Conclusions: We suggest that the newly constructed neural circuits we observed are conducive to the treatment of facial nerve palsy.

1. Introduction

The treatment and concepts of nerve injury described in animal models of palsy involve physical transection and are very different from those of neurodegenerative diseases such as Bell's palsy, in which the axons degenerate instead of being physically damaged.

Numerous reports on the treatment of facial nerve palsy (FP) show that the approach to treatment must distinguish between total and partial FP. For total FP, surgeons need not hesitate to transect the facial nerve stem for direct suturing to other motor nerves end-to-end (ETE) or end-to-side (ETS). For example, the cut end of the facial nerve is sutured to the transected hypoglossal nerve stump (Hypoglossal facial anastomosis; HFA) [1,2]; in other cases, however, the cross-face nerve graft is used [3,4].

In partial FP, however, transection of the facial nerve sacrifices its remaining functions, and the purpose of facial nerve reconstruction must be to enhance the electric signal intensity reaching the musculature. With this in mind, one may totally or partially transect the hypoglossal nerve and anastomose the cut end to the facial nerve by ETS neurorrhaphy or use an interpositional graft between the hypoglossal and facial nerves [5,6]. These surgical techniques provide fair results by retaining facial muscle tone, with the drawbacks of synkinesis of facial muscle movement and total or partial sacrifice of the functions of the hypoglossal nerve.

In general, total or partial functional sacrifice of the donor motor nerve is unavoidable in end-to-side neurorrhaphy. In the neural supercharge (here called "cross-link"; CL) type of nerve reconstruction [7], double end-to-side neurorrhaphy is made on both termini of the grafted nerve, which is a sensory nerve

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Peer review under responsibility of the Japanese Society for Regenerative Medicine.

http://dx.doi.org/10.1016/j.reth.2015.05.003

harvested for this purpose. For example, a harvested great auricular nerve may be transplanted and sutured to the facial nerve and the hypoglossal nerve with double end-to-side neurorrhaphy. The purpose of CL in facial nerve palsy is reinforcement of the weakened function of the facial nerve with hypoglossal axonal invasion as a motor source. In fact, some clinical cases have shown improvement of facial muscle kinesis, and an axonal supercharge from the hypoglossal nerve to the facial nerve has been proposed to account for this [7]. Some questions remain, however. Does axonal supercharge actually take place? If it does, is it caused by collateral sprouting, by terminal sprouting, or both? If not from the facial nerve, where does the axonal supercharge originate? We here created a rat CL model and analyzed the formation of the associated neural network by anterograde and retrograde tracer methods to gain insight into the mechanism of CL.

2. Materials and methods

2.1. Animals

Adult female rats purchased from a local supplier were housed at about 24 °C on a 12/12-h L/D cycle in acrylic cages with wood-chip bedding and unlimited access to normal laboratory chow and water. All experiments were carried out with the approval of

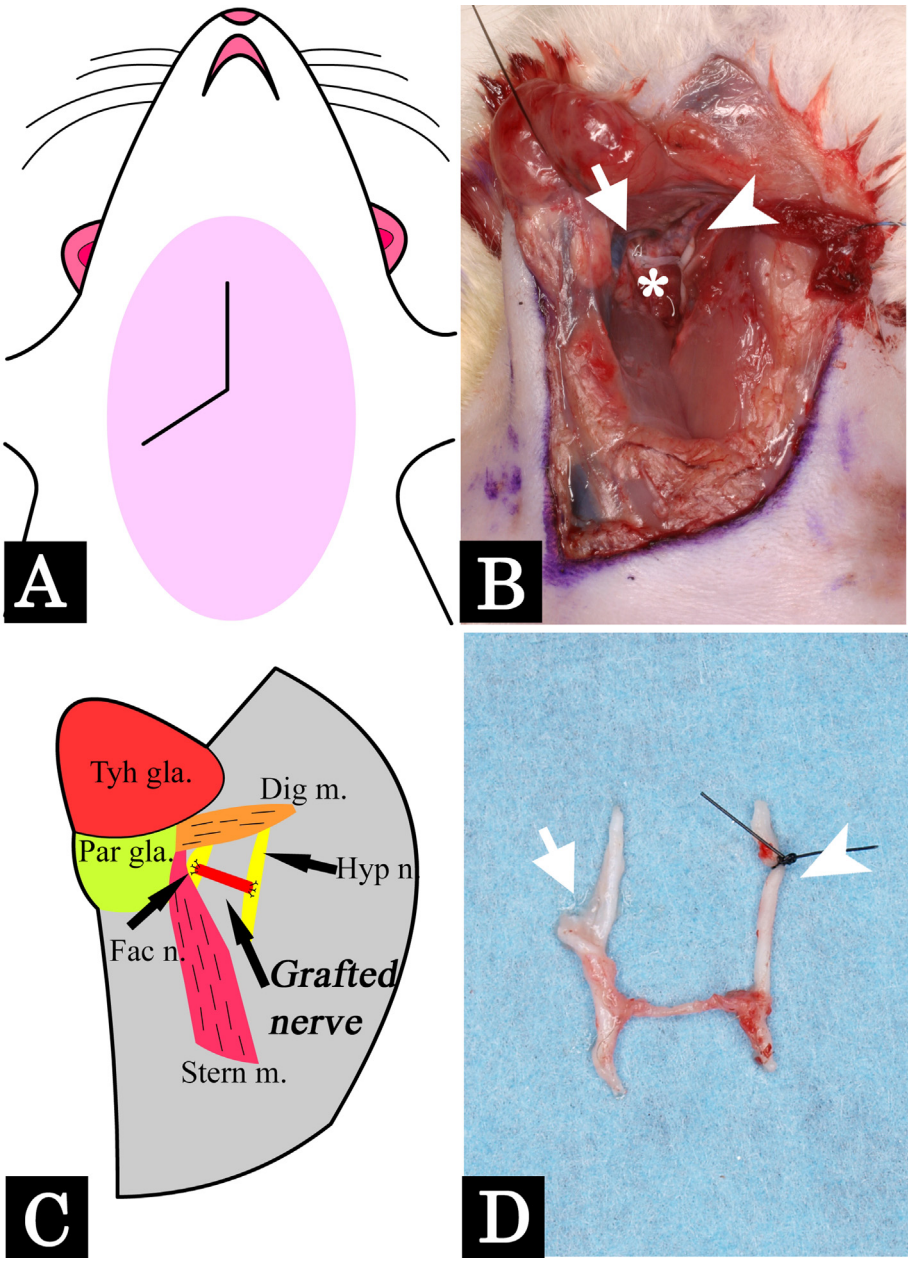

Fig. 1. "Cross-link" operation. A–C) A rat is fixed in the supine position and the saphenous nerve is grafted between the hypoglossal nerve and facial nerve in the "cross-linking" arrangement. D) The grafted nerve is harvested together with the hypoglossal and facial nerves, presenting an overall "H" shape. Arrow indicates facial nerve, arrowhead indicates hypoglossal nerve, and * indicates the grafted nerve. Thy gla, thyroid gland; Par gla, parotid gland; Stern m, sternocleidomastoid muscle; Dig m, digastric muscle; Fac n, facial nerve; Hyp n, hypoglossal nerve.

the Committee on Animal Care and Welfare, Kobe University School of Medicine.

2.2. Cross-linking between facial and hypoglossal nerves

The animals were deeply anesthetized with pentobarbital (47 mg/kg) administrated by intraperitoneal injection, fixed in a supine position, and operated to expose the facial nerves just beneath the parotid gland (Fig. 1A). The hypoglossal nerves were then exposed medial to the digastric muscle. About 2 cm of the saphenous nerve on the femur was then harvested and trimmed (Fig. 1B). Double end-to-side neurorrhaphy of the grafted saphenous nerve, the facial nerve, and the hypoglossal nerve was carried out under the microscope using epineural windows, with 11-0 black nylon suture (Fig. 1C).

2.3. Anterograde labeling of facial and hypoglossal nerves

Five weeks after the cross-link operation, the animals were anesthetized with pentobarbital (47 mg/kg) and clamped in a stereotactic apparatus (Narishige, Tokyo). A small burr hole was made with a dental drill in the medial part of the occipital bone and 1 μL of 10% fluoro-ruby was injected into the nucleus of the hypoglossal nerve with a Hamilton syringe wear on the glass tip (n = 3). Five days thereafter, the animals were transcardially perfused with 10% buffered formalin and their hypoglossal, facial, and grafted nerves were harvested as an "H"-shaped tissue piece (Fig. 1D). This was immersed in 5% buffered sucrose (pH 7.4) at 4 °C overnight then in 20% buffered sucrose and frozen in a 2:1 mixture of 20% buffered sucrose-OCT compound (Sakura Tek, Japan) as previously described [8]. The nerves were then sliced into 10-μm-thick sections in a cryostat.

2.4. Retrograde labeling of facial and hypoglossal nerves

Two months after the cross-link operation, tracers were injected directly into the distal parts of the nerve sheaths (n = 2). A bolus of 1 μL of 10% fluoro-ruby (Invitrogen, USA; Cat D1817) diluted with distilled water was injected into the facial nerve, and the same quantity of fluoro-emerald (Invitrogen, USA; Cat D1820) was injected into the hypoglossal nerve. Five days thereafter, the animals were killed by perfusion with 10% formalin in 0.1 M phosphate buffer (PB) at room temperature, and the brains removed. The brains were immersed overnight in 0.1 M PB at 4 °C ontaining 20% sucrose for cryoprotection, and then sliced into 40-mm-thick

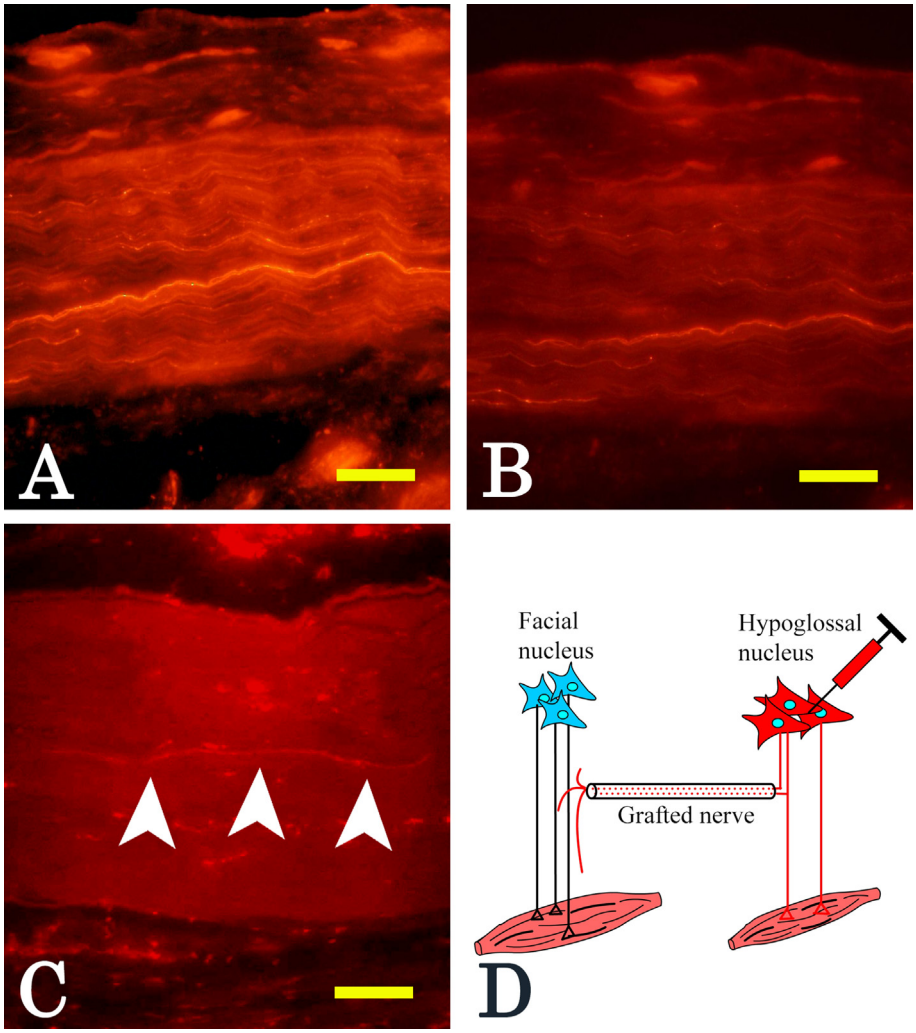

Fig. 2. Anterograde labeling of hypoglossal axons. Labeled axons are seen as red fluorescence. A) Hypoglossal nerve stem. B) Grafted nerve. C) Facial nerve stem. Arrowheads, labeled axons derived from hypoglossal nuclei; Scale bars, 100 μm. D) Schematic drawing shows axonal sprouting of the hypoglossal nerve revealed by the red fluorescence.

sections on a freezing microtome. The sections were mounted on gelatin-coated slides and cover slipped.

3. Results

3.1. Anterograde labeling

The small volume of fluoro-ruby solution injected into the right nucleus of the hypoglossal nerve (nHGN) sufficed to show anterogradely labeled fibers exhibiting red fluorescence running along the HGN (Fig. 2A), a small number crossing in the grafted nerve (Fig. 2B), and a few entering the facial nerve (Fig. 2C). Scheme is shown in Fig. 2D.

3.2. Retrograde labeling

In both nuclei, neurons were retrogradely labeled ipsilaterally. The neurons in the nHGN were mainly labeled fluorescent green (Fig. 3A), and those in the nucleus of the facial nerve (nFN), fluorescent red (Fig. 3B). A few neurons in nHGN were labeled red (Fig. 3A, arrow), and a few in nFN green (Fig. 3B, arrow). Furthermore, a small number of neurons in nHGN were labeled yellow (Fig. 3A, arrowhead), resulting from a merging of red and green fluorescence, indicating that these neurons extended axon collaterals into the facial nerve through the grafted nerve. Scheme is shown in Fig. 3C.

4. Discussion

In this study, axonal invasion (axonal supercharge) was observed from the hypoglossal nerve to the facial nerve through the grafted nerve. Although the reverse was not observed, some nFN neurons were labeled retrogradely with the green fluorescent material injected into the distal part of the hypoglossal nerve, indicating that facial nerve axons also invaded the hypoglossal nerve through the grafted nerve. Moreover, some neurons in the hypoglossal nucleus were labeled yellow, a merging of the red and green fluorescence, suggesting the presence of collateral sprouting. For reasons unknown, no yellow-labeled neurons were observed in the facial nucleus. Since non-operated (not cross-linked) animals showed no crossed nuclear uptake of tracer between facial and hypoglossal nerves (data not shown), our results indicate that the neural circuit responsible for crossed uptake had been newly formed by cross-linking.

Peripheral nerves are known to be capable of regeneration after injury, if properly repaired; however, the methods of repair differ depending on the extent of injury. The simplest method is end-to-end neurorrhaphy, although in many cases, suturing the cut ends

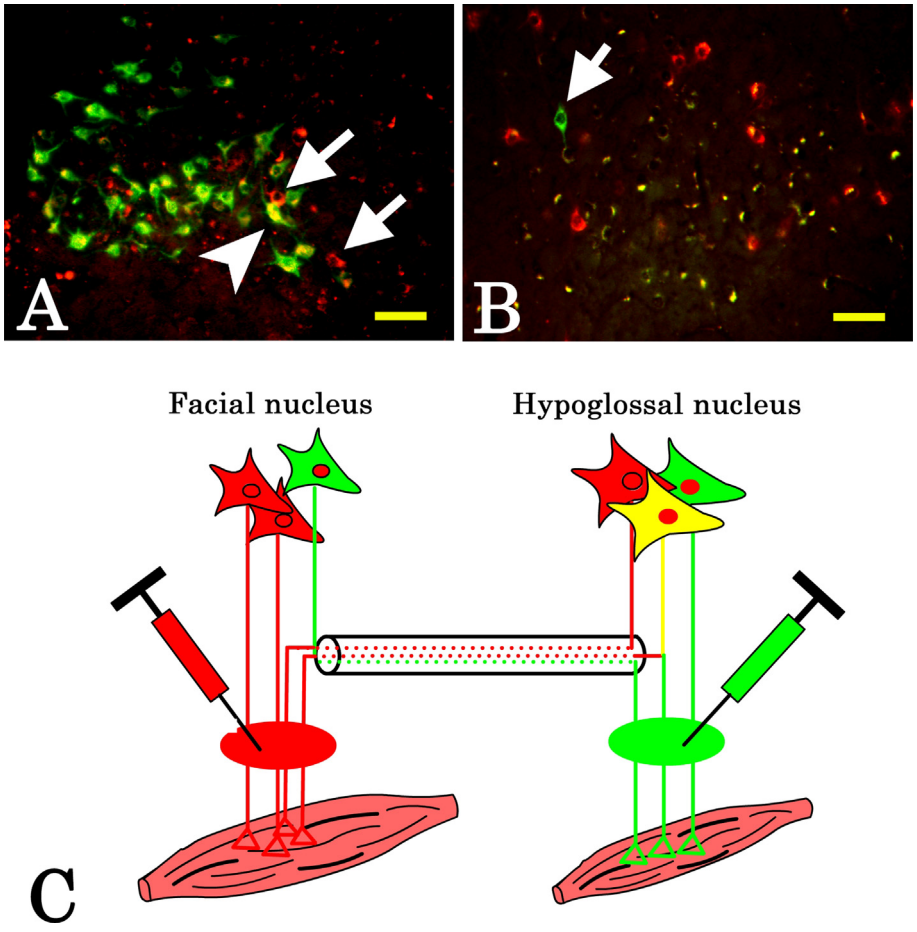

Fig. 3. Retrograde labeling of hypoglossal and facial nucleus neurons. A) Neurons in the hypoglossal nuclei are mainly labeled fluorescent green and a small number are labeled red (arrow). A small number of neurons are labeled both green and red, the superposition of which is observed as an apparent yellow fluorescence (arrowhead). B) Neurons in the facial nuclei are mainly labeled red with some labeled green (arrow). Scale bars, 50 μm. C) As shown in the schematic drawing, the neuron that takes up both green and red dye shows a merged fluorescence (yellow).

directly is challenging because of nerve defects attributed to soft tissue injury. In such cases, nerve grafting is a possible solution, but sacrificing donor nerve function is unavoidable. The recently reintroduced technique of end-to-side (ETS) neurorrhaphy [9,10] incorporates two forms of axonal regeneration: terminal sprouting and collateral sprouting [11]. Collateral sprouting via epineural windows occurs more frequently than terminal sprouting via perineural windows. However, the latter involves partial neurotomy or axonal damage [12,13]. Therefore, reconstructing damaged nerves by ETS is preferable in that sacrifice of donor nerve function is minimal.

In our experimental model, exclusively epineural windows were created on both the facial and hypoglossal nerves. The necessity of the perineural window remains controversial. The perineurium prevents axonal sprouting [14,15]. However, many laboratories have reported axonal sprouting without a perineural window, and some have shown sprouting without an epineural window [16]. In our study, epineural windows were created in the cross-linking operation and our result therefore supports the view that an epineural window is sufficient for axonal sprouting.

Our results showed axonal sprouting from both the facial and hypoglossal nerves. Although the detailed mechanism of CL is still unknown, axonal supercharge from the hypoglossal nerve to the facial nerve plays a predominant part in clinical cases [7], where axonal sprouting from weakened facial nerves is unlikely. We speculate that surgeons may be able to regulate the direction of axonal supercharge by making different types of windows at the termini of the transplanted nerve (for example, an epineural window on the facial nerve and a perineural window on the hypoglossal nerve, or no window on the facial nerve and an epi- or a perineural window on the hypoglossal nerve).

Axons extended by collateral sprouting are seen to degenerate at long observation times [17]. Indeed, Shichinohe et al. showed a few retrograde-labeled neurons in both nFN and nHGN after a CL [18] but could not detect double-labeled neurons. However, they had injected the neural tracer 3 months after the CL operation, which is a longer postsurgical waiting period than ours is. In these situations, 2 or 3 months is long enough to permit the sprouted collateral axons to degenerate. In addition, their experimental number was small ($n = 4$) and they only analyzed every third section, so that the possibility of seeing double staining was further reduced. Our study showed collateral extension at 2 months with a small number of cases ($n = 2$). Therefore, further experiments with shorter and longer waiting periods are needed.

Our CL model was created with healthy rats without facial palsy; others have created facial palsy models with the use of hemostatic forceps. Hayashi et al. showed axonal sprouting proximal at some distance to the forceps-damaged region [17], where regeneratively sprouting axons may extend into a grafted nerve. Consequently, our model is inadequate for demonstrating the validity of the CL method in clinical cases of chronic facial palsy.

Although our CL model is based on clinical cases of facial nerve palsy, it may be relevant to upper- and lower-limb nerve reconstruction. When damage to the nerve is total, surgeons need not hesitate in creating end-to-side neurorrhaphy by transecting the paralyzed nerve. When nerve damage is partial, however, transection of the damaged nerve is suboptimal because of loss of precious residual function and axonal supercharge with CL would be a better therapeutic choice.

5. Conclusions

"Cross-linking" of the facial and hypoglossal nerves with a nerve graft provides a newly constructed neural circuit conducive to the treatment of facial nerve palsy. Because our study is based advantageously on an uninjured animal model of facial nerve palsy, further study with this model is desirable.

Conflict of interest

The authors have declared no conflicts of interest.

Acknowledgments

This research was supported by "Japan Society for the Promotion of Science," Grant-in-Aid for Scientific Research (C) 19592075.

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