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HYPOGLOSSAL-FACIAL 'SIDE'-TO-SIDE NEURORRHAPHY COMBINED WITH ELECTRICAL MYOSTIMULATION FOR FACIAL PALSY IN RATS

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Abstract

Introduction This study investigated the effect of combining hypoglossal-facial nerve "side"-to-side neurorrhaphy and electrical myostimulation in a rat model of facial palsy.

Methods Rats with facial nerve crush injury were subjected to control condition, monotherapy of either neurorrhaphy or electrical myostimulation, or bitherapy of the two treatments. After 1, 3, and 6 months, rats were performed the facial symmetry evaluation, electrophysiological examination and the retrograde labeling of motor neurons.

Results As early as 3 months after injury, face symmetry significantly improved in rats of the bitherapy group. At 3 or 6 months after injury, either the parameters of electrophysiological examination or the number of labeled motor neurons were significantly increased in the bitherapy group than in any other group.

Discussion The combination of neurorrhaphy and electrical myostimulation effectively promoted the functional recovery after facial nerve crush injury.

Keywords

Facial palsy • Neurorrhaphy • Electrical myostimulation • Motor action potential • Retrograde labeling • Animal model

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Introduction

Facial palsy, a commonly encountered syndrome in the clinic, arises from various causes including inflammation, cranial trauma and the removal of cerebellopontine angle tumors [1]. Although the injured facial nerve (FN) remain anatomically intact in most clinical cases, the capacity of spontaneous axonal regeneration is difficult to anticipate [2,3]. This uncertain prognosis represents an obstacle to the physician's decision making. Generally, most surgeons recommend neurorrhaphy to patients only if no satisfactory functional recovery is achieved after a period of conservative treatments [4]. However, this delay results in the atrophy of the denervated target muscles, which prevents their functional reinnervation when a remedied neurorrhaphy is performed [5,6]. Even when repair surgery is performed early, the long duration of axonal regeneration, which takes up to 2 years in patients, increases the risk of facial muscle degeneration [7]. Hence, interventions that would simultaneously promote axonal regeneration and maintain facial muscle activation represent an interesting therapeutic option.

Electrical stimulation, first used for pain relief, has become a widely used method to treat various neuromuscular disorders [8,9]. With the development of electrotherapy, several new stimulating techniques, such as brief electrical stimulation (BES) and electrical myostimulation (EMS), have been introduced into neurological practice, rehabilitation medicine, sports medicine and psychiatry [10-13]. They have been shown to confer significant functional benefits by promoting axonal regeneration in both animal and clinical studies. Mendez et al via implantation stimulator after facial nerve stem cutting and neuroanastomosis demonstrated that BES could accelerate the recovery of whisker muscle movement, whereas Foecking et al following a facial nerve crush injury animal models demonstrated that the application of a single 30 min BES immediately will further reduce the time to complete recovery of facial paralysis. [14,15]. Moreover, Gordon et al. showed that BES combined with *in situ* neurorrhaphy between the common peroneal nerve and the tibial nerve could improve nerve regeneration and functional outcomes [16]. Additionally, several clinical units have used transcutaneous electrical nerve stimulation to achieve satisfactory functional improvement of the facial muscles of patients suffering from facial palsy [17].

Over the past years, interest has been growing for protecting denervated muscles from atrophy. For example, EMS has been introduced for preventing sarcopenia, defined as muscle weakness and mass loss [18]. Many studies have shown that EMS can significantly improve muscle functions in patients suffering from end-stage renal disease, chronic heart failure, chronic obstructive pulmonary disease and other severe conditions [19-23]. Furthermore, in trained athletes, EMS has shown effectiveness in enhancing the strength

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and function of the lower leg muscles [24]. There are also studies indicating that the regular EMS increase the oxidative capacity and enhance glucose disposal of the paralyzed muscles, to partly counteract the neurotrophic deficiency after the nerve injury [25,26] .

In the present study, considering both axonal regeneration and the status of the target muscles as crucial elements for the successful recovery of facial motor control, we combined neurorrhaphy with EMS after crush injury of the rat facial nerve. The aim was to develop a new bitherapy for incomplete facial palsy. We have previously reported a surgical procedure of "side"-to-side neurorrhaphy between the hemisectioned hypoglossal nerve (HN) and the injured FN through a predegenerated nerve graft (PNG). An advantage of this surgical method is to preserve spared epineurium and to allow facial axons' regeneration. It was first developed in a rat model of FN crush injury, and was then successfully translated to the clinic for patients with facial palsy [27,28]. Here, we show experimentally that simultaneous EMS significantly improves the recovery of facial symmetry and functions. After FN crush injury and hemi-HN-FN "side"-to-side neurorrhaphy, rats underwent repeated daily EMS of the whisker pad muscle. Functional outcomes and motoneuron survival were evaluated at 1, 3 and 6 months after injury.

Methods

Animals

Seventy-two adult male Wistar rats (weight 180-200 g) were used in this study. All rats were kept under an artificial cycle of 12 h of light and 12 h of darkness and were fed standard laboratory food and water. The experimental project was approved by the local Animal Ethics Committee (KY2016-056-01).

Rats were randomly divided into four groups (n=18), receiving different treatments after crush injury of the FN: 1) hemi-HN-FN neurorrhaphy alone, 2) hemi-HN-FN neurorrhaphy combined with routine EMS (bitherapy), 3) routine EMS alone or 4) no treatment (control group). At 1, 3, and 6 months after the surgery, each group was randomly divided into three subgroups of 6 rats each.

Surgical procedures

Surgeries were performed by an experienced surgeon under a neurosurgical microscope (M400-E, Leica, Germany). Eight rats across the 4 groups (2 rats per group) underwent surgery each day. The rats were randomly selected from each group, and the order of the surgeries was randomized.

Rats were placed under general anesthesia by intra-peritoneal injection of sodium pentobarbital (54 mg/kg). The right FN region was chosen as the surgical site. The whole FN trunk was exposed, beginning at its emergence from the stylomastoid foramen and continuing to the bifurcation. A crush lesion was inflicted at the very beginning of the FN trunk using microforceps and pressure was maintained for 1 minute. This procedure resulted in the section of all axons but spared the epineurium, leaving 1 mm-wide gap. Preservation of the epineurium allowed spontaneous regeneration of the injured FN axons to imitate the clinical incomplete facial palsy in which the FN was anatomically intact **(Fig. 1A)**.

For hemi-HN-FN neurorrhaphy which has been introduced in our previous study [27], the right HN trunk was exposed and hemisectioned at the site closest to the FN. At the level of the FN, an epineurial window 5 mm distal to the lesion site was carefully created using microscissors. Thereafter, a PNG prepared 1 week beforehand from the same animal (procedure was described below) was harvested. The proximal stump of the PNG was joined in an end-to-'side' ('side' represents here the hemisection of HN trunk with maintaining its continuity) neurorrhaphy with the hemisectioned HN, while its distal stump was anastomosed to the epineurial window of the FN in an end-to-side fashion. The surgical wound was then closed with 4-0 nylon sutures **(Fig. 1B, 1C)**.

The PNG was prepared 1 week before its removal for neurorrhaphy. Rats underwent the same general anesthesia as described above. The right sural nerve was exposed through a skin incision on the right medial thigh followed by dissection of the septum between the semi-membranosus and the gracilis. A crush

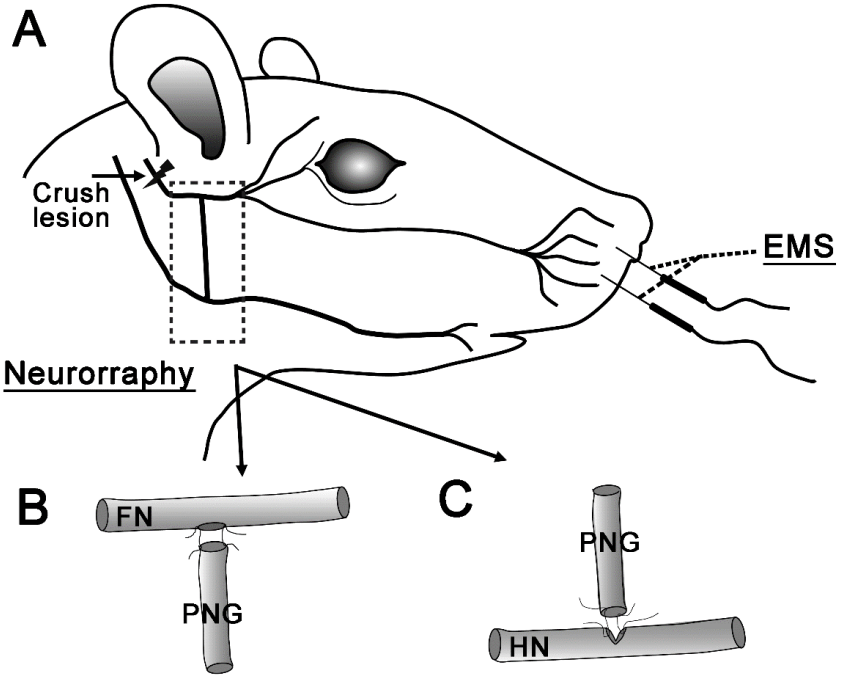

Figure 1. Schematic drawings showing the neurorrhaphy and the EMS procedure. **A.** A crush injury was made at the initial site where the FN emerges from the stylomastoid foramen. A hemi-HN-FN 'side'-to-side neurorrhaphy through a PNG was then performed immediately after the injury. Routine EMS was performed once a day using two electrodes inserted into the whisker pad muscle. **B.** The anastomosis between the FN and the PNG. An epineurial window was unilaterally created on the FN trunk, which was then anastomosed with the distal site of PNG in an end-to-side fashion. **C.** The anastomosis between the PNG and the HN. The HN was hemisectioned, and the proximal site of the PNG was anastomosed with the sectioned site of the HN in an end-to-'side' fashion.

injury was inflicted by compressing the initial level of the sural nerve with microforceps for 1 minute. It was verified under a microscope that all axons in the sural nerve were sectioned and the epineurium was maintained. The surgical wound was closed with 4-0 nylon sutures, and rats were returned to their cages. The mobility of the right hindlimb was preserved after the surgical intervention.

After the surgical procedures, buprenorphine (0.05mg/kg) is subcutaneously injected 3 times with interval of 8 hours, while the amitriptyline (15 mg/kg/d) is orally administrated in the first postsurgical week for rats, aiming to reduce the pain due to the surgeries and prevent the possible neuropathic pain caused by the nerve injury.

Electrical myostimulation (EMS) therapy

In groups assigned to daily EMS therapy, rats were anesthetized by respiration of isoflurane every morning (initial dose of 4.0% for 3 min, then a maintaining dose of 2.0%, gas flow rate of 2 L/min). Stimulation (0.1 ms, 10 mA) was continuously delivered by an electrical stimulator (Neuro-MEP-micro, Neurosoft, Russia) at a frequency of 1 Hz through two electrodes (0.2-mm diameter) inserted into the right whisker pad **(Fig. 1A)**. EMS was performed once per day, lasting 10 minutes per session. The daily treatment order of the rats was randomly decided by another researcher, who was blind to the group information. After recovering consciousness, rats were returned to their cages.

Evaluations

1. Facial symmetry

The face of each rat was photographed at the following time points: before FN lesion and 1 week, 1 month, 3 months and 6 months after FN lesion. Angle α, which was defined as the acute angle formed by a line extending from the fold on the bridge of the nose and a line linking the two outer canthi, was measured for each rat based on the photos. Facial symmetry and its recovery were evaluated by comparing angle α between groups (α = 90° corresponds to perfect symmetry).

2. Electrophysiological examination

To evaluate functional reinnervation by the FN, muscle action potentials (MAPs) were recorded from the right whisker pad muscle while the PNG or FN trunk was electrically stimulated using a Neuro-MEP-micro electromyogram (Neurosoft, Russia). Recordings were collected at 1 month, 3 months and 6 months after injury. Rats were reanesthetized with pentobarbital as for the initial surgical procedure. A single stimulation (0.1 ms, 1 mA) was delivered through two electrodes (0.2 mm diameter) placed on the middle of the PNG or on the FN trunk very distal to the neurorrhaphy site. MAPs were recorded by two electrodes (0.2 mm diameter) inserted into the right whisker pad muscle at the most proximal and distal sites of the muscle belly, thus covering the entire muscle. The amplitude and the area under the curve (AUC) were measured from the recorded potentials and used to represent the quantity of synchronic conductive axons and the quality of the conductive synchronism, respectively.

3. Retrograde labeling

The neural tracer CTB-Alexa 555 (Molecular Probes, Eugene, Oregon, USA) was used to retrogradely label axon regrowth from the facial or hypoglossal nucleus to the paralyzed facial muscles, either via the initial FN or via the reconstructed PNG pathway.

At 1, 3, and 6 months after surgery, 6 rats were randomly selected from each group for retrograde labeling. After the electrophysiological test, while the rats were still under pentobarbital anesthesia, a 10-µl volume of 1% CTB-Alexa 555 solution was injected at multiple points into the right whisker pad muscle using a 10-µl Hamilton syringe. Thereafter, rats recovered consciousness and were returned to their cages. Five days later, rats were euthanized by an anesthetic overdose (pentobarbital 120 mg/kg) and underwent intracardiac perfusion with 300 ml of phosphate-buffered saline (0.1 M, pH 7.2) followed by 200 ml of 4% paraformaldehyde (PFA) solution. The brainstem was harvested and postfixed in the 4% PFA solution for 3 hours.

After the gradual dehydration in successive <20%, 30% and 40% sucrose solutions, all specimens were immersed in optimal cutting temperature (OCT) compound and then cross-sectioned at a thickness of 30 µm on a cryostat (Leica CM1950, Germany). The labeled motor neurons in the facial nucleus and hypoglossal nucleus were identified and quantified under a fluorescence microscope (Nikon Diaphot, Japan) by another researcher blinded to the group assignments.

Statistical analysis

The data are all presented as the mean ± standard error of mean (SEM). GraphPad Prism 7.0 (USA) was used for statistical analysis. The data were analyzed by two-way ANOVA with factors of treatment and time. Intergroup differences were analyzed by performing post hoc Bonferroni tests. P values ≤ 0.05 were considered statistically significant (### or ***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05).

Results

During the follow-up period, no rat suffered from either the surgeries or the routine treatments, based on our observations of their physical status and activities in their home cages. The surgical wounds healed well.

Recovery of facial symmetry

Angle α was measured to evaluate changes in facial symmetry after FN injury and in response to treatments. It corresponds to the acute angle between a line extending from the fold on the bridge of the nose and a line linking the outer corners of the two canthi, as defined before. The average of α is about 90° before FN injury **(Fig. 2B)**. Interaction between the treatment and time after the FN lesion was shown by two-way ANOVA ($F_{(12, 215)} = 12.36$; $p < 0.001$). One week after FN lesion, α decreased to approximately 71.9° in all four groups, without statistical inter-group differences. One month after treatment, α slightly increased to 73.04 ± 0.36° in the bitherapy group, but there was still no statistically significant difference compared with other groups. At 3 months after surgery, α increased in all groups except the control group, but to different extents. In particular, there was a significant difference between the bitherapy group (α = 75.07 ± 0.33°) and the control group

(α = 71.68 ± 0.30°; p < 0.001). At the end of the 6-month follow-up period, facial symmetry had markedly recovered in the bitherapy group (α = 80.78 ± 0.30°), but also significantly improved in the in the neurorrhaphy and EMS monotherapy groups, although to a lesser extent, with α reaching approximately 76°. In contrast, the angle value in the control group remained low at 71.68 ± 0.31 °. Statistical analysis showed that the bitherapy group significantly differed from the monotherapy groups and the control group (p < 0.001). Moreover, statistically significant differences also existed between the monotherapy groups and the control group (p < 0.001) **(Fig. 2A)**.

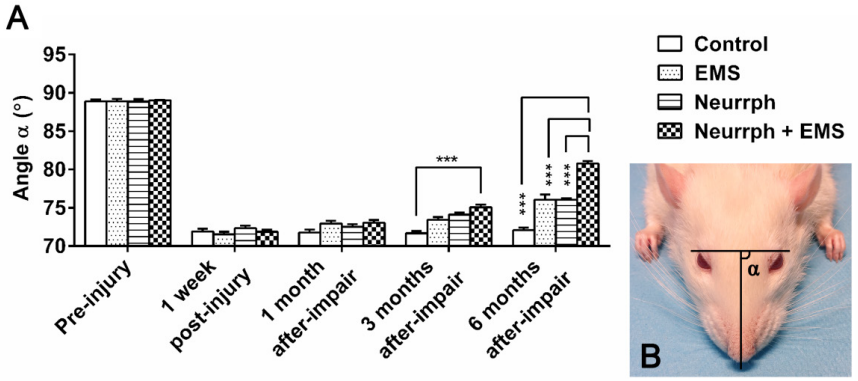

Figure 2. The measure of angle α for the assessment of facial symmetry. **A.** Angle α was measured for all rats before injury and 1 week after FN injury, 1 month, 3 months and 6 months after FN repair. Results are presented as the means ± SEM and were analyzed by two-way ANOVA. The effects of time ($F_{(4, 215)} = 1087$; $p < 0.001$), treatment ($F_{(3, 215)} = 33.23$; $p < 0.001$) and the interaction between time and treatment ($F_{(12, 215)} = 12.36$; $p < 0.001$) were all significant. Post hoc multiple comparisons with a Bonferroni correction were performed between groups at different time points. ***, $p < 0.001$ between the angle α in the bitherapy group and the values in the other groups at the same time point. **B.** The angle α was defined as the acute angle formed by a line extending from the fold on the bridge of the nose and a line linking the two outer canthi.

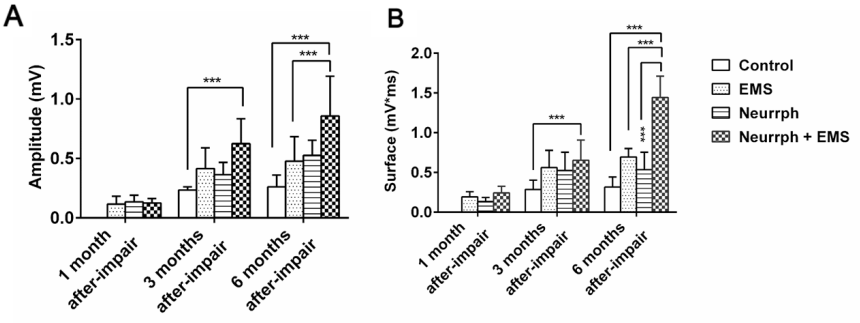

Figure 3. The two-way ANOVA analysis followed post hoc multiple comparisons with a Bonferroni correction for amplitude and AUC of MAPs, which were collected at the paralyzed whisker pad muscle during electrical stimulation of the FN trunk at 1, 3, and 6 months after FN repair. **A.** The amplitude values. Time effect ($F_{(2, 44)} = 45.6$; $p < 0.001$), treatment effect ($F_{(3, 44)} = 15.68$; $p < 0.001$) and interaction between time and treatment ($F_{(6, 44)} = 2.67$; $p < 0.05$) were significant. *, $p < 0.05$ between the bitherapy group and the EMS group at 6 months. **, $p < 0.01$ between the bitherapy group and the control group at 3 months. ***, $p < 0.001$ between the bitherapy group and the control group at 6 months. **B.** The AUC values. Time effect ($F_{(2, 49)} = 60.97$; $p < 0.001$), treatment effect ($F_{(3, 49)} = 30.89$; $p < 0.001$) and interaction between time and treatment ($F_{(6, 49)} = 9.03$; $p < 0.001$) were significant. ***, $p < 0.001$ between the bitherapy group and the other groups at 3 or 6 months.

Electrophysiological examination

In order to measure electrical conduction in both residual FN and reconstructed PNG nerve pathways, MAPs were recorded from the right whisker pad muscle in response to electrostimulation of either the FN or the PNG trunk. The amplitude and the area under the curve (AUC) of the recorded potentials were measured to evaluate the effectiveness of nerve conduction.

Regarding the FN pathway, two-way ANOVA showed significant interactions between treatment and time after the FN lesion for both MAP amplitude ($F_{(6, 44)} = 2.671$; $p < 0.05$) and AUC ($F_{(6, 49)} = 9.028$; $p < 0.001$). These two parameters increased in all groups with time elapsed since injury. At one month after injury, FN stimulation produced weak MAPs in the 3 treated groups, with an amplitude of 0.12 ± 0.01 mV and an AUC of approximately 0.20 ± 0.03 mV•ms, while no signal was recorded in the control group. After 2 months, the amplitude and AUC values of the bitherapy group reached 0.62 ± 0.09 mV and 0.65 ± 0.09 mV•ms, respectively. Post hoc Bonferroni tests indicated significant differences when compared with the control group for amplitude (0.36 ± 0.05 mV; $p < 0.05$) and AUC (0.28 ± 0.03 mV•ms; $p < 0.01$), respectively. There were no significant differences in amplitude or AUC between the bitherapy group and the monotherapy groups ($p > 0.05$).

At the end of 6 months after injury, post hoc Bonferroni tests showed significant differences in amplitude when the bitherapy group (0.94 ± 0.26 mV) was compared with the EMS only group (0.75 ± 0.19 mV) ($p < 0.05$) and the control group (0.35 ± 0.05 mV) ($p < 0.001$) **(Fig. 3A)**. Regarding the AUC, the bitherapy group (1.25 ± 0.16 mV•ms) showed a significantly higher value than either of the two monotherapy groups (0.74 ± 0.15 mV•ms and 0.59 ± 0.09 mV•ms) or the control group (0.47 ± 0.12 mV•ms) ($p < 0.001$) **(Fig. 3B)**.

After stimulation of the PNG in the neurorrhaphy groups, both amplitude and AUC of MAPs increased with the duration of the follow-up period. Two-way ANOVA indicated that there was an interaction between treatment and time for AUC ($F_{(2, 43)} = 30.35$; $p < 0.001$), but not for amplitude ($F_{(2, 36)} = 1.467$; $p = 0.244$). Bonferroni post hoc tests showed significant difference in AUC at 6 months after surgery between the two groups (1.68 ± 0.08 mV•ms versus 0.68 ± 0.14 mV•ms, $p < 0.001$) **(Fig. 4)**.

Retrograde labeling

At each evaluated time point, motor neurons in both facial nucleus and hypoglossal nucleus were retrogradely labeled in 6 rats per group by injecting the tracer CTB-Alexa 555 in the right whisker pad muscle **(Fig. 5A, 5B)**.

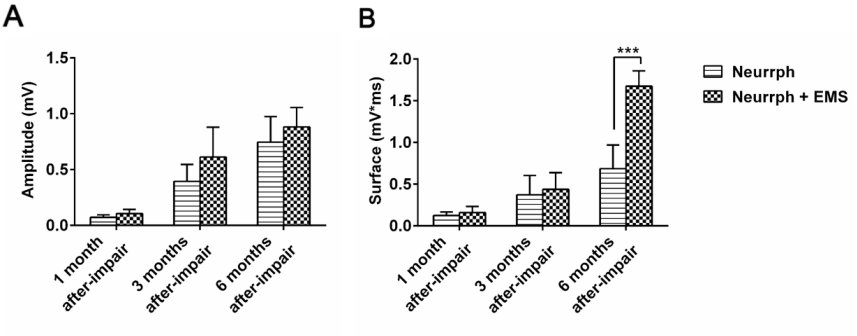

Figure 4. The two-way ANOVA analysis followed post hoc multiple comparisons with a Bonferroni correction for amplitude and AUC of MAPs, which were collected at the paralyzed whisker pad muscle during electrical stimulation of the PNG at 1, 3, and 6 months after FN repair. **A.** The amplitude values. Time effect ($F_{(2, 36)} = 79.5$; $p < 0.001$) and treatment effect ($F_{(1, 36)} = 7.12$; $p < 0.01$) were significant, but there was no interaction between time and treatment ($F_{(2, 36)} = 1.467$; $p = 0.244$). No significant difference between the two groups was found at each time point, however the bitherapy group presented the higher amplitude values. **B.** The AUC values. Time effect ($F_{(2, 43)} = 130.1$; $p < 0.001$), treatment effect ($F_{(1, 43)} = 51.99$; $p < 0.001$) and interaction between time and treatment ($F_{(2, 43)} = 30.35$; $p < 0.001$) were significant. ***, $p < 0.001$ between the bitherapy group and the neurorrhaphy group at 6 months.

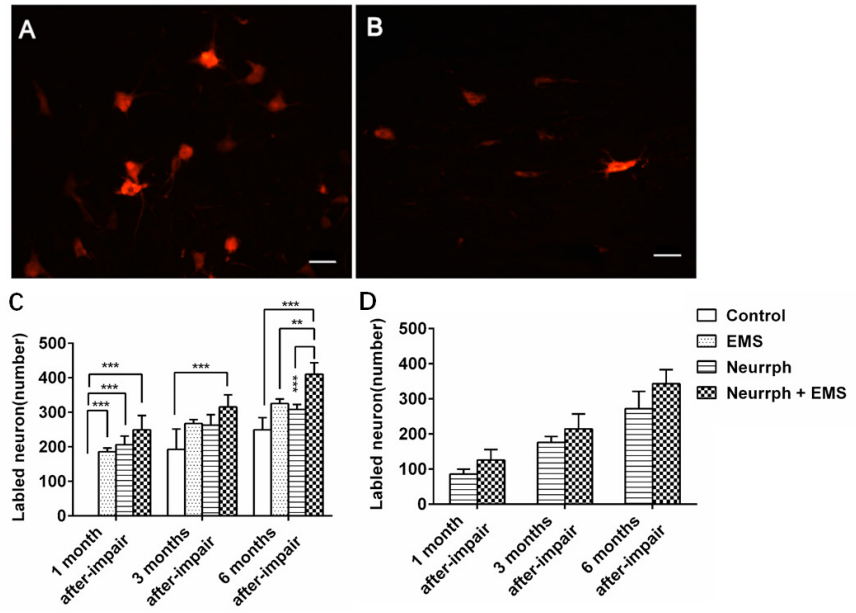

Figure 5. The CTB-Alexa 555 retrogradely labeled motor neurons in the facial nucleus and the hypoglossal nucleus. **A.** Labeled motor neurons (in red) in the facial nucleus at 6 months. **B.** Labeled motor neurons (in red) in the hypoglossal nucleus at 6 months. Bar = 50 µm. **C.** The two-way ANOVA followed Bonferroni's post hoc multiple comparisons of number of labeled neurons in the facial nucleus for each group at 1, 3, and 6 months after repair. Time effect ($F_{(2, 37)} = 120.8$; $p < 0.001$), treatment effect ($F_{(3, 37)} = 73.02$; $p < 0.001$) and interaction between time and treatment ($F_{(6, 37)} = 6.06$; $p < 0.001$) were significant. **, $p < 0.01$ between the bitherapy group and the EMS group at 6 months. ***, $p < 0.001$ between the bitherapy group and the other corresponding groups at 3 or 6 months. ###, $p < 0.001$ between the control group and the treated groups at 1 month. **D.** The same statistical analysis of number of labeled neurons in the hypoglossal nucleus for each group. No interaction between time and treatment was found ($F_{(2, 20)} = 0.64$; $p = 0.538$), while time effect ($F_{(2, 20)} = 74.34$; $p < 0.001$) and treatment effect ($F_{(1, 20)} = 13.74$; $p < 0.001$) were significant. There was no significant difference of labeled neuron number between the two groups, while the bitherapy group presented more labeled neurons than the neurorrhaphy group at each time point.

Regarding the number of labeled neurons in the hypoglossal nucleus, which corresponds to innervation of the whisker pad via the PNG pathway, no interaction was shown by a two-way ANOVA between EMS therapy and time ($F_{(2, 20)} = 0.64$; $p = 0.538$). Moreover, Bonferroni post hoc tests did not show significant differences between the bitherapy and the EMS monotherapy groups for the number of labeled hypoglossal motoneurons at each time point ($p > 0.05$) **(Fig. 5D)**.

Spontaneous reinnervation via the FN pathway, represented by the number of labeled neurons in the facial nucleus, showed a significant interaction between treatment and time by two-way ANOVA ($F_{(6, 37)} = 6.061$; $p < 0.001$). At 1 month after repair, no retrograde-labeled neurons were found in the control group, revealing the absence of spontaneous reinnervation at this early time point. In contrast, about 200 labeled facial motor neurons were observed in the three treated groups, with no significant differences between them ($p > 0.05$ by post hoc Bonferroni tests). However, at 3 and 6 months after repair, about 200 labeled neurons were also observed in the control group, reflecting a marked delay in spontaneous axonal regeneration when compared with the treatment groups. At 3 months after repair, the number of labeled neurons in the bitherapy group (316 ± 18) was significantly higher than in the control group (192 ± 20; $p < 0.001$). More apparently, at 6 months after injury, the bitherapy group showed more labeled neurons (410 ± 15) than the neurorrhaphy group (308 ± 7; $p < 0.001$), the EMS group (326 ± 6; $p < 0.01$) or the control group (275 ± 15; $p < 0.001$). These differences were significant as indicated by a post hoc Bonferroni tests. **(Fig. 5C)**

Discussion

After injury to the facial nerve, the facial muscles become weak and may undergo degeneration [29]. Muscle atrophy becomes not only a particularly serious problem when the decision making of repair surgery is delayed because of uncertain prognosis, but also remains an issue after early neurorrhaphy because it may take up to two years to regain satisfactory facial recovery in clinic [30,31]. For this reason, paralyzed facial muscle training is often recommended [32].

Here, we experimentally demonstrate an additive beneficial effect of EMS and hemi-HN-FN "side"-to-side neurorrhaphy in a rat model of facial nerve crush injury. Of note, the combined positive effects of EMS and neurorrhaphy only

became evident after a follow-up period of 3 months, and they became particularly important after 6 months, indicating that the maintenance of muscle activity during the entire process of FN recovery is very important. Thus, angle α, which reflects facial symmetry, was significantly higher for the combined treatment when compared with neurorrhaphy or EMS alone only at 6 months after injury. A similar situation was observed for MAP amplitudes (corresponding to the number of synchronic discharging axon terminals) and AUCs (reflecting the quality of axonal discharging synchronism and muscle reinnervation) measured at the whisker pad muscle after electrical stimulation of the FN. Interestingly, already at 3 months after injury, facial symmetry and MAPs were significantly improved by the bitherapy when compared with the control group, whereas at this earlier time point, the monotherapies still did not differ from the control. Thus, combining EMS and neurorrhaphy accelerates the process of functional recovery.

Importantly, benefits of combined EMS and neurorrhaphy were observed long before functional improvements for retrogradely labeled facial motor neurons innervating the whisker pad muscle. Already at 1 month after injury, their number was highest in the group receiving bitherapy. The bithepapy's advantage was maintained between 1 and 6 months after injury, during the progressive increase in the number of retrogradely labeled facial motor neurons. This result indicates that it is important to start regular myostimulation as soon as possible after repair surgery.

An intriguing observation was that EMS did not improve the number of retrogradely labeled motor neurons of the hypoglossal nucleus innervating the whisker pad muscle after hemi-HN-FN neurorrhaphy, at any time point studied. Indeed, the number of retrogradely labeled motor neurons increased progressively and to a comparable level in both neurorrhaphy alone and bitherapy groups. Likewise, MAP amplitude after electrical stimulation of the PNG did not differ between the neurorrhaphy alone and bitherapy groups. Thus, between 1 and 6 months after hemi-HN-FN neurorrhaphy, the number of connecting hypoglossal motoneurons and the amplitudes of MAPs after stimulating the hypoglossal reinnervation pathway progressively increased, whether the target whisker pad muscles were electrically stimulated or not. Taken together, our results suggest quantitively that combined EMS and neurorrhaphy is mainly beneficial for the regeneration of the initial facial axons. However, the whole FN conductive pathway, from the motor cortex to the target muscle, was regularly activated by routine EMS, which also promoted transformation of cortical function between the tongue and face areas when combining EMS with HN-FN neurorrhaphy [33]. Therefore, in spite of a similar number of hypoglossal axons innervating the whisker pad muscle, revealed by retrograde labeling and the measure of MAP amplitudes, the longer-term quality of the hypoglossal innervation of facial muscles was improved by their electrical stimulation. Indeed, after 6 months, AUC values were strongly improved by the bitherapy.

Another important finding was that hemi-HN-FN neurorrhaphy alone accelerated the reinnervation of whisker pad muscles by facial motor neurons. At 1 month, about 200 retrogradely labeled motor neurons were counted in the facial nucleus after neurorrhaphy, whereas no retrogradely labeled neurons were observed in the control group. This result suggests that repair surgery should be performed as early as possible after incomplete facial nerve injury in order to promote the regeneration of spared facial axons.

Rapidly after incomplete facial nerve injury, hemi-HN-FN "side"-to-side neurorrhaphy can be proposed as the most advantageous surgical procedure. In contrast to "side"-to-end neurorrhaphy, with transection of remaining facial nerve tissue, "side"-to-side neurorrhaphy preserves the remaining facial axons. Our present results show that their regeneration is promoted by an early surgical intervention and the rapid start of target muscle stimulation.

Crush injury of the FN of course does not perfectly reflect all clinical cases of incomplete FN injury. After FN crush, all axons are sectioned. However, as the epineurium is preserved, a significant percentage of them has the potential of regenerating and remaking functional connections with the whisker pad muscle. This explains why for some of the outcome measures the EMS alone was as efficient as the neurorrhaphy alone in our study. However, this model allowed us to demonstrate that regenerating facial neurons can make a significant contribution to the recovery of facial functions, and that their regenerative capacity is supported by *ex situ* neurorrhaphy and target muscle stimulation.

The study has other limitations, which may be addressed in subsequent studies. First, the angle α used to evaluate facial muscle symmetry cannot be simply correlated with the House-Brackmann (H-B) score system in humans. The angle α mainly depends on the function of the whisker pad muscle, but fails to provide information concerning functions of the orbicularis oculi muscle; hence, it does not globally reflect the symmetry of the facial muscles as the H-B score does. Second, EMS as performed in this study, with two needle electrodes inserted into the target muscle, can barely activate the trigeminal-cortex-facial pathway (compared with adhering cutaneous chip electrodes to the paralyzed face). Whether the additional activation of the trigeminal-cortex-facial pathway is more effective for functional transformation could be investigated in further studies. Furthermore, on account of the effect of EMS, different levels of the stimulation parameters, such as the intensity and frequency, would probably need to be studied in order for clinical translation.

Conclusions

This study shows that a hemi-HN-FN 'side'-to-side neurorrhaphy via a PNG combined with EMS effectively improves the functional recovery of the crush-injured FN in adult rats. The neurorrhaphy provides sufficient regenerated axonal resources, while the EMS enhances the target muscle's reinnervation and activates the conductive pathway of the nerve. This optimal bitherapy presents an additive effect that is more effective than treatment with either of the two interventions alone.

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Abbreviations:

AUC: area under the curve;
BES: brief electrical stimulation;
EMS: electrical myostimulation;
HN: hypoglossal nerve;
FN: facial nerve;
MAP: muscle action potential;
PFA: paraformaldehyde;
PNG: predegenerated nerve graft.

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