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# Functional, Structural and Molecular Analyses Indicate that Injections of Trophic Factors into the Paralyzed Whiskerpad Muscles after Facial Nerve Injury Improve Vibrissal Motor Performance in Rats

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Polyinnervation of neuro-muscular junctions (NMJs) in reinnervated mimic muscles is a major reason for poor motor recovery (vibrissal whisking) after transection and suture of the facial nerve. Recent own molecular biological analyses showed parallelism between better recovery of motor performance and increased amounts of brain derived neurotrophic factor (BDNF) and fibroblast growth factor 2 (FGF2) in denervated vibrissal muscles. The aim of the present study was therefore to test the effects of substitutive therapies with intramuscular injections of these trophic factors. Following transection and end-to-end suture of the buccal branch of the rat facial nerve (buccal-buccal anastomosis, BBA), we injected the paralyzed vibrissal musculature with different concentrations of BDNF and FGF2 at different postoperative periods. Video-based motion analysis of vibrissal whisking followed. We found that rats receiving BDNF over 14-28 days after injury showed the highest whisking amplitude.

*Key words:* nerve injury, motor recovery, motor endplates, BDNF, FGF2

# Introduction

### *Three components of the misdirected regrowth of transected axons*

Peripheral nerve transection results in poor function restoration and the inevitable development of a "post-paralytic syndrome" (paresis, synkinesis, and dysreflexia). It is widely accepted that incorrect reinnervation of the muscle targets is the cause of this insufficient recovery [43].

First, transected and regenerating axons are misrouted and fail to re-join their original nerve fascicles [47].

Second, each transected axon gives off up to 25 "collateral" branches within the nerve itself. Excessive collateral branching leads to reinnervation of several muscle groups, often with antagonizing action, by one single motoneuron or innervation of one muscle by more than one motoneuron, i.e. polyneuronal innervation [72]. It is thought that this kind of axonal misdirection is mostly responsible for abnormally associated movements.

The intramuscular axonal sprouting is a third issue. Axons divide again after they reach their target, reinnervating a large number of muscle fibers [39, 69]. Sprouting is thought to be an adaptive response to a decrease in functional capacity. It does, however, also have a "maladaptive" side, characterized by the enlargement of the motor units [21] and by the reinnervation of neuro-muscular junctions (NMJ) by more than one axon, a state known as "polyinnervation" [58].

### *Improved pathfinding by transected axons to reach the original fascicle*

Little is currently known about potential solutions for this issue. The degree of collateral axonal branching at the lesion site was only slightly reduced by surgically guided axonal regrowth into a 3-way-conduit. However, neither an improvement in whisking performance nor a decrease in NMJ-polyinnervation accompanied this trend. When combined, the findings imply that the 3-way-conduit does not provide any further functional benefit following surgical reconstruction of the facial nerve, even if it is not an impediment for axonal regeneration [8].

### *Reduction of collateral branching does not enhance recovery*

Utilizing a well-established technique for measuring vibrissae motor performance [27, 30], Streppel et al. investigated the possibility that a decrease in collateral axonal branching at the lesion site [70] may enhance function recovery. They discovered that whisking did not improve despite this reduction, indicating that functional performance was not primarily hampered by post-transectional collateral branching.

### *Reduced polyinnervation of NMJ promotes motor recovery*

Further research revealed that the percentage of muscle endplates with polyneuronal reinnervation strongly correlated with the degree of functional recovery [29].

It has been demonstrated that motor endplate polyinnervation and intramuscular sprouting are reduced when denervated muscles are mechanically stimulated [3, 46]. Probably by activating cAMP signaling [52] or by restoration of neurotrophins and synaptic plasticity [73].

### *Muscle reinnervation's cellular correlates: the function of terminal Schwann cells*

The issue which is currently addressed is, how mechanical stimulation reduces intramuscular axonal sprouting. Whereas it might be expected that the reduced number of axon terminals reaching one motor endplate after stimulation is a consequence of fewer cell processes emanating from the terminal Schwann cells (TSC; cellular correlate), the question about possible reduction of sprouting-inducing stimuli (molecular correlates) generated by the denervated muscle fibers and TSC is still open. This is why, current knowledge about the role(s) of TSC and expression of trophic factors/cytokines by denervated muscles are briefly reviewed in the following sections.

Adjacent still denervated motor endplates can be reached by processes that enlarge and sprout from just reenervated TSC [22]. TSC can reach, draw, and guide intramuscular axonal sprouts toward the denervated endplates by means of these bridges [33, 38, 57]. Interestingly, research has demonstrated that TSC processes emerge before sprouts from growing intramuscular axons do; in other words, TSC initiate intramuscular axonal sprouting [68]. Hence, by obstructing TSC processes' expansion and capacity to bridge between endplates, stimulation may have a positive effect on muscle reinnervation. Recent reports have shown similar findings with disrupted TSC bridge generation, albeit following running exercise [71] or electrical stimulation [46]. Therefore, any type of mechanically stimulated muscle contraction may prevent TSC from forming bridges and lessen intramuscular sprouting following a lesion.

### *Muscle reinnervation and molecular correlates: BDNF is a stimulus inducing sprouting*

The low percentage of polyinnervated motor endplates found in the vibrissal muscles (e.g., *m. levator labii superioris*) following mechanical stimulation may be explained by decreased levels of sprouting-inducing stimuli [29]. Short-range diffusible sprouting stimuli have been demonstrated to be produced by denervated muscles [18, 54, 66, 75]. Numerous neurotrophic factors have been suggested as potential contenders for this function [23, 56, 63]. Muscle activity has an inverse relationship with their amount [10, 11]. Ultimately, peripheral nerve fibroblasts that produced high levels of BDNF were shown to accumulate in tissues from the proximal and distal nerve stumps following nerve transection, according to mRNA sequencing [34].

The brain-derived neurotrophic factor (BDNF) influences the dynamic branching of axonal arbors [14, 36, 75] and effectively promotes axonal outgrowth [17, 48, 49, 51, 67]. Application of BDNF has been shown to prevent neuronal cell death after nerve lesion [64] improving functional recovery of injured motor nerve after root avulsion [32], but not after transection of the rat sciatic nerve [65]. Three days after sciatic nerve transection in rats, mRNA sequencing showed that peripheral nerve fibroblasts accumulated in the proximal and distal nerve stumps and expressed large amounts of BDNF. *In vitro*, BDNF secreted from peripheral nerve fibroblasts increased the expression of **β**-actin and F-actin through the extracellular regulated protein kinase and serine/threonine kinase pathways, and enhanced motoneuron neurite outgrowth [34]. The neutralization of BDNF reduces the arborization of axons [15] and the length of regenerated nerves [74], decreases the average axon length [20, 50] and diminishes the nerve conduction velocity and muscle action potential duration [12].

The basic fibroblast growth factor (FGF-2) "stimulates *in vivo* neurite outgrowth from the proximal stump of transected peripheral nerves and contributes to the enlargement of axon caliber" [1, 2, 4, 24, 25, 37, 42, 53, 60]. Its neutralization causes a significant decrease in the number of regenerating axons [13]. Following transection of the buccal branch of rat's facial nerve there occurs a rapid upregulation of bFGF-immunoreactivity in the distal nerve stump and in the target muscles of the whisker pad at 1 day post lesion (DPL). This immunoreactivity reaches a first peak at 2 DPL, declines at 4 DPL, and climbs to a second peak at 5-6 DPL. A gradual decline follows at 8 DPL [70].

### *Quality of muscle reinnervation is decisive for recovery of function*

To elucidate the mechanisms that may promote recovery of motor function the expression of trophic factors in denervated (after transection and suture of the buccal branch of the facial nerve) rat facial muscles has been analyzed by immunofluorescence and by *in situ* hybridization. As a rapid and strong increase was found, it has been supposed that this may correlate with the collateral axonal branching at the lesion site and hypothesized that a neutralization of those trophic factors could reduce collateral branching. After transecting the facial nerve trunk, Streppel et al. (2002) placed both ends into a silicon tube filled with collagen gel and antibodies to NGF, BDNF, FGF2, IGF-I, CNTF, and GDNF at neutralizing concentrations. The percentage of motor neurons, whose axons had split and projected concurrently into three primary fascicles of the facial trunk, was estimated two months later using retrograde labeling. Neutralizing concentrations of anti-neurotrophins, anti-FGF-2 and anti-IGF-I significantly reduced this collateral axonal branching [70].

Immediately thereafter another study checked whether reduced collateral branching would promote a better functional recovery. Surprisingly, the results of vibrissae whisking video-based motion analysis (VBMA) did not reveal any beneficial effects, indicating that collateral axonal branching at the lesion site is not the essential limiting factor for function recovery. This is why researchers decided to concentrate their work on the level of polyinnervation of NMJ. It was found that polyinnervated NMJ comprised 51%, whereas in blind RCS animals with a perfect recovery of motor performance they were only 10%. The conclusion learned from these experiments was that the quality of muscle reinnervation (poly- or mono-innervated NMJ) plays a decisive role for the recovery of function [29].

### *Manual stimulation of paralyzed muscles reduces NMJ-polyinnervation and improves recovery of function*

Denervated muscles have few clinical alternatives for treatment. Electrical stimulation (ES) is one option, albeit there is a lot of debate over its application. Electrical stimulation of the denervated soleus muscle reduces motor-end plate polyinnervation and prevents intramuscular sprouting [10, 46]. Nevertheless, ES of denervated muscle fibers inhibits the generation of chemical mediators necessary for an axon branch to reconnect with its NMJ. It also lessens the spontaneous electrical activity of orphaned muscle fibers, known as fibrillation, which is believed to be a stimulus for the motor nerve that is starting to grow again. For the reasons listed above, ES is not a recommended treatment for facial paralysis and has not even been applied extensively.

We therefore decided to try a novel approach and used manual stimulation of muscles after nerve injury. Based on clinically established positive benefits of soft

tissue massage, supposed to promote muscle blood flow and to keep it in optimum condition whilst awaiting nerve regrowth, we gently stroked the vibrissal, the suprahyoid-sublingual and the orbicularis oculi muscles by hand for 5 minutes daily for two months after nerve injury. Video-based motion analysis showed that daily manual stimulation resulted in full recovery of whisking, tongue position and eye closure. Polyneuronal reinnervation of motor end-plates was reduced to 10% [3, 9, 19, 28]. This treatment's success was confirmed by other studies [31, 35, 41, 45].

***Trophic factors play a key role in NMJ-reinnervation***

Looking for the reasons for this very beneficial effect of manual stimulation we started to explore the role of trophic factors. We focused on IGF-1, BDNF and FGF-2 and studied the quality of reinnervation of vibrissal muscles and recovery of whisking function after FFA in mice deficient in

– IGF-1+/- (STOCKIgf1tmTs/ImJ,003258, Jacksons Laboratory),
– BDNF$^{+/-}$ (STOCK *Bdnf*$^{tm1Jae}$/J, 002267, Jackson Laboratory), and
– FGF2$^{-/-}$ (strain *Fgf2*$^{tm1Zllr}$ C57/Bl6).

Controls were wild-type (WT) littermates and intact animals. We quantified vibrissal motor performance [5] and determined the percentage of NMJ bridged by S100-positive terminal Schwann cells (TSC).

We found that IGF-1 [40], BDNF [67] and FGF2 [61] are required for proper target muscle reinnervation and recovery of whisking function.

***Orchestrated trophic factors´ expression regulates muscle reinnervation and the recovery of motor whisking function after FFA in blind rats***

*In situ* hybridization studies showed that, till 1 week after the lesion, the main source of trophic factors´ release at the lesion site were the adjacent Schwann cells. Thereafter blood-borne immunoreactive macrophages invaded the lesioned nerve [70].

Likewise, in paralyzed muscles, the terminal Schwann cells (TSC) have been shown to produce short-range diffusible neurotrophic factors. To learn more about their effects we decided to determine which trophic factors may be responsible for the reinnervation of the neuro-muscular junctions (NMJs) in:

(i) the facial muscles in Sprague Dawley (SD)-rats with poor recovery of facial motor function (whisking) after facial nerve injury (50% of NMJs are poly-innervated) and in

(ii) the facial muscles of the blind SD/Royal College of Surgeons (RCS) rats with complete recovery of motor function (only 10% of NMJs are poly-innervated).

Our quantitative measurements showed that functioning reinnervation of mimic muscles in the blind SD/RCS rats was accompanied by (1) an early increase in FGF2 and IGF2 at 2 days after FFA, (2) reduced NGF between 2 and 14 days after FFA, (3) a late rise in BDNF at 14-28 days after FFA and (4) reduced IGF1 at 28 days [23]. These findings show that recovery of motor function after peripheral nerve injury is associated with a precisely orchestrated expression of regeneration-associated neurotrophic factors and cytokines in the denervated muscles [7]. The increase of FGF-2 protein and concomitant decrease of NGF during the first week following FFA in SD/

RCS blind rats possibly prevent the intramuscular (terminal) sprouting of regenerating axons resulting in reduced poly-innervation of motor endplates [23].

To investigate this hypothesis, we performed a buccal branch of the facial nerve transsection and suture (buccal-buccal anastomosis, BBA), and administered combinations of BDNF, anti-BDNF, and FGF2 at several intervals and doses following BBA operation into the levator labii superioris muscle, which moves the vibrissae. By surgically transecting and reconstructing just the buccal branch of the facial nerve, we were able to investigate the impact of BDNF suppression and FGF2 enhancement on NMJ reinnervation within a specific subset of axons.

## Materials and Methods

### *Animals*

For this investigation, thirty three-month-old female rats weighing 200–250 g (RjHan:WI-Wistar, Janvier Labs) were utilized, six in each group. Subsequent to unilateral transection and suture of the right buccal branch of the facial nerve (buccal-buccal anastomosis, BBA) under aseptic surgical settings, the rats were subcutaneously injected with various combinations and quantities of BDNF and FGF2.

Rats were given tap water at will and normal laboratory food (Sniff, Soest, Germany) both before and after surgery. They were also kept in an artificial light-dark cycle with 12 hours of light and 12 hours of darkness. The University of Cologne's Animal Welfare Committee gave its approval to all experimental methods, which were carried out in compliance with international law regarding animal protection.

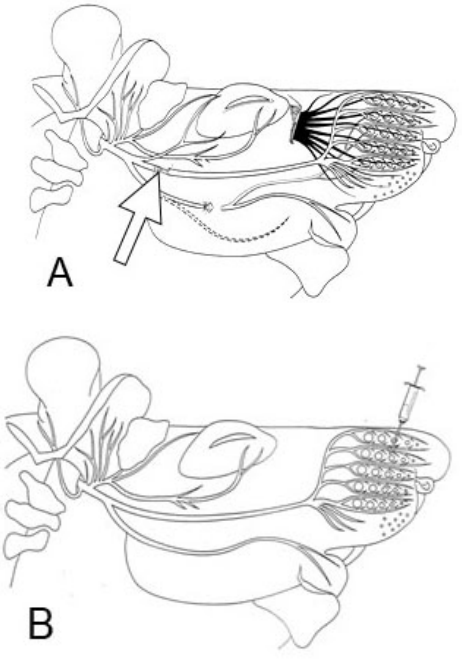


**Fig. 1. A:** Schematic drawing illustrating the site of transection and suture in the buccal branch (arrow) and of the transection and ligature of the marginal mandibular branch of the facial nerve. The cervical branch of the facial nerve is indicated by a dotted line. Adapted from Semba and Egger (1986) [62]. **B:** Schematic drawing of the extratemporal rat facial nerve indicating the site of intramuscular injections.

### *Buccal-buccal anastomosis surgery*

Two 11-0 atraumatic sutures (Ethicon, Norderstedt, Germany) were used to suture the exposed buccal branch of the right facial nerve after an intraperitoneal injection of 200 µl of Xylazin (20 mg/kg body weight) and Ketamine (120 mg/kg body weight). To prevent subsequent reinnervation of the whisker muscles by nerve fibers from this branch of the facial nerve, the marginal mandibular branch was transected, and the proximal stump ligated (**Fig. 1**).

***Post-operative injections of BDNF and FGF2***

Daily subcutaneous injections of placebo, neurotrophic factors, and antibodies to brain-derived neurotrophic factor (anti-BDNF 2.0 µg/ml; PeproTech, 500-P84BT) were given 1-28, 1-13, and 14-28 days after BBA. Isoflurane (1.8% volume, Forene, Abbott), 0.6 l/min $O_2$ (Conoxia, Linde), and inhalation anesthesia with 1.2 l/min $N_2O$ (Niontix, Linde) were also administered. The injections were performed using a micro-fine insulin syringe [U-100 (0.3 ml), 0.3 mm (30G) x 8.0 mm (Becton Dickinson, 324826)] at the same site halfway between the two dorsal vibrissal rows A and B of the whisker pad [6]. 30 µl of a saline placebo, fibroblast growth factor 2 (FGF2, PeproTech, 100-18C), or BDNF (PeproTech, 450-02) at low to high doses were injected. As an extension of our previous work with injections of trophic factors in 6 groups of rats [59], we performed a pilot study with the following 5 groups:

**Group 1 Prelim "Placebo"** consisted of 6 rats that received daily injections with 30 µl 0.9% NaCl from day 1 till day 28 after BBA.

**Group 2 Prelim "anti-BDNF x 5"** consisted of 6 rats that received daily injections with 30 µl anti-BDNF (10 µg/ml; Peprotech, Cat. Nr. 500-P84) into the LLS from day 1 till day 13 after BBA. Justification: the concentration of anti-BDNF was 5 times higher than the one used for Group 6 Publ of Rink et al., 2020 [59].

**Group 3 Prelim "Late BDNF x 20"** consisted of 6 rats that received daily injections with 30 µl BDNF (20 µg/ml; PeproTech, Cat. Nr. 450-02) into the LLS from day 14 till day 28 after BBA. Justification: since the dosage of 1 µg/ml did not improve whisking after BBA (amplitude of 21 ± 8 degrees for Group 3 Puble in Rink et al., 2020) [59], we tested the effect of 20 times higher dosage.

**Group 4 Prelim "FGF-2 x 10 throughout "** consisted of 6 rats that received daily injections with 30 µl FGF2 (100 µg/ml; PeproTech, Cat Nr. 100-18C) into the LLS from day 1 till day 28 after BBA. Justification: this concentration was ten times higher than the one which yielded in very good results in our pilot experiment (s. Group 10 Publ in Rink et al., 2020) [59].

**Group 5 Prelim "early anti-BDNF x 5 plus late FGF-2 x 10"** consisted of 6 rats that received daily injections with 30 µl anti-BDNF (10 µg/ml; Peprotech, Cat. Nr. 500-P84) into the LLS from day 1 till day 13 after BBA. From day 14 till day 28 rats received daily injections with with 30 µl FGF2 (100 µg/ml; PeproTech, Cat Nr. 100-18C) into the LLS. Justification: this combination yielded in the best results of our pilot experiment (s. Group 9 Publ in Rink et al., 2020) [59]. We repeated this experiment with increased dosages for anti-BDNF (2 times) and FGF2 (10 times).

***Analysis of vibrissae motor performance during exploration***

Video-based motion analysis (VBMA) of whisking behavior was conducted 56 days following BBA surgery to investigate the hypothesis that BDNF enhanced motor function.

Protraction (**Fig. 2a**) and retraction (**Fig. 2b**) are the two main vibrissal movements. As previously mentioned, just two of the C-row's big vibrissae on either side of the face were subjected to VBMA [16, 30]. Using a Panasonic NV DX-110 EG digital camcorder, the rats were videotaped while actively exploring for three to five minutes.

After watching the videos, 1.5-second segments featuring each animal were chosen for examination, with the animal's head posture being the determining factor. The following whisking parameters were assessed:

i) frequency, i.e. the number of times per second that an active forward vibrissal movement (protraction) and a passive backward movement (retraction) occur;

ii) the angle at maximal protraction of the whiskers, meaning the angle (in degrees) that is open rostrally between the mid-sagittal plane and the hair shaft. Maximal protractions have low angle values;

iii) amplitude - the difference between maximal retraction and maximal protraction (in degrees);

iv) angular velocity during protraction measured in degrees per second;

v) angular acceleration during protraction measured in degrees per second$^2$.

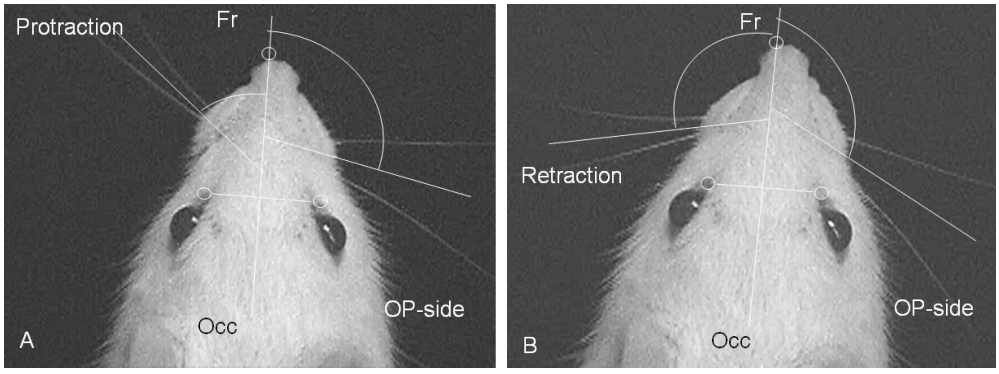


**Fig. 2.** "When the vibrissae are being protracted **(a)** and retracted **(b)**, the created spatial model enables accurate measurement of angles, angular velocity, and angular acceleration on the intact (left) and operated side (right). On the unaffected side, notice the marked change in angle between the sagittal line Fr-Occ during protraction and retraction. The operated side's vibrissae remain spastic. Figure and text adopted from Guntinas-Lichius et al. (2002) [30].

### *Tissue preparation for neuromuscular immunocytochemistry*

The rats in all groups were thoroughly anesthetized 56 days after BBA, and their vessels were washed with phosphate buffered saline pH 7.4, transcardially, by perfusion. Tissues were then fixed by perfusion with 4% paraformaldehyde in the same buffer. After being dissected, the *levator labii superioris* (LLS) muscle was cryoprotected in sucrose and then sectioned longitudinally, 30 µm, using a cryostat (32–37 sections per muscle).

Every third section was used for immunostaining with a rabbit polyclonal antibody against neuronal class III β-tubulin (Covance, Richmond, CA, USA, No. PRB-435P, 1:1000; Cy3-conjugated anti-rabbit IgG; 1:400; Sigma), according to the fractionator section selection approach [26]. The sites of post-junctional acetylcholine receptors, labeled with Alexa Fluor 488-conjugated α-bungarotoxin (Molecular Probes, AZB13422, 1:500), were used to visualize the boundaries of the neuromuscular junctions (NMJ) in the LLS muscle.

As previously mentioned, the degree of non-, poly-, and mono-innervation of NMJs was assessed [29]. Mono-innervated NMJs are defined as muscle endplates innervated by a single axonal branch, whereas poly-innervated NMJs are defined as those innervated by two or more axonal branches (**Fig. 3**). The endplates without

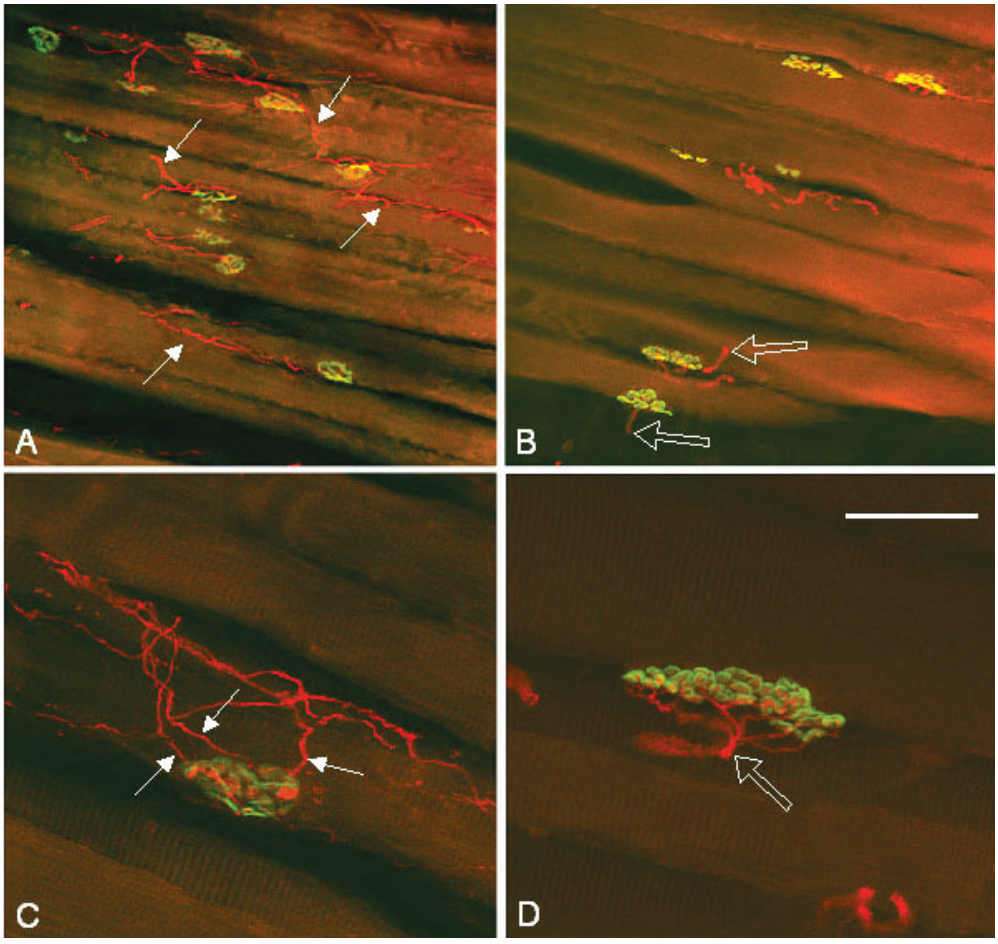


**Fig. 3 A-D.** "Stacks of superimposed confocal images of the end-plates in reinnervated LLS muscles visualized by staining of the motor endplates with Alexa Fluor 488 α-bungarotoxin (green fluorescence) and immunostaining of the intramuscular axons for neuronal class III β-tubulin (Cy3 red fluorescence). The innervation pattern is visible in the low magnification images presented in panels A and B. Note that while panel B lacks extensive intramuscular axonal branches, panel A exhibits them amongst endplates (arrows). Furthermore, it appears that the muscle fiber diameters in A have a smaller diameter than those in B. Panels C and D show examples of a polyinnervated and a monoinnervated endplate, respectively. The polyinnervated endplate boundaries, as indicated by the alpha-bungarotoxin staining, are reached by three axonal branches (arrows in C). On the other hand, a single axon with many preterminal rami (empty arrow in D) reaches the monoinnervated endplate. In both examples, the whole endplates are within the stack of confocal images. Scale bar shown in D indicates 125 μm for A, B and 40 μm for C, D." Adopted from Guntinas-Lichius et al. (2005) [29].

an apparent axonal branch were considered non-innervated. Observers blinded to allocation counted NMJs with and without innervation under a microscope at 40× magnification. Sections were examined using a Zeiss Axioskop 50 epifluorescence microscope and either "fluorescein" (Nr. 9, Carl Zeiss) or "rhodamine" (Nr. 15, Carl Zeiss) filter. An analysis was conducted on the number of β-tubulin positive axonal branches that enter or exit the borders of individual α-bungarotoxin positive endplates.

***Statistical evaluation***

For a given parameter, the data from all experimental groups were tested in a one-way analysis of variance (one-way ANOVA) procedure for overall experimental effects. If significant effects were detected ($p < 0.05$), comparisons of all groups with one control group (placebo) were performed using the post-hoc test of Dunnett at a significance level of 0.05. Statistica 6.0 software (StatSoft, Tulsa, OK, USA) was used for the analysis.

## Results

In the following lines we place the suffix "Publ" to all groups groups which we have already published [59]. This is supposed to help to differentiate them from the groups of our preliminary results (indicated by the suffix "Prelim").

***Quantitative estimates of vibrissal motor performance before and after buccal-buccal anastomosis (BBA) surgery and BDNF treatments***

***Pre-operative performance in intact rats***

Mystacial vibrissae swept back and forth at ~6-7 Hz during active exploration, with a maximum protraction of ~50º, which is the rostrally open angle between the vibrissa shaft and the median sagittal plane. The difference between the maximal protraction and retraction in degrees, or the whisking amplitude, was around 60º. The movements were executed at approximately 1200º/sec sagittal angular velocity and ~40,000°/sec$^2$ sagittal angular acceleration [59].

***Vibrissal motor function at 56 days after BBA surgery***

The published results indicate that the mean amplitude of whisking was significantly higher in the groups receiving intramuscular injections of anti-BDNF alone for 1 to 13 days post-surgery (Group 6), anti-BDNF in combination for 28 days (Group 9), or FGF2 alone for 28 days (Group 10), compared to the placebo group 1 (ANOVA $F(9/50) = 7.6$; $p < 0.0001$) [59].

The present preliminary results on recovery of the whisking amplitude (histology is not ready yet) can be summarized as follows:

i) Injections with anti-BDNF in concentration 5x higher than in the previous study [59] did not result in an increased amplitude at 56 days after BBA (see Group 1 Prelim in **Fig. 4**).

ii) Injections with FGF-2 in concentrations 10x higher than in the previous study [59] also failed to promote further recovery of the whisking amplitude at 56 days after BBA (see Groups 4 Prelim and 5 Prelim in **Fig. 4**).

iii) Only the injections with BDNF in concentration 20 times higher than in the previous study [59] improved the amplitude significantly when compared to the one in Group 3 (see Group 3 Prelim in **Fig. 4**).

***Quality of target reinnervation***

With this evaluation, we anticipate to demonstrate whether daily trophic factor injections into the whisker pad muscles during the initial two weeks following BBB surgery, effect intramuscular axon sprouting at the NMJ by decreasing polyinnervation of the junctions and, consequently, enhancing the restoration of whisking behavior. Till the day of the EFEM lecture held on 30.09.2023 at the Congress of the Bulgarian Anatomical Society in Sofia this quantification was not completed.

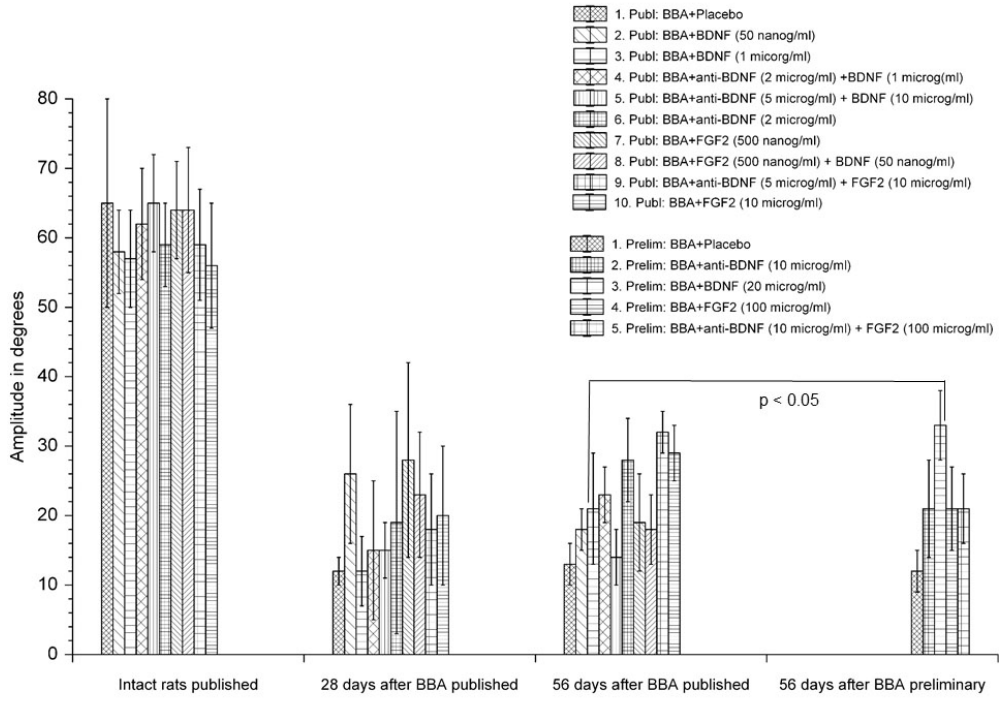


**Fig. 4.** Mean amplitude of vibrissal whisking in intact rats as well as on rats 28 and 56 days after transection and suture of the buccal branch of the facial nerve (buccal-buccal anastomosis, BBA). The amplitude in group 3 significantly increased. Prelim: BBA+ 20 μg/ml BDNF when compared to that in group 3 Publ; BBA+ 1 μg/ml BDNF is indicated.

## Discussion

### *Justification of the approach*

The application of the proposed trophic factors is justified by the results of three earlier published studies of our group [23, 59, 70]. Due to the rather short half-time life of the trophic factors, e.g. neurotrophins [55] injections will be performed every day.

The largest LLS muscle functioned as the main representative of the group whiskerpad muscles, even though it's possible that the injected solutions diffused towards the intrinsic follicular muscles that are innervated by the facial nerve as well as the extrinsic *maxillolabialis*, *transversus nasi*, *nasalis*, and *dilator naris muscles*.

Of course, we cannot claim that daily application will provide the optimal concentration (the amount of trophic elements that remain in the muscles of the whisker pad following injection is not quantifiable), but a 24 h interval between the injections is the minimum that was allowed by the Animal Welfare Committee (Az. 84-02.04.2021. A101 of 13.07.2021).

Unfortunately, the manufacturer (PeproTech) does not determine the half-life of its products under live conditions. Nevertheless, the activity bioassays are performed at 37°C with no media change. This is why, they know that the proteins are stable for: Human/Murine/Rat BDNF (Cat# 450-02) – 7 days, Anti-Human/Murine/Rat BDNF (Cat# 500-P84) – 7 days, Human IGF-II (Cat# 100-12) – 4 days and Human FGF-basic (Cat# 100-18C) – approx. 45 hours. We feel thus confident, that the proteins which we use will be long enough in an active state and exert their effects on the reinnervation of the vibrissal muscles.

### *Methodological considerations*

Three possibilities are considered: The exogenous BDNF that was acquired from PeproTech (450-02) might be not effective at all or it might have been metabolized improperly or supplied in incorrect doses. Given that the identical BDNF formulation doses were utilized in a prior investigation that showed both in vitro and in vivo regeneration of motor and sensory axons, the first explanation is improbable [60]. Regarding the second argument, daily injections were administered to offset the growth factors' brief half-lives (30 minutes to 2 hours), at least in part [55]. Nevertheless, we cannot be sure that the daily application supplied the ideal concentrations since we were unable to determine the level of trophic elements still present in the whiskerpad muscles following injections. Furthermore, ethical constraints limited us to 24-hour intervals between injections. Although conditional measures would be needed to permit administration throughout certain time periods, delivery through viral transfection may be a future alternative [44].

We began with the lowest concentration of 50 ng BDNF/ml of distilled water, since this is the first study to inject neurotrophic factors into denervated muscles and as advised by the manufacturer (PeproTech) and Santos, et al. (2016) [60]. We also tested the effects of 1 µg/ml and 10 µg/ml. Lastly, it's possible that the 14–28-day window for applying BDNF following BBA surgery was not long enough.

## Conclusions

The results of this study enable us to draw the conclusion that intramuscular administration of a complex mixture of trophic factors at particular post-operative times and concentrations can restore optimal muscle target re-innervation and vibrissal function following facial nerve injury and surgical repair in rats. To identify the best therapeutic approaches, further experimental research is needed.

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