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# POOR FUNCTIONAL RECOVERY AND MUSCLE POLYINNERVATION AFTER FACIAL NERVE INJURY IN FIBROBLAST GROWTH FACTOR-2$^{-/-}$ MICE CAN BE IMPROVED BY MANUAL STIMULATION OF DENERVATED VIBRISSAL MUSCLES

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**Abstract—Functional recovery following facial nerve injury is poor. Adjacent neuromuscular junctions (NMJs) are "bridged" by terminal Schwann cells and numerous regenerating axonal sprouts. We have recently shown that manual stimulation (MS) restores whisking function and reduces polyinnervation of NMJs. Furthermore, MS requires both insulin-like growth factor-1 (IGF-1) and brain-derived neurotrophic factor (BDNF). Here, we investigated whether fibroblast growth factor-2 (FGF-2) was also required for the beneficial effects of MS. Following transection and suture of the facial nerve (facial-facial anastomisis, FFA) in homozygous mice lacking FGF-2 (FGF-2$^{-/-}$), vibrissal motor performance and the percentage of poly-innervated NMJ were quantified. In intact FGF-2$^{-/-}$ mice and their wildtype (WT) counterparts, there were no differences in amplitude of vibrissal whisking (about 50°) or in the percentage of polyinnervated NMJ (0%). After 2 months FFA and handling alone (i.e. no MS), the amplitude of vibrissal whisking in WT-mice decreased to 22±3°. In the FGF-2$^{-/-}$ mice, the amplitude was reduced further to 15±4°, that is, function was significantly poorer. Functional deficits were mirrored by increased polyinnervation of NMJ in WT mice (40.33±2.16%) with polyinnervation being increased further in FGF-2$^{-/-}$ mice (50.33±4.33%). However, regardless of the genotype, MS increased vibrissal whisking amplitude (WT: 33.9°±7.7; FGF-2$^{-/-}$: 33.4°±8.1) and concomitantly reduced polyinnervation (WT: 33.9%±7.7; FGF-2$^{-/-}$: 33.4%±8.1) to a similar extent. We conclude that, whereas lack of FGF-2 leads to poor functional recovery and target reinnervation, MS can nevertheless confer some functional benefit in its absence.** 

**Key words: facial nerve, axotomy, motor end-plate, whisking, polyinnervation.**

Full recovery function is rare following peripheral nerve injury (Lundborg, 2003; Tang et al., 2004; Xin et al., 2008; Hadlock et al., 2010b). The common symptoms of the "post-paralytic syndrome" (paresis, synkinesis, and dysreflexia) even worsen over time (Kerrebijn and Freeman, 1998).

Although axon regrowth is robust, it is highly inaccurate. This feature is a major cause for the lack of functional recovery (Sumner, 1990; Ito and Kudo, 1994; Dai et al., 2000; Moran and Graeber, 2004; Angelov et al., 2005; English, 2005; Robinson and Madison, 2009). Innacuracy occurs at a number of locations along the axis facial nucleus-facial-nerve trunk-facial nerve fascicles-facial muscles. Within the nerve itself, each transected axon emits several regenerating branches (Shawe, 1955; Morris et al., 1972; Mackinnon et al., 1991). Both excessive branching, as well as gross misalignment of the original fascicles, results in axons failing to rejoin their original nerve fascicles with subsequent misdirection *en route* to target tissue (Anonsen et al., 1986; Baker et al., 1994). Axonal branches thus reach the target tissue with one muscle fiber being re-innervated by more than one motoneuron (polyneuronal innervation), often with antagonizing actions (Vleggeert-Lankamp et al., 2005). Once in the vicinity of motor end-plates, regenerating axons also undergo i.m. (terminal) axon sprouting and simultaneously reinnervate multiple end-plates (Grimby et al., 1989; Trojan et al., 1991; Son et al., 1996). In addition, terminal Schwann cells respond to injury by extending numerous processes that form bridges between motor endplates, thereby enabling terminal sprouts to reach multiple adjacent, rather than one single, motor endplate (Kang et al., 2003; Magill et al., 2007; Griffin and Thompson, 2008; Madison et al., 2009).

The quality of peripheral nerve regeneration, both within the nerve and at the motor end-plate/terminal

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*Abbreviations:* BDNF, brain-derived neurotrophic factor; FFA, facial-facial anastomisis; FGF-2, fibroblast growth factor 2; IGF-1, insulin-like growth factor-1; LLS, levator labii superioris muscle; MS, manual stimulation; NMJs, neuromuscular junctions; TBS, tris–buffered saline; WT, wildtype.

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Schwann cell complex, can be improved by various non-invasive therapies. Muscles with flaccid paralysis can be stimulated electrically or by exercise, procedures which inhibit i.m. axonal sprouting and diminish motor-end-plate polyinnervation thereby improving reinnervation quality (Brown and Ironton, 1977; Brown and Holland, 1979).

With respect to functional improvements, we have recently shown that, after facial nerve injury, manual stimulation (MS) of denervated whisker pads reduces the proportion of polyinnervated neuro-muscular junctions (NMJs). Furthermore, the shift towards the normal monoinnervated state is associated with improved whisking function and blink reflexes (Angelov et al., 2007; Bischoff et al., 2009; Hadlock et al., 2010a). Interestingly, a number of key growth factors appear to be required during MS to both decrease polyinnervation and improve whisking function. Both insulin-like growth factor-1 (IGF-1) and brain-derived neurotrophic factor (BDNF) are up-regulated after facial-facial anastomisis (FFA) and, although regeneration is robust in IGF-1- and BDNF-deficient (heterozygous) mice, MS neither reduces polyinnervation nor improves motor performance of reinnervated muscles (Kiryakova et al., 2010; Sohnchen et al., 2010).

Denervated muscles have been shown to produce numerous short-range diffusible sprouting stimuli (Slack and Pockett, 1981; Pockett and Slack, 1982; English, 2003; Zhao et al., 2004). Various neurotrophic factors have been identified as possible candidates for this role (Sendtner, 1998; Raivich and Makwana, 2007). This is why, now we investigate whether another growth factor, fibroblast growth factor 2 (FGF-2) also mediates the beneficial effects of MS.

FGF-2 is up-regulated after facial nerve injury with immunoreactivity in the distal nerve stump and target muscles of the whisker pad first peaking at 2 days post-lesion, declining at 4 days, peaking again at 5–6 days and finally declining to zero by 8 days (Chen et al., 1999; Streppel et al., 2002). FGF-2 also stimulates neurite regrowth *in vivo* and contributes to the enlargement of axon calibre (Aebischer et al., 1989; Laquerriere et al., 1994; Piehl et al., 1998). Homozygous FGF-2 knock-out ($FGF^{-/-}$) mice were used comparing those receiving MS to age-matched counterparts which received handling but no MS; wildtype (WT) animals were also used.

## EXPERIMENTAL PROCEDURES

Animals were fed standard laboratory food (Ssniff, Soest, Germany), provided tap water *ad libitum* and kept in an artificial light-dark cycle of 12 h light on, 12 h off. Experiments conformed to German Law on the Protection of Animals and all procedures were approved by the local Animal Care Committee, University of Cologne. Guidelines were identical to those of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, the UK Animals (Scientific Procedures) Act 1986 and associated guidelines and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

### Animals

Homozygous mice constitutively deficient in FGF-2 (strain *Fgf2*$^{tm1Zllr}$ C57/Bl6, Jackson Laboratories, Bar Harbor, ME, USA) were used as well as wildtype (WT) animals (C57/Bl6).

Genotyping was performed by polymerase chain reaction (PCR) (Cycling conditions: 30 min 95 °C, 30 cycles: 30 s 95 °C, 30 s 61 °C, 90 s 72 °C, final elongation for 10 min 72 °C) using the HotStarTaq Master Mix Kit (Qiagen, Hilden, Germany) from mouse tail desoxyribonucleic acid and subsequent Agarose-Gel-electrophoresis. To differentiate between wild type (WT) and transgenes the following primers were used:

-neo6: 5′-GAT CTG GAC GAA GAG CAT CAG GGG-3′
-wt6: 5′-CAA GTT TCT AAC TTT CTC CGC TCC TGC-3′
-wt5: 5′-CAA TCT ATT GGG GTC AAG CCT ATT GGG-3′

The transgene allele yielded an amplicon of 750 bp and the wild type 344 bp.

Mice were randomized to six groups, eight in each (Table 1). Groups 1 and 2 were intact WT or homozygous knockouts ($FGF^{-/-}$). Groups 3–6 comprised WT (Groups 3 and 4) or $FGF^{-/-}$ (Groups 5 and 6) mice which underwent FFA. Following FFA, animals either received MS (see below, Groups 4 and 6) or served as "handling" controls (see below; Groups 3 and 5).

### Surgery

FFA involved transection and end-to-end suture of the right facial nerve under surgical anaesthesia (Ketamin/Xylazin; 100 mg

**Table 1.** Biometrics of whisking behavior in aged-matched WT ($FGF\text{-}2^{+/+}$) and $FGF\text{-}2^{-/-}$ female mice after facial-facial anastomosis (FFA) with manual stimulation (MS) of the vibrissal muscles and without MS (i.e. handling animals only)

| Animal group | Frequency (in Hz) | Angle at maximal protraction (in degrees) | Amplitude (in degrees) | Angular velocity during protraction (in degrees/s) | Angular acceleration during protraction (in degrees/s$^2$) |
|---|---|---|---|---|---|
| 1. WT intact | 6.8±1.9 | 55.7°±11.7 | 50.8°±8.3 | 883°±458 | 62,780°±7720 |
| 2. $FGF\text{-}2^{-/-}$ intact | 6.4±2.3 | 58.2°±7.2 | 59.1°±7.1 | 1128°±105 | 52,768°±2060 |
| 3. WT+FFA+handling | 5.2±1.1 | 88.1°±29.8 | 22.3°±3.1$^{\#,\S,*}$ | 170°±122$^{\#,*}$ | 4248°±1197$^{\#,*}$ |
| 4. WT+FFA+MS | 5.3±3.1 | 96.2°±20.3 | 33.9°±7.7$^{\#,*}$ | 492°±144$^{\#,*}$ | 23,158°±9447$^{\#,*}$ |
| 5. $FGF\text{-}2^{-/-}$+FFA+handling | 5.1±2.3 | 72.4°±10.8 | 15.3°±4.1$^{\#,\S,*}$ | 194°±105$^{\#,*}$ | 2374°±1046$^{\#,*}$ |
| 6. $FGF\text{-}2^{-/-}$+FFA+MS | 6.1±2.1 | 70.4°±17.2 | 33.4°±8.1$^{\#,*}$ | 402°±165$^{\#,*}$ | 17,681°±11,648$^{\#,*}$ |

The post-operative survival time was 8 wks. Each value is a mean±SD of eight mice. Significantly different values (ANOVA and post-hoc Tukey's test, $P<0.05$) obtained in surgically treated mice (Groups 3–6) when compared to those from intact mice (Groups 1 and 2) are indicated by $^{\#}$ Significant differences between values obtained from WT-mice when compared to those from $FGF\text{-}2^{-/-}$ animals (Group 3 vs. Group 5 and Group 4 vs. Group 6) are indicated by $^{\S}$ Finally, significant differences between non-stimulated and manually stimulated WT- (Group 3 vs. Group 4) or $FGF\text{-}2^{-/-}$-mice (Group 5 vs. Group 6) are indicated by $^{*}$ .