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Comparison of trophic factors' expression between paralyzed and recovering muscles after facial nerve injury. A quantitative analysis in time course

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ABSTRACT

After peripheral nerve injury, recovery of motor performance negatively correlates with the poly-innervation of neuromuscular junctions (NMJ) due to excessive sprouting of the terminal Schwann cells. Denervated muscles produce short-range diffusible sprouting stimuli, of which some are neurotrophic factors. Based on recent data that vibrissal whisking is restored perfectly during facial nerve regeneration in blind rats from the Sprague Dawley (SD)/RCS strain, we compared the expression of brain derived neurotrophic factor (BDNF), fibroblast growth factor-2 (FGF2), insulin growth factors 1 and 2 (IGF1, IGF2) and nerve growth factor (NGF) between SD/RCS and SD-rats with normal vision but poor recovery of whisking function after facial nerve injury. To establish which trophic factors might be responsible for proper NMJ-reinnervation, the transected facial nerve was surgically repaired (facial-facial anastomosis, FFA) for subsequent analysis of mRNA and proteins expressed in the *levator labii superioris* muscle. A complicated time course of expression included (1) a late rise in BDNF protein that followed earlier elevated gene expression, (2) an early increase in FGF2 and IGF2 protein after 2 days with sustained gene expression, (3) reduced IGF1 protein at 28 days coincident with decline of raised mRNA levels to baseline, and (4) reduced NGF protein between 2 and 14 days with maintained gene expression found in blind rats but not the rats with normal vision. These findings suggest that recovery of motor function after peripheral nerve injury is due, at least in part, to a complex regulation of lesion-associated neurotrophic factors and cytokines in denervated muscles. The increase of FGF-2 protein and concomittant decrease of NGF (with no significant changes in BDNF or IGF levels) during the first week following FFA in SD/RCS blind rats possibly prevents the distal branching of regenerating axons resulting in reduced poly-innervation of motor endplates.

1. Introduction

A major reason for the poor recovery of motor function after severe peripheral nerve injury is the terminal sprouting of regenerating axons. Upon reaching a denervated target, the axons undergo intramuscular sprouting to reinnervate many muscle fibers that compensate for limited axon regeneration; the upper limit of this sprouting is ~3–5-fold (Fu and Gordon, 1995; Rafuse and Gordon, 1996; Son et al., 1996). Sprouting has been regarded as an adaptive mechanism to compensate for reduced functional capacity but the reduced numbers of motor units, despite their enlargement having compensated for the reduction, cannot function as well as the full complement of motor units in their progressive recruitment during movement (Gordon et al., 2004). This intensive intramuscular sprouting is considered to be a prerequisite for reinnervation of neuromuscular junctions (NMJ) by more than one axon, a state known as "polyinnervation" (Rich and Lichtman, 1989). The designation "polyinnervated" NMJ is used to indicate similarity to a morphological abnormality in adult skeletal muscle of mammals observed in pathological conditions such as, e.g., nerve damage or intoxication (Brown et al., 1981). However, the term polyinnervated does not imply that all end-plates in this category

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are polyneuronally innervated by two or more motoneurons (Guntinas-Lichius et al., 2005).

Using a standard method for precise quantitative assessment of vibrissae motor performance, 'whisking' (Tomov et al., 2002), we found that the whisking remains poor even when collateral axonal branching at the lesion site was reduced (Streppel et al., 2002). Hence, the post-transectional collateral branching is not a significant factor contributing to limited functional performance after misdirection of regenerating nerves. Our subsequent investigations showed that the degree of functional recovery corresponded directly to the proportion of reinnervated NMJs that were polyinnervated by the regenerated facial axons (Guntinas-Lichius et al., 2005).

It is known that intramuscular sprouting after injury is controlled by a special population of Schwann cells, the terminal Schwann cells (TSCs). Denervated TSCs extend processes which reach adjacent innervated NMJs (Griffin and Thompson, 2008; O'Malley et al., 1999; Reynolds and Woolf, 1992). Using these bridges, the TSCs attract and direct intramuscular axonal sprouts towards the denervated NMJs (Kang et al., 2003; Love and Thompson, 1999; Reddy et al., 2003). Because the outgrowth of TSC processes precedes the outgrowth of sprouts from the intact intramuscular axons, the TSCs are able to initiate intramuscular axonal sprouting (Son and Thompson, 1995; Trachtenberg and Thompson, 1996).

Since the TSCs of denervated muscles have been shown to produce short-range diffusible sprouting stimuli (English, 2003; Pockett and Slack, 1982; Slack and Pockett, 1981; Zhao et al., 2004) many of which have been identified as neurotrophic factors (Raivich and Makwana, 2007; Sendtner, 1998), we wished to determine which trophic factors may be responsible for the reinnervation of NMJs after nerve transection and surgical repair in rats with (i) poor recovery of motor function (50% of NMJs are polyinnervated) and (ii) complete recovery of motor function (10% of NMJs are polyinnervated).

2. Materials and methods

2.1. Animals and overview of experiments

2.1.1. Two major groups of rats (SD and RCS) were used

The SD major group consisted of 60 adult female Sprague–Dawley rats (SD, purchased from Charles River, Germany) distributed in 10 groups (SD1–SD10). These rats have normal visual perception and demonstrate poor recovery of facial (whisking) function after facial nerve transection and suture (facial–facial anastomosis, FFA, see below).

The RCS major group consisted of 60 blind adult SD female rats (substrain Royal College of Surgeons, RCS). Animals were kindly provided by Dr. Andreas Teubner, Institut für Versuchstierkunde, Medical Faculty, University of Aachen, Germany). The SD/RCS rats lose their photoreceptor cells after birth due to a genetic defect of the retinal pigment epithelium (D'Cruz et al., 2000; Sheedlo et al., 1991). Thus, the mystacial vibrissae of these animals are their only available means to resolve spatial information (Brecht et al., 1997). Following FFA, the SD/RCS rats recover whisking function completely (Guntinas-Lichius et al., 2005; Tomov et al., 2002).

Both major groups of rats were subdivided into 10 groups of 6 rats each (SD1-SD10 and RCS1-RCS10) for RT-PCR semiquantitative analysis (1–5) and ELISA semiquantitative protein analysis (6–10) of neurotrophic factors as a function of time after the facial-facial anastomosis over a 28 day period. Groups SD1 and RCS1 consisted of intact (non-operated) control animals.

Before and after surgical treatment, the rats were kept on standard laboratory food (Ssniff, Soest, Germany) and tap water ad libitum with an artificial light–dark cycle of 12 h light on, 12 h off. All experiments were conducted in accordance with the German Law on the Protection of Animals, and the procedures used were approved by the Animal Welfare Committee (Az. 26.203.2K 35, 35/01).

2.2. Surgery

2.2.1. Facial–facial anastomosis (FFA)

Following an intraperitoneal injection of Ketamine/Xylazine, the main trunk of the right facial nerve was exposed and transected close to its emergence out of the foramen stylomastoideum. The proximal stump was then microsurgically reconnected to the distal stump with two 11–0 atraumatic sutures (Ethicon, Norderstedt, Germany). Finally the wound was closed by three 4–0 skin sutures (Ethicon).

2.3. Tissue preparation

At the designated postoperative survival periods of 2, 7, 14 and 28 days post-facial nerve transection and anastomosis, the rats were deeply anesthetized and their vascular system rinsed by transcardial perfusion with phosphate buffered saline pH 7.4. Under an operation microscope the levator labii superioris (LLS) muscle was identified. Thereafter a narrow band of tissue just behind the last column of vibrissal hairs was rapidly dissected free and immediately snap-frozen in liquid nitrogen. We selected this part of LLS because most of the NMJs are situated in it (long-time own observations).

2.4. RNA extraction and real-time Polymerase Chain Reaction (RT-PCR)

RNA Extraction and Reverse Transcription The RNeasy Mini Kit from Qiagen (Cat. No. 74,106) was used to purify total RNA (up to 100 μg total RNA per sample) from 30 mg frozen tissue samples (from the levator labii superioris) as per manufacturer's instructions. High quality RNA was eluted in 30–100 μl RNAse-free water. Isolated RNA was transferred into cDNA via reverse transcription using random primers and the Super Script III Reverse Transcriptase (RT) Kit from Invitrogen (Cat. No. 18,080–044). The cDNA was stored at −80 °C.

Quantitative real-time PCR The extent of up- or downregulation of neurotrophic factors (NGF, BDNF, NT-3, FGF2, IGF1, and IGF2) in the muscle part with most numerous NMJs of the levator labii superioris muscle was determined by quantitative PCR. Real-time PCR was conducted in 40 cycles on Mx3000p (Stratagene) utilizing the RT^2 qPCR Primer Assay from SABiosciences with the RT^2 SYBR Green/Rox qPCR Master Mix according to the manufacturers' instructions (Cat. No. PA-112). All samples were measured in triplicate using primers listed in Table 1. For data analysis we used the ΔΔCt method with ß-Actin serving as housekeeping gene as described in the User Bulletin No. 2 (Applied Biosystems, P/N 4303859).

2.5. ELISA

Frozen tissue samples were crushed using a pebble mill with additional ultrasound treatment on ice in lysis buffer made of complete Mini (Roche, CatNo.11836153001), HALT Protease Inhibitor Cocktail (Thermo Scientific, CatNo. 78410) and PMSF (Applichem, CatNo. A0999) according to the manufacturers' instructions. After centrifugation the total protein content was measured in the supernatant using a Bradford-Assay (Serva, CatNo. 39222). The transcription of the proteins, NGF (Promega, CatNo. G7630), BDNF (Promega, CatNo. TB257),

Table 1
List of the primers used for triplicate measurements.

Primer	Forward	Reverse
ß-actin	5′-AGCCATGTACGTAGCCATCC-3′	5′-CTCTCAGCTGTGGTGGTGAA-3′
FGF-2	5′-GAACCGGTACCTGGCTATGA-3′	5′-CCGTTTTGGATCCGAGTTTA-3′
BDNF	5′-GCGGCAGATAAAAAGACTGC-3′	5′-GCCAGCCAATTCTCTTTTTG-3′
NGF	5′-TGTTGCTAGAGTCCGCCTTT-3′	5′-CAGTGATGAGGACCAGAGCA-3′
IGF-1	5′-CAGTTCGTGTGTGGACCAAG-3′	5′-TCAGCGGAGCACAGTACATC-3′
IGF-2	5′-GTCGATGTTGGTGCTTCTC-3′	5′-AAGCAGCACTCTTCCACGAT-3′