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Recovery of whisking function promoted by manual stimulation of the vibrissal muscles after facial nerve injury requires insulin-like growth factor 1 (IGF-1)

S. Kiryakova [a], J. Söhnchen [a], M. Grosheva [b], U. Schuetz [c], Ts. Marinova [d], R. Dzhupanova [d], N. Sinis [e], C.U. Hübbers [f], E. Skouras [g], J. Ankerne [a], J.W.U. Fries [h], A. Irintchev [i,1], S.A. Dunlop [j,1], D.N. Angelov [a,*,1]

[a] Department of Anatomy I, University of Cologne, Cologne, Germany
[b] Department of Oto-Rhino-Laryngology, University of Cologne, Cologne, Germany
[c] Department of Anatomy II, University of Cologne, Cologne, Germany
[d] Department of Biology, Medical Genetics and Microbiology, Medical Faculty, "St. Kliment Ohridski," University Sofia, Bulgaria
[e] Department of Hand-, Plastic-, and Reconstructive Surgery with Burn Unit, BG-Trauma Centre, University of Tuebingen, Tuebingen, Germany
[f] Jean-Uhrmacher Institut for clinical ENT-Research, University of Cologne, Cologne, Germany
[g] Department of Trauma-, Hand- and Reconstructive Surgery, University of Cologne, Cologne, Germany
[h] Department of Pathology, University Hospital of Cologne, Cologne, Germany
[i] Department of Oto-Rhino-Laryngology, University of Jena, Germany
[j] School of Animal Biology Institute for Medical Research, The University of Western Australia

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ABSTRACT

Recently, we showed that manual stimulation (MS) of denervated vibrissal muscles enhanced functional recovery following facial nerve cut and suture (FFA) by reducing poly-innervation at the neuro-muscular junctions (NMJ). Although the cellular correlates of poly-innervation are established, with terminal Schwann cells (TSC) processes attracting axon sprouts to "bridge" adjacent NMJ, molecular correlates are poorly understood. Since quantitative RT-PCR revealed a rapid increase of IGF-1 mRNA in denervated muscles, we examined the effect of daily MS for 2 months after FFA in IGF-$1^{+/-}$ heterozygous mice; controls were wild-type (WT) littermates including intact animals. We quantified vibrissal motor performance and the percentage of NMJ bridged by S100-positive TSC. There were no differences between intact WT and IGF-$1^{+/-}$ mice for vibrissal whisking amplitude (48° and 49°) or the percentage of bridged NMJ (0%). After FFA and handling alone (i.e. no MS) in WT animals, vibrissal whisking amplitude was reduced (60% lower than intact) and the percentage of bridged NMJ increased (42% more than intact). MS improved both the amplitude of vibrissal whisking (not significantly different from intact) and the percentage of bridged NMJ (12% more than intact). After FFA and handling in IGF-$1^{+/-}$ mice, the pattern was similar (whisking amplitude 57% lower than intact; proportion of bridged NMJ 42% more than intact). However, MS did not improve outcome (whisking amplitude 47% lower than intact; proportion of bridged NMJ 40% more than intact). We conclude that IGF-I is required to mediate the effects of MS on target muscle reinnervation and recovery of whisking function.

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Introduction

Restoration of function after transection of peripheral nerves is poor (Ferreira et al., 1994; Lundborg, 2003; Tang et al., 2007; Xin et al., 2008) and the occurrence of "post-paralytic syndrome" (paresis, synkinesis and dysreflexia) inevitable (Kerrebijn and Freeman, 1998). Poor recovery is due, at least in part, to inaccurate reinnervation of muscle targets (Sumner, 1990; Dai et al., 2000; Moran and Graeber, 2004).

Substantial evidence indicates three levels of inaccuracy. First, axons are misrouted and fail to rejoin their original nerve fascicles (Anonsen et al., 1986; Baker et al., 1994). Second, each transected axon gives off up to 25 "collateral" branches within the nerve itself (Shawe, 1954; Morris et al., 1972; Mackinnon et al., 1991). Excessive collateral branching in turn leads to re-innervation of several muscle groups, often with antagonizing action, by one single motoneuron ("polyinnervation") or reinnervation of one muscle by more than one motoneuron (polyneuronal innervation) (Vleggeert-Lankamp et al., 2005). Third, upon reaching a paralyzed muscle, axons undergo additional "terminal axon sprouting" and simultaneously reinnervate multiple motor end-plates (Grimby et al., 1989; Trojan et al., 1991; Son et al., 1996). Inaccurate reinnervation is considered to be the main factor underpinning abnormal function after peripheral nerve injury

* Corresponding author. Institut für Anatomie der Universität zu Köln, Joseph-Stelzmann-Strasse 9, D-50924 Köln, Germany. Fax: +49 221 478 87893.
E-mail address: angelov.anatomie@uni-koeln.de (D.N. Angelov).
[1] Contributed equally and share senior authorship.

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(Ito and Kudo, 1994; Angelov et al., 2005; English, 2005; Robinson and Madison, 2009).

A number of factors have been identified which improve the accuracy of reinnervation. Stimulating muscles with flaccid paralysis by a variety of means (e.g. electrical stimulation, exercise) inhibits intramuscular sprouting and diminishes motor-end-plate polyinnervation (Brown and Ironton, 1977; Brown and Holland, 1979). Similarly, we have recently shown that manual stimulation (MS) of denervated whisker pads after facial nerve injury reduces the amount of terminal sprouting; more accurate reinnervation patterns are associated with improved whisking function and blink reflexes (Angelov et al., 2007; Bischoff et al., 2009). Another factor involves terminal Schwann cells which, after injury, extend numerous processes that form bridges within target muscles and act as a substrate for terminal sprouts to reach multiple adjacent (rather than single) motor endplates (Kang et al., 2003; Magill et al., 2007; Madison et al., 2009). Moreover, both running exercise and electrical stimulation limit the formation of such bridges and therefore improve the accuracy of reinnervation (Tam and Gordon, 2003; Love et al., 2003).

A number of molecular factors have been identified which underpin the above structural correlates with Insulin-like Growth Factor-1 (IGF-1) being pivotal. It induces muscle regeneration (Caroni and Grandes, 1990; Glazner and Ishii, 1995; Keller et al., 1998; Di Guilio et al., 2000; Iida et al., 2004; Hayashi et al., 2004) and prevents muscle atrophy (Levinovitz et al., 1992; Messi and Delbono, 2003; Urushiyama et al., 2004; Okano et al., 2005). It is also involved in Schwann cell viability and myelination (Liang et al., 2007; Chattopadhyay and Shubayev, 2009). In addition, it is closely correlated with neuronal responses to injury being up-regulated at the time of axonal sprouting and elongation (Pu et al., 1999; Zochodne and Cheng, 2000; Tiangco et al., 2001; Streppel et al., 2002; Aberg et al., 2006). However, addition of exogenous IGF-1 fails to increase the accuracy of regeneration or functional outcomes such as muscle power, motor evoked potentials and conduction velocity (Welch et al., 1997; Lutz et al., 1999).

Although IGF-1 mediates exercise-induced anabolic changes in muscle tissue (Philippou et al., 2007; Velloso, 2008; Vale et al., 2009), it is not known whether this neurotrophic factor is involved in stimulation-induced changes within regenerating peripheral nerve axons. Here, we first examined IFG-1 expression after facial nerve injury and then used our manual stimulation (MS) protocol in IGF-1 deficient mice to examine whether MS could improve accuracy of reinnervation and recovery of function when IGF-1 was diminished.

Animals, materials and methods

Up-regulation of IGF-1 after facial nerve injury

Overview

IGF-1 mRNA was analysed using RT-PCR at 2, 7, 14 and 28 days after facial nerve injury. The postoperative survival times were selected because semi-quantitative immunohistochemistry for several growth factors in the proximal and distal stumps of the transected buccal branch of the facial nerve revealed that most trophic factor expression occurs at the lesion site by 2 days with levels returning to normal by 28 days post lesion (Streppel et al., 2002; Angelov et al., 2005). Marked IGF-1 mRNA upregulation at 2 days was detected using RT-PCR and confirmed using immunohistochemistry for IGF-1 and its receptor IGF-1Rα.

Animals

For RT-PCR, we used adult (12 weeks old) female Sprague Dawley (SD) rats, a strain with normal visual perception. In addition, we examined SD/Royal College of Surgeons (SD/RCS) rats which lose photoreceptors 2 months after birth due to a genetic defect of the retinal pigment epithelium and are blind (D'Cruz et al., 2000; Sheedlo et al., 1991). We have previously shown that, after facial nerve cut and anastomosis (FFA), recovery of vibrissal motor performance is superior in SD/RCS compared to SD rats (Tomov et al., 2002). Mystacial vibrissae in RCS rats are their only available means to resolve spatial information (Brecht et al., 1997) and our previous data suggest that increased vibrissal use in these blind animals promotes whisking function which is accompanied by increased accuracy of re-innervation (reduced poly-innervation) within vibrissal muscles (Guntinas-Lichius et al., 2005a). SD/RCS rats thus provide a model to examine recovery following facial nerve injury in the absence of MS. We therefore reasoned that comparison of IGF-1 expression in SD (no recovery) with SD/RCS (normal recovery) rats would indicate whether IGF-1 *per se* was associated with improved accuracy of target re-innervation.

There were 5 groups of SD and SD/RCS rats ($n = 4$ in each). Groups SD-1 and SD/RCS-1 were intact control animals. Groups SD2-5 and SD/RCS2-5 received unilateral transection and suture of the right facial nerve (see below) and were sacrificed respectively at 2, 7, 14 and 28 days after surgery. The portion of the extrinsic vibrissal muscle levator labii superioris immediately behind the last column of vibrissal hairs which contains the most motor end-plates was snap-frozen and processed for RT-PCR to examine neurotrophic factors expression quantitatively (see below).

Surgery

Transection and end-to-end suture of the right facial nerve (facial-facial anastomosis, FFA) was performed by a trained surgeon (M. Grosheva) after an intraperitoneal injection of Ketamin/Xylazin (100 mg Ketanest®, Parke-Davis/Pfizer, Karlsruhe, Germany, and 5 mg Rompun®, Bayer, Leverkusen, Germany, per kg body weight; i.p.). Under an operating microscope, the trunk of the facial nerve was exposed and transected close to its emergence from the foramen stylomastoideum (Fig. 1a). Immediately thereafter, the proximal stump was reconnected to the distal stump with two 11-0 atraumatic sutures (Ethicon, Norderstedt, Germany).

RT-PCR

Tissue preparation. Rats were deeply anesthetized and their vascular system rinsed by transcardial perfusion with phosphate buffered saline pH 7.4. The levator labii superioris muscle was dissected free. A narrow band of tissue immediately behind the last column of vibrissal hairs (Fig. 2) was rapidly dissected using an operating microscope and immediately snap-frozen in liquid nitrogen.

RNA extraction. The RNeasy Mini Kit from Qiagen (Cat. No. 74106) was used to purify total RNA (up to 100 μg total RNA per sample) from 30 mg samples of frozen tissue as per manufacturer's instructions. High quality RNA was eluted in 30–100 μl RNAse-free water.

Reverse transcription. Isolated RNA was transferred into cDNA via reverse transcription using the Super Script III Reverse Transcriptase (RT) Kit from Invitrogen (Cat. No. 18080-044). cDNA was stored at −80 °C using 500 ng RNA for RT-PCR.

Qualitative polymerase chain reaction (PCR). 500 ng cDNA was incubated in 15 μl Vol. including Mastermix (Taq Polymerase, Magnesium buffer and nucleotides). A hot start (enzyme activation at 95 °C) was initiated followed by a series of 40 cycles. Each cycle consisted of a brief denaturation of 15 s. followed by the annealing of the primer pair at 55 °C also for 15 s and finally primer extension at 72 °C for 30 s. β-actin served as a housekeeping gene (Fries et al., 1990).

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