Neuroscience 170 (2010) 372–380

RECOVERY OF WHISKING FUNCTION AFTER MANUAL STIMULATION OF DENERVATED VIBRISSAL MUSCLES REQUIRES BRAIN-DERIVED NEUROTROPHIC FACTOR AND ITS RECEPTOR TYROSINE KINASE B

J. SÖHNCHEN,[a1] M. GROSHEVA,[b1] S. KIRYAKOVA,[a] C. U. HÜBBERS,[c] N. SINIS,[d] E. SKOURAS,[e] J. ANKERNE,[a] K. KAIDOGLOU,[f] J. W. U. FRIES,[g] A. IRINTCHEV,[h] S. A. DUNLOP[i] AND D. N. ANGELOV[a]*

[a]*Department of Anatomy I, University of Cologne, Joseph-Stelzmann-Strasse 9, D-50924 Cologne, Germany*

[b]*Department of Oto-Rhino-Laryngology, University Hospital of Cologne, Joseph-Stelzmann-Strasse 9, D-50924 Cologne, Germany*

[c]*Jean-Uhrmacher Institut for clinical ENT-Research, University Hospital of Cologne, Joseph-Stelzmann-Strasse 9, D-50924 Cologne, Germany*

[d]*Plastic Surgery Clinic, Martin-Luther-Hospital, Caspar-Theyβ-Strasse 27-3114193 Grunewald, Berlin*

[e]*Department of Orthopedics and Trauma Surgery, University Hospital of Cologne, Joseph-Stelzmann-Strasse 9, D-50924 Cologne, Germany*

[f]*Laboratory of Histology and Embryology, Medical School, Aristotle University of Thessaloniki, Greece*

[g]*Department of Pathology, University Hospital of Cologne, Joseph-Stelzmann-Strasse 9, D-50924 Cologne, Germany*

[h]*Department of Oto-Rhino-Laryngology, Lessingstrasse 2, University of Jena, D-07743 Jena, Germany*

[i]*Experimental and Regenerative Neurosciences, School of Animal Biology, The University of Western Australia*

Abstract—Functional recovery following facial nerve injury is poor. Neuromuscular junctions (NMJs) are "bridged" by terminal Schwann cells and numerous regenerating axonal sprouts. We have shown that this poly-innervation of NMJs can be reduced by manual stimulation (MS) with restoration of whisking function. In addition, we have recently reported that insulin-like growth factor-1 (IGF-1) is required to mediate the beneficial effects of MS. Here we extend our findings to brain derived neurotrophic factor (BDNF). We then examined the effect of MS after facial-facial anastomosis (FFA) in heterozygous mice deficient in BDNF (BDNF$^{+/-}$) or in its receptor TrkB (TrkB$^{+/-}$). We quantified vibrissal motor performance and the percentage of NMJ bridged by S100-positive terminal Schwann cells. In intact BDNF$^{+/-}$ or TrkB$^{+/-}$ mice and their wild type (WT) littermates, there were no differences in vibrissal whisking nor in the percentage of bridged NMJ (0% in each genotype). 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 (27% more than intact). MS improved both the amplitude of vibrissal whisking (not significantly different from intact) and the percentage of bridged NMJ (11% more than intact). After FFA and handling in BDNF$^{+/-}$ or TrkB$^{+/-}$ mice, whisking amplitude was again reduced (53% and 60% lower than intact) and proportion of bridged NMJ increased (24% and 29% more than intact). However, MS failed to improve outcome in both heterozygous strains (whisking amplitude 55% and 58% lower than intact; proportion of bridged NMJ 27% and 18% more than intact). We conclude that BDNF and TRkB are required to mediate the effects of MS on target muscle reinnervation and recovery of whisking function.

Key words: mice, facial nerve, axotomy, motor end-plate, polyinnervation, terminal Schwann cells.

Following injury to peripheral nerves, functional recovery seldom occurs (Lundborg, 2003; Tang et al., 2004; Xin et al., 2008; Hadlock et al., 2010). Rather, a range of conditions develop, an indeed worsen, over time including paresis, synkinesis and dysreflexia with "post-paralytic syndrome" often being the norm (Kerrebijn and Freeman, 1998).

Cellular responses following peripheral nerve injury have been extensively documented with inaccurate reinnervation being considered the main feature that leads to abnormal function (Sumner, 1990; Ito and Kudo, 1994; Dai et al., 2000; Moran and Graeber, 2004; English, 2005; Maegele et al., 2005; Robinson and Madison, 2009). The axons themselves undergo extensive branching with each transected axon giving off up to 25 "collateral" branches within the nerve itself (Shawe, 1955; Morris et al., 1972; Mackinnon et al., 1991). Partly as a consequence of this excessive sprouting, regenerating axons are misrouted, failing to rejoin their original nerve fascicles (Anonsen et al., 1986; Baker et al., 1994). Once regenerating axons reach target muscles, the excessive number of collateral branches can result in one muscle being reinnervated by more than one motoneuron (polyneuronal innervation), often with antagonizing actions (Vleggeert-Lankamp et al., 2005). Finally, once regenerating axons reach a paralyzed muscle, they undergo additional "terminal axon sprouting" and simultaneously reinnervate multiple motor end-plates (Grimby et al., 1989; Trojan et al., 1991; Son et al., 1996). Furthermore, after injury, Schwann cells extend numerous processes that form bridges within target muscles (terminal Schwann cells) and act as a substrate for axon terminal sprouts to reach multiple adjacent (rather than single) motor endplates (Kang et al., 2003; Magill et al., 2007; Griffin and Thompson, 2008; Madison et al., 2009).

A number of factors have been identified which improve the accuracy of motor end plate reinervation both at the level of the regenerating axon and the terminal

[1] Contributed equally and share first authorship.

*Corresponding author. Tel: +49-221-478-5654; fax: +49-221-478-87893.
E-mail address: angelov.anatomie@uni-koeln.de (D. N. Angelov).

Abbreviations: BDNF, brain derived neurotrophic factor; FFA, facial-facial anastomosis; IGF-1, insulin-like growth factor-1; mRNA, messenger ribonuclein acid; MS, manual stimulation; NMJ, neuromuscular junction; TrkB, tyrosine kinase B; WT, wild type(mice).

0306-4522/10 $ - see front matter © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2010.06.053

Schwann cells. Stimulating muscles with flaccid paralysis by, for example, electrical stimulation or exercise, inhibits intramuscular axonal sprouting and diminishes motor-end-plate polyinnervation (Brown and Ironton, 1977; Brown and Holland, 1979). Similarly, after facial nerve injury, we have shown that manual stimulation (MS) of denervated whisker pads reduces polyinnervation which is associated with improved whisking function and blink reflexes (Angelov et al., 2007; Bischoff et al., 2009). Moreover, both running exercise and electrical stimulation limit the formation of terminal Schwann cell bridges thereby also improving reinnervation accuracy (Love et al., 2003; Tam and Gordon, 2003b).

The above structural features have been underpinned by several trophic factors. Among them, the brain-derived neurotrophic factor (BDNF) has been shown to act directly on motoneurons (Oppenheim et al., 1992; Sendtner et al., 1992; Yan et al., 1992) including rat facial motoneurons (Kobayashi et al., 1996) and thus play an important role (Sendtner, 1998). Also, terminal Schwann cells in chronically denervated muscle express BDNF indicating the potential to participate in repair processes under appropriate conditions (Dedkov et al., 2002). In addition, previous work has shown that accelerated motor reinnervation of muscle targets after brief electrical stimulation is associated with an accelerated and enhanced up-regulation not only of the neurotrophin BDNF, but also of its cognate receptor TrkB, in motor neurons (Al-Majed et al., 2000b, 2004).

Although exercise-induced upregulation of BDNF has been shown to facilitate recovery of locomotion following spinal cord injury (Gomez-Pinilla et al., 2002, 2004; Ying et al., 2005), it is not known whether this neurotrophic factor or its receptor is involved in stimulation-induced changes within regenerating peripheral nerves. Here, we first examined BDNF expression in denervated muscles in wild type mice after facial nerve injury, showing significant upregulation. To evaluate whether this growth factor and its receptor are required to mediate the beneficial effects of MS, we used our MS protocol in heterozygous mice deficient in BDNF ($BDNF^{+/-}$) or its receptor tyrosine kinase B ($TrkB^{+/-}$). Data were compared to heterozygous littermates for each genotype which received handling but no MS and to intact wild type (WT) mice.

EXPERIMENTAL PROCEDURES

Before and after surgical treatment, 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. All animal experiments were carried out in accordance in accordance with the German Law on the Protection of Animals, and the procedures were approved by the local Animal Care Committee. These guidelines are identical with those of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 or the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, or the European Communities Council Directive of 24 November 1986 (86/609/EEC). The formal approval to conduct the experiments described has been obtained from the animal subjects review board of the University of Cologne (Az.) and can be provided upon request.

Manual stimulation after facial nerve injury in BDNF deficient mice

Animals. Homozygous BDNF mutant animals die perinatally and we therefore derived breeding pairs of either heterozygote×heterozygote or wildtype×heterozygote from strain STOCK *Bdnf* ^{tm1Jae}/J, (Stock Number 002267, Jackson Laboratories, Bar Harbor, ME, USA). Heterozygotes were used as well as WT littermates as controls.

Genotyping was performed by PCR (Cycling conditions: 15 min 95 °C, 30 cycles: 30 s 95 °C, 30 s 64 °C, 60 s 72 °C, final elongation for 7 min 72 °C) using the HotStarTaq Master Mix Kit (Qiagen) from mouse tail DNA and subsequent Agarose-Gel-electrophoresis. The following primers were used to distinguish transgenics from WT: oIMR0132 (5′GGG AAC TTC CTG AGG GG-3′), oIMR133 (5′ATG AAA GAA GTA AAC GTC CAC-3′) and oIMR134 (5′CCA GCA GAA AGA GTA GAG GAG-3′). The transgene allele yielded an amplicon of 340 bp and the wild type 275 bp.

Mice were randomized to six groups, 10 in each (Tables 1 and 2). Groups 1 and 2 were intact WT or heterozygotes ($BDNF^{+/-}$). Groups 3–6 comprised WT (Groups 3 and 4) or $BDNF^{+/-}$ (Groups 5 and 6) and underwent FFA. Following FFA, animals either received MS (see below) (Groups 4 and 6) or served as "handling" controls which were held by the experimenter in exactly the same way as occurred during MS but without MS being given (Groups 3 and 5).

Surgery. Transection and end-to-end suture of the right facial nerve (facial-facial anastomosis, FFA) was performed by a trained surgeon (M. Grosheva) under surgical anaesthesia (Ketamin/Xylazin; 100 mg Ketanest®, Parke-Davis/Pfizer, Karlsruhe, Germany, and 5 mg Rompun®, Bayer, Leverkusen, Germany, per kg body weight; i.p.). The trunk of the facial nerve was exposed and transected close to its emergence from the foramen stylomastoideum (Fig. 1a) and the proximal and distal stumps imme-

Table 1. Biometrics of whisking behaviour in aged-matched WT ($BDNF^{+/+}$) and BDNF $^{+/-}$ female mice after facial-facial anastomosis, (FFA) with and without manual stimulation (MS) of the vibrissal muscles. The postoperative survival time was 8 wk. Each value is a mean±SD of 10 mice

Animal group	Frequency (in Hz)	Angle at maximal protraction (in degrees)	Amplitude (in degrees)	Angular velocity during protraction (in degrees/sec)	Angular acceleration during protraction (in degrees/sec^2)
1. WT intact	9.0±2.0	36.0°±17.0	63.0°±21.0	442°±172	24517°±5521
2. $BDNF^{+/-}$ intact	4.1±2.0	42.0°±13.0	49.0°±17.1	393°±210	19046°±5014
3. WT+FFA+handling	5.1±2.1	64.1°±15.3	25.2°±5.1$^{\#}$	297°±102$^{\#}$	6029°±2955$^{\#}$
4. WT+FFA+MS	7.1±2.1	63.2°±17.3	40.4°±9.1*	420°±150$^{\#}$	14188°±2003
5. $BDNF^{+/-}$+FFA+handling	7.1±2.1	63.4°±15.1	23.3°±5.1§	240°±93§	4695°±2491§
6. $BDNF^{+/-}$+FFA+MS	7.1±2.3	55.0°±25.1	22.1°±6.1§,*	290°±192§	5247°±1980§

Significantly different values (ANOVA and post-hoc Tukey's test, $P<0.05$) obtained in surgically treated WT-mice (groups 3, 4) when compared to those from intact WT-mice (group 1) are indicated by $^{\#}$. Likewise, significantly different values obtained in surgically treated $BDNF^{+/-}$-mice (groups 5, 6) when compared to those from intact $BDNF^{+/-}$-mice (group 2) are indicated by §. Finally, significant differences (ANOVA and post-hoc Tukey's test, $P<0.05$) between WT- and $BDNF^{+/-}$-mice subjected to handling (group 3 vs. group 5) or manual stimulation (group 4 vs. group 6) are indicated by *.