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DEFICIENT FUNCTIONAL RECOVERY AFTER FACIAL NERVE CRUSH IN RATS IS ASSOCIATED WITH RESTRICTED REARRANGEMENTS OF SYNAPTIC TERMINALS IN THE FACIAL NUCLEUS

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Abstract—Crush injuries of peripheral nerves typically lead to axonotmesis, axonal damage without disruption of connective tissue sheaths. Generally, human patients and experimental animals recover well after axonotmesis and the favorable outcome has been attributed to precise axonal reinnervation of the original peripheral targets. Here we assessed functionally and morphologically the long-term consequences of facial nerve axonotmesis in rats. Expectedly, we found that 5 months after crush or cryogenic nerve lesion, the numbers of motoneurons with regenerated axons and their projection pattern into the main branches of the facial nerve were similar to those in control animals suggesting precise target reinnervation. Unexpectedly, however, we found that functional recovery, estimated by vibrissal motion analysis, was incomplete at 2 months after injury and did not improve thereafter. The maximum amplitude of whisking remained substantially, by more than 30% lower than control values even 5 months after axonotmesis. Morphological analyses showed that the facial motoneurons ipsilateral to injury were innervated by lower numbers of glutamatergic terminals (−15%) and cholinergic perisomatic boutons (−26%) compared with the contralateral non-injured motoneurons. The structural deficits were correlated with functional performance of individual animals and associated with microgliosis in the facial nucleus but not with polyinnervation of muscle fibers. These results support the idea that restricted CNS plasticity and insufficient afferent inputs to motoneurons may substantially contribute to functional deficits after facial nerve injuries, possibly including pathologic conditions in humans like axonotmesis in idiopathic facial nerve (Bell's) palsy.

Key words: cholinergic synapses, microglia, peripheral nerve regeneration, synaptic plasticity, vesicular glutamate transporter 2, whisking.

INTRODUCTION

The central nervous system (CNS) of adult mammals is enriched in molecules inhibiting axonal growth and ample evidence indicates that this inhibition limits neural plasticity and functional recovery after CNS injuries (Loers and Schachner, 2007; Fawcett, 2009; Giger et al., 2010; Irintchev and Schachner, 2012). The role of CNS plasticity after injuries of peripheral nerves is by far less elucidated. It is well recognized that nerve lesions induce reorganization of cortical and subcortical CNS projections (Chen et al., 2002; Navarro et al., 2007) and it has been proposed that the outcome of upper extremity nerve repair in humans is strongly dependent on cortical reorganizations (Lundborg, 2003). A few experimental studies have provided direct evidence that manipulations attenuating inhibition in the CNS are beneficial. Removal of glycosaminoglycan chains, responsible for axon growth inhibition, by delivery of the bacterial enzyme chondroitinase ABC into the spinal cord leads to improved functional recovery (Galtrey et al., 2007) and promotes plasticity of spinal reflexes after forelimb nerve repairs in rats (Bosch et al., 2012). Genetic ablation of the extracellular glycoprotein tenascin-R (TNR), known also to inhibit axon growth, leads to enhanced rearrangements of perisomatic synaptic terminals after spinal cord injury (Apostolova et al., 2006) and improved functional outcome of both spinal cord injury (Apostolova et al., 2006) and peripheral nerve repair (Guntinas-Lichius et al., 2005a). Recently we identified synaptic terminal aberrations after nerve repair in rats which might be considered as potential factors contributing to poor functional restitution (Raslan et al., 2013). Surgical repair (transection and suture) of the facial nerve is followed by poor functional recovery which reaches maximum at 8 weeks after lesion and does not improve thereafter (Angelov et al., 2005; Raslan et al., 2013). At time points of maximum recovery (8 and 16 weeks) we found reduced, compared with sham-operated control animals, glutamatergic and cholinergic input to motoneurons and reduced ratio of excitatory to inhibitory terminals (Raslan

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Abbreviations: ANOVA, analysis of variance; ChAT, choline acetyltransferase; CNS, central nervous system; Iba1, ionized calcium binding adaptor molecule 1; LLS, levator labii superioris muscle; *N*, number; N_v, numerical density; SD, standard deviation; TNR, tenascin-R; *V*, volume; VGLUT, vesicular glutamate transporter.

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et al., 2013). These aberrations were correlated with the functional deficits and associated with persistent microgliosis in the facial nucleus. We speculated that the deficient afferent inputs attenuate the motor output and, thus, negatively influence the functional performance after muscle reinnervation. Surgical nerve repair is, however, also associated with another major abnormality considered detrimental for functional recovery, i.e. false rewiring of muscle fibers as a result of axonal sprouting, misdirected axonal regrowth and polyneuronal innervation (Choi and Raisman, 2002; Guntinas-Lichius et al., 2002, 2005b; Streppel et al., 2002; Angelov et al., 2005).

Here we were interested whether the deficits in afferent inputs observed after facial nerve repair are also present after nerve crush when muscle reinnervation is expected to be precise since the lesion results in Wallerian degeneration but leaves uninterrupted the connective tissue sheaths projecting to the appropriate muscle targets. Eight and 20 weeks after facial nerve crush in rats we performed (1) retrograde labeling of facial motoneurons to assess precision of reinnervation, (2) functional analysis of whisking to estimate recovery of function and (3) stereological counts of cholinergic and glutamatergic terminals in the facial nucleus to measure the degree of restoration of motoneuron afferent connections. Our results indicate that despite precise target reinnervation after nerve crush, a significant degree of motoneuron deafferentation persists and this deficit is correlated with insufficient recovery of function.

EXPERIMENTAL PROCEDURES

Animals and experimental design

Male Wistar Unilever rats were obtained from Harlan Laboratories (Harlan Winkelmann, Borchen, Germany) at the age of 2 months and used for experiments after a 2-week period of adaptation to the local housing conditions. Two experiments were performed. In Experiment 1, rats were subjected to either crush or cryogenic lesion ($N = 9$ per group) and video recorded for motion analysis at 8 and 20 weeks after injury. After the second recording, these animals and a control group of previously uninjured age-matched rats ($N = 6$) were used for retrograde labeling of motoneurons. In Experiment 2, rats ($N = 8$) were subjected to unilateral facial nerve crush. Eight weeks later, the animals were video recorded for vibrissal motion analysis and subsequently euthanized for stereological analyses of the injured and intact contralateral facial nuclei, as well as analyses of endplate innervation. The animals were housed in groups of 3–4 under standard conditions and received food and water *ad libitum*. All experiments were performed according to the German and European Community laws on protection of animals including permission of the ethics committee of the State of Thuringia.

Surgery

Unilateral facial nerve lesions were done under neurolept anesthesia with fentanyl (Fentanyl Janssen, Janssen, Neuss, Germany, 0.005 mg/kg i.m.), midazolam (Dormicum-R, Roche, Basel, Switzerland, 2 mg/kg i.m.) and medetomidine (Domitor-R, Orion Pharma, Espoo, Finland, 0.15 mg/kg i.m.). The trunk of the right facial nerve was exposed under an operation microscope and injured close to its emergence from the stylomastoid foramen. Two types of injury were performed: (1) nerve crush produced by manual pressing of the nerve between the tips of Dumont forceps #2 (Fine Science Tools, Heidelberg, Germany) for 30 s (Fey et al., 2010; Hadlock et al., 2010) or (2) freezing of approximately 8-mm long segment of the nerve trunk by applying, for 7 s, a metal cryode precooled in liquid nitrogen (Irintchev et al., 1990, 1991). The metal cryode was made out of a copper rod (70 mm long, 4 mm in diameter) and had a flattened tip ($4 \times 4 \times 1$ mm, length $\times$ width $\times$ thickness) which was attached to the nerve trunk during freezing. After injury, the skin was closed with 4–0 sutures (Ethicon, Norderstedt, Germany) and an antidote cocktail containing atipamezole (Antisedan, Orion Pharma, 0.75 mg/kg), flumazenil (Anexate, Roche, 0.2 mg/kg) and naloxone (Naloxon, CuraMed Pharma, Karlsruhe, Germany, 0.12 mg/kg) was injected subcutaneously to achieve rapid (within 5 min) recovery from the anesthesia. Immediately after crush or cryogenic lesion the vibrissae of all animals were motionless and oriented backward (retracted) and these signs of paralysis persisted, as previously observed by Hadlock and colleagues (2010), during the first 10 post-operative days.

Video recordings and analysis of whisking

The video analyses were performed according to the method of Tomov and colleagues (2002) as described (Raslan et al., 2013). All vibrissae were clipped except two large vibrissae in row C (C1–2) on each side. The animals were placed into a day–light illuminated arena (60×40 cm) and recorded twice for 3–4 min during active exploration using a video camera (Pike F-032, Allied Vision Technologies, Stadtroda, Germany) located at about 60 cm above the arena. The camera was equipped with an 8–48 mm manual TV zoom lens (Pentax C60812, Pentax Rocoh Imaging Systems, Hamburg, Germany) and operated at 100 frames per second using a motion analysis software (SIMI Motion, Simi Reality Motion Systems, Unterschleißheim, Germany). After 2-D calibration, manual tracking of the vibrissae in selected video sequences (2×1 s) was performed using the SIMI Motion software. The amplitude of movement was estimated as difference between protraction and retraction angles measured with respect to the sagittal plane. For each animal, the maximum amplitude was calculated as mean of the three largest values.

Retrograde labeling of motoneurons

Triple labeling of facial motoneurons in Experiment 1 was performed under deep neurolept anesthesia (see above). The main branches of the facial nerve were transected approximately midway between the facial nerve trunk