Annals of Anatomy 193 (2011) 286–303

Contents lists available at ScienceDirect

Annals of Anatomy

journal homepage: www.elsevier.de/aanat

Intrinsic and therapeutic factors determining the recovery of motor function after peripheral nerve transection

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ARTICLE INFO

Article history:
Received 28 December 2010
Received in revised form 3 February 2011
Accepted 4 February 2011

Keywords:
Facial nerve
Axotomy
Facial palsy
Motor endplates
Polyinnervation
Physiotherapy
Electrical stimulation
Mechanical stimulation

SUMMARY

Insufficient recovery after peripheral nerve injury has been attributed to (i) poor pathfinding of regrowing axons, (ii) excessive collateral axonal branching at the lesion site and (iii) polyneuronal innervation of the neuromuscular junctions (NMJ). The facial nerve transection model has been used initially to measure restoration of function after varying therapies and to examine the mechanisms underlying their effects. Since it is very difficult to control the navigation of several thousand axons, efforts concentrated on collateral branching and NMJ-polyinnervation. Treatment with antibodies against trophic factors to combat branching improved the precision of reinnervation, but had no positive effects on functional recovery. This suggested that polyneuronal reinnervation – rather than collateral branching – may be the critical limiting factor. The former could be reduced by pharmacological agents known to perturb microtubule assembly and was followed by recovery of function. Because muscle polyinnervation is activity-dependent and can be manipulated, attempts to design a clinically feasible therapy were performed by electrical stimulation or by soft tissue massage. Electrical stimulation applied to the transected facial nerve or to paralysed facial muscles did not improve vibrissal motor performance and failed to diminish polyinnervation. In contrast, gentle stroking of the paralysed muscles (vibrissal, orbicularis oculi, tongue musculature) resulted in full recovery of function. This manual stimulation was also effective after hypoglossal–facial nerve suture and after interpositional nerve grafting, but not after surgical reconstruction of the median nerve. All these findings raise hopes that clinically feasible and effective therapies could be soon designed and tested.

1. Foreword: generally acknowledged reasons for the poor recovery of function after peripheral nerve transection

Peripheral nerve transection initiates a complex series of changes. About 24 h after disconnection, the axons in the distal fragment begin to lyse. When resorption of debris is complete, the Schwann cells re-arrange in the chains of Büngner (1891) which bridge the interfragmentary gap and form guiding channels for the regenerating axons to their target(s). This so-called Wallerian degeneration creates an environment that is highly supportive of axonal re-growth and ensures that the vast majority of axons enter the distal stump (Bisby, 1995). Nevertheless, complete recovery of function is only rarely achieved. Despite the use of modern microsurgical techniques for nerve repair an aberrant re-innervation of targets inevitably occurs and is expressed clinically by abnormally associated movements and altered reflexes (Anonsen et al., 1986; Baker et al., 1994; Kimura et al., 1975).

Minimal recovery has been attributed to

(i) *misdirected regrowth of axons* at the transection site: due to malfunctioning axonal guidance, a muscle gets reinnervated by a "foreign" axon which has been simply misrouted along the "wrong" nerve fascicle (Aldskogius and Thomander, 1986; Esslen, 1960; Ito and Kudo, 1994);
(ii) presence of supernumerary branches from all transected axons (*collateral axonal branching*; (Friede and Bischhausen, 1980; Morris et al., 1972; Shawe, 1955). In this way, a given muscle can be reinnervated by branches stemming from several motoneurons, a state known as "polyneuronal innervation" (Brown et al., 1981; Rich and Lichtman, 1989) or "hyperinnervation" (Angelov et al., 1996). Though claimed to be transient (Hennig and Dietrichs, 1994), this excessive axonal branching may persist for extended periods (Mackinnon et al., 1991;

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doi:10.1016/j.aanat.2011.02.014

Madison et al., 1999) with deleterious effects on synchronized motor function;

(iii) vigorous "*intramuscular" or "terminal" sprouting* of regenerationg axons at their terminals in the target muscle (English, 2003; Fu and Gordon, 1997; Gordon et al., 2004; Grimby et al., 1989; Hayworth et al., 2006; Son et al., 1996; Tam and Gordon, 2003; Trojan et al., 1991) such that the majority of motor endplates become poly-, rather than mono-, innervated (Guntinas-Lichius et al., 2005).

Whereas numerous aspects of the post-transectional aberrant reinnervation have been extensively documented (Brushart, 1993), little is known how this phenomenon could be prevented. Attempts to act on the malfunctioning axonal pathfinding, achieving a "fascicular" or "topographic" specificity" (Evans et al., 1991; Mackinnon et al., 1986) have failed: so far, it is technically impossible to correctly guide the growth cones of several thousands of axons (plus their branches) originating from the proximal stump of a transected nerve. This is why most of the basic research laboratories have concentrated their efforts on the reduction of the postlesional collateral axonal branching and on diminution of the NMJ-polyinnervation.

2. Attempts to reduce collateral branching of axons at the lesion site

2.1. General biological significance of the process

Injury to the peripheral nerve initiates a complex series of changes distal to the site of injury, collectively known as Wallerian degeneration. Within 24 h after lesion, the axonal content begins to necrotize and axonal debris is phagocytosed by blood–borne macrophages and proliferated Schwann cells (Hirata and Kawabuchi, 2002; McPhail et al., 2004; Perry and Brown, 1992). When resorption is complete, the Schwann cells form long chains of cells (bands of Büngner), which bridge the interfragmentary gap and form guiding channels for the regenerating branches on their way to the target(s). The architectural pattern of the Büngner's bands in the peripheral stump remains unchanged for 3 months, after which progressive distorsion by proliferating connective tissue occurs. The process of Wallerian degeneration creates an environment that is highly supportive of axonal growth. The preference for axonal growth into a degenerating nerve ensures that the vast majority of axons will regrow into the distal stump if it remains in continuity with the proximal stump (Bisby and Tetzlaff, 1992).

In spite of that, regenerating axons do not merely elongate towards the distal stump, but respond with axonal branching (sprouting) by lateral budding mainly at the nodes of Ranvier, up to 6–8 mm proximal to the injury site in the rat cervical sympathetic trunk. As regeneration proceeds, some of these supernumerary branches are pruned off over a period of up to 12 months (Bray and Aguayo, 1974). There are, however, persistently higher numbers of myelinated and unmyelinated axons in regenerated segments of peripheral nerves than in intact nerves.

What is the general biological significance of branching? To answer this question one needs more information about the structural and biochemical events which accompany the process of axonal sprouting. The majority of the recent reports suggest that axonal branching is part of the neuronal response to injury within a complex programme directed towards regeneration. This attempt is associated with substantial cytoskeletal reorganization (King et al., 2001), resulting in the elaboration of fine protrusions (sprouts) into and across lesion sites (McHale et al., 1995).

Observations *in vitro* show that axonal branching begins from the end-bulb within 3 h after injury (Sjoberg and Kanje, 1990). The regenerating branches initially lie on the surface of the Schwann cells. Later, these branches increase in diameter and get surrounded by Schwann cell processes. The guidance of these immature axons to their final destination can be considered as a series of short-range projections to intermediate targets under the influence of local guidance cues. Neurons respond to these cues by means of motile sensory apparatus at the tip of the advancing axon termed the "*growth cone*", which very often does not emerge from the axon at the precise site of injury, but proximal to it. The initial formation of growth cones occurs before the necessary newly synthesized proteins would have time to arrive at the site of axon injury, i.e. too rapidly to be dependent on metabolic changes in the cell body (Borgens, 1988; Ziv and Spira, 1997).

The growth cone borne by neurites is shaped like a webbed foot (Fawcett and Keynes, 1990). There is a swollen central core from which flattened processes called *lamellipodia* and numerous stiff fine processes called *filopodia* extend. Current studies have identified 3 major intracellular cytoskeletal components responsible for the cytomechanical forces in the leading edge of elongating axons: actin microfilaments, myosin and microtubules (Challacombe et al., 1996).

A microtubule is a long, hollow cylinder that is made of a polymer of α- and β-tubulins and has a diameter of 25 nm. It has intrinsic polarity, with a fast-growing 'plus end' and an opposite, slow-growing 'minus end'. In axons, microtubules run in a longitudinal orientation and serve as rails along which membranous organelles and macromolecular complexes can be transported; they are unipolar, with the plus end pointing away from the cell body (Hirokawa and Takemura, 2005). Facilitating the fusion of vesicles with the plasma membrane, microtubules have been shown to promote the extension of growth cone lamellipodia (Kalil and Dent, 2005; Spira et al., 2003). Upregulated levels of tubulin in the perikarya and increased delivery of mictotubules to regrowing axon tips have been considered essential for regeneration (Tetzlaff et al., 1988a,b, 1991, 1996). Accordingly, Schaefer et al. (2002) and Fukata et al. (2002) have shown that the population of microtubules that invade the peripheral domain via filopodia are highly dynamic, suggesting functional specializations, perhaps in exploratory and/or signaling capacity.

2.2. Proof of principle: would pharmacologically reduced collateral axonal branching at the lesion site promote improved recovery of function?

Drugs that attenuate either microtubule or actin dynamics (inhibition of actin polymerization with cytochalasin, stabilization of microtubules with taxol, or damping of microtubule dynamics with vinblastine) have been shown to inhibit *in vitro* axonal branching but not elongation (Baas and Ahmad, 1993; Challacombe et al., 1997; Tanaka et al., 1995; Williamson et al., 1996). Treatment with vincristine, an inhibitor of microtubule formation blocks the outgrowth of some axons and delays the regeneration of others (Pan et al., 2003).

Accordingly Grosheva et al. (2008) proved whether a similar treatment *in vivo* would also increase the rate of neurite regrowth and improve recovery of muscle function: they applied established pharmacological agents to perturb microtubule assembly towards stabilization (enhanced polymerization with 10 μg/ml taxol) or increased synthesis (challenged by destabilization with 100 μg/ml nocodazole and 20 μg/ml vin-