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Strategies to promote peripheral nerve regeneration: electrical stimulation and/or exercise

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Abstract

Enhancing the regeneration of axons is often considered a therapeutic target for improving functional recovery after peripheral nerve injury. In this review, the evidence for the efficacy of electrical stimulation (ES), daily exercise, and their combination in promoting nerve regeneration after peripheral nerve injuries in both animal models and in human patients, is explored. The rationale, effectiveness, and molecular basis of ES and exercise in accelerating axon outgrowth are reviewed. In comparing the effects of ES and exercise in enhancing axon regeneration, increased neural activity, neurotrophins, and androgens are considered common requirements. Similar, gender-specific requirements are found for exercise to enhance axon regeneration in the periphery and for sustaining synaptic inputs onto injured motoneurons. ES promotes nerve regeneration after delayed nerve repair in humans and rats. The effectiveness of exercise is less clear. Although ES, but not exercise, results in a significant misdirection of regenerating motor axons to reinnervate different muscle targets, the loss of neuromuscular specificity encountered has only a very small impact on resulting functional recovery. Both ES and exercise are promising experimental treatments for peripheral nerve injury that seem ready to be translated to clinical use.

Keywords

electrical stimulation; peripheral nerve regeneration; exercise and nerve regeneration; exercise or electrical stimulation; nerve stimulation

Introduction

Since 2000 when Al-Majed and colleagues demonstrated that brief (one hour) low frequency (20 Hz) electrical stimulation (ES) accelerated axon regeneration after nerve transection and microsurgical repair (Al-Majed et al 2000b), there has been increasing interest in using ES to improve the capacity of injured peripheral nerves to regenerate their axons and in turn, to promote functional recovery. The comparison of the efficacy of ES and daily exercise first examined in detail by Asensio-Pinilla et al (Asensio-Pinilla et al 2009; Udina et al 2011) later fueled interest in daily exercise in promoting peripheral nerve regeneration and

functional recovery. Of particular interest is that the exercise regimens needed for successful promotion of axon regeneration are gender-specific, providing both rationales for patient treatment as well as insights into mechanisms by which nerve regeneration may be enhanced with improved functional outcomes.

In this review, we explore the evidence for the efficacy of ES, daily exercise, and the combination in promoting nerve regeneration after different nerve injuries in both animal models and in human patients. Further, we provide evidence for a key role of neurotrophic factors as well as androgens in the efficacy of both ES and exercise in both genders.

Electrical stimulation and peripheral nerve regeneration

Wallerian degeneration and the molecular response of peripheral nerves to injury

The Schwann cells of the peripheral nervous system support nerve regeneration after injury whilst the analogous myelinating glial cells of the central nervous system, the oligodendrocytes, do not (Fenrich and Gordon 2004). When axons are isolated from their neuronal cell bodies, Wallerian degeneration of these axons proceeds only after axonal transport within the axons aborts and the Schwann cells lose their myelin cover and begin to proliferate (Fu and Gordon 1997;Gaudet et al 2011;Grafstein 1975;Lieberman 1971). Interestingly, the transport continues for hours in the isolated nerve stump accounting for the ability of the nerves to conduct action potentials and elicit muscle contractions in isolated nerve-muscle preparations for several hours as the degeneration spreads longitudinally from the injury site (Lubinska 1977;Mackenzie et al 2012;Miledi and Slater 1970). In human nerves, the ability to conduct electrical impulses distally in the severed nerve persists for days (Mackenzie et al 2012). Schwann cells initially phagocytose the myelin and axon debris but it is the macrophages that penetrate the leaky blood-nerve barrier days after the injury that are the prime phagocytic cells that remove the debris within ~3 weeks (Avellino et al 1995;Stoll et al 2002). As the Schwann cells proliferate, they undergo a phenotypic change from myelinating to a pre-myelinating form that is permissive for axonal growth: many regeneration associated genes are expressed, including neurotrophic factors such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), glial derived neurotrophic factor (GDNF) and pleiotrophin (Boyd and Gordon 2003;Hoke et al 2006), and transcription factors such as c-Jun and Notch (Fontana et al 2012;Jessen and Mirsky 2005;Jessen et al 2008) (Fig. 1). The injured neurons also undergo growth-related changes as their nuclei move to an eccentric position in the soma with many of the morphological changes, referred to as chromatolysis (Lieberman 1971), reflecting the change of the neurons from a transmitting to a growth mode. Regeneration associated genes are upregulated, including those that transcribe the cytoskeletal proteins, tubulin and actin and several neurotrophic factors whilst transmitter related genes such as acetylcholine and choline-acetyltransferase are downregulated (Fu and Gordon 1997;Zigmond 2012). Neurofilament protein expression is downregulated accounting for the decline in diameter of the axons in the proximal nerve stump (Gordon and Stein 1982;Holmquist et al 1993;Kerns et al 1993). The altered RAG expression allows for the outgrowth of axon sprouts with transport of tubulin and actin being central to this capacity (Tetzlaff et al 1988). Intriguingly, axon sprouts are emitted even from isolated axons *in vitro* (Kato and Ide 1994;Shaw and

Bray 1977) but materials transported from the cell body, including the cytoskeletal proteins, tubulin and actin, are the major source for axonal elongation (Kato and Ide 1994).

Axon regeneration after nerve injury

Sprouts are emitted by each of the axons in continuity with the neuronal cell bodies. Ramon Y Cajal described these sprouts more than a century ago using silver staining (Cajal 1928) as illustrated in Fig. 2. The sprouts wander within the site of surgical repair of a transected nerve and may even double back for a short distance with bizarre coiling of axons within the proximal nerve stump (Fig. 2A). As a result, the growth cones that do cross the surgical site are not aligned but rather they 'stagger' as they progress through the distal nerve stump (Fig. 2B, C). The staggered regeneration of axons across the surgical site and into the distal nerve stump was recently described by Brushart and colleagues who, using transgenic mice expressing green fluorescent protein (GFP) in their neurons reported a highly disorganized extracellular matrix with few Schwann cells within the site in the first 7 days (Witzel et al 2005). At this point in time very few neurons have regenerating their axons across the surgical repair site as determined from counts of motoneurons backlabelled with fluorescent dyes applied just distal to the surgical repair site (Fig. 2D). Indeed these findings illustrate that a latent period of a few days established in experiments where the regenerated nerve was exposed at progressively longer intervals after the nerve injury to elicit a reflex withdrawal in response to crush of the regenerated axons in distal nerve stump, does not reflect the actual process of nerve regeneration (Holmqvist et al 1993; Kerns et al 1993).

It is only when the distribution of the extracellular matrix protein, laminin, becomes organized into parallel arrays within ~10 days after the nerve transection and surgical repair, that Schwann cells move into the surgical site and align in parallel as the Bands of Büngner to guide the progressively increasing number of axon sprouts across the surgical repair site: the neurons regenerating their axons across the site increase to a maximum four weeks after the surgical repair (Fig. 2D). This slow progression of axons across the surgical gap was referred to as 'staggered axonal regeneration' that accounted for a protracted period of 8–10 weeks for all motoneurons to regenerate their axons over a 25 mm distance within the denervated distal nerve stump after microsurgical repair of the transected rat femoral nerve (Al-Majed et al, 2000) (Fig. 2E). This staggering was particularly striking when motoneurons that had regenerated their axons into the appropriate motor branch to the quadriceps muscle and into the inappropriate saphenous sensory nerve branch were backlabelled with two different dyes at different times after the femoral nerve transection and surgical repair (Fig. 2F). Of particular interest was the finding that the regeneration of the axons into the two nerve branches was random two weeks after surgical repair, as had previously been shown by Brushart (1993) *and* that the described preferential reinnervation of the appropriate motor nerve branch emerged because thereafter, *all* the motoneurons regenerated their axons into the appropriate motor nerve branch. Those motoneurons with axons in the inappropriate sensory nerve branch and with axons in both nerve branches did not change in numbers after two weeks (Fig. 2E).

Staggered axon regeneration is quite evident in the longitudinal images shown on the left of Fig. 3C of regenerating axons from the proximal stump of a transgenic mouse of the H strain

of the *thy1-YFP* transgenic mouse into a denervated distal nerve stump of a non-transgenic wild type litter mate. In axons of the common peroneal nerve of these mice, yellow fluorescent protein (YFP) is expressed in a subpopulation of ~35 motor and sensory neurons, assumed to represent a sample of all axons. After two weeks, few axons have grown across the surgical repair site and into the distal nerve stump where the ongoing Wallerian degeneration of the isolated distal axons that do not express YFP does not obscure the ingrowth of the YFP-positive regenerating axons from the proximal nerve stump (English et al 2007). Within the distal nerve stump, the axons follow the extended processes of the Schwann cells beneath the basal lamina, the axons coursing between the cells and the extracellular matrix at a regeneration rate of 1–3 mm/day through the distal nerve stumps (Brushart 2011; Haftek and Thomas 1968; Sunderland 1947; Sunderland 1978).

ES accelerates nerve regeneration

The enticing findings in the 1980's that 1) continuous 4 Hz ES of the proximal stump of a nerve to the lateral gastrocnemius and soleus muscles of the rabbit accelerated the recovery of soleus muscle force after a crush injury (Nix and Hopf 1983) and 2) brief (15–60 minutes) 20Hz ES accelerated the appearance of the plantar extensor reflex (Pockett and Gavin 1985) was the basis for the later experiments of Al-Majed and colleagues who used the backlabeling techniques to examine whether the low frequency ES actually affected nerve regeneration (Al-Majed et al 2000b). The experimental design was to initially examine whether continuous ES of the proximal nerve stump of the transected and surgically repaired femoral nerve at 20Hz for two weeks, influenced the ability of the motoneurons to regenerate axons into motor and sensory nerve branches. The 20 Hz frequency of ES was chosen following on from the findings of Pockett and Gavin (1985), this frequency also being the mean frequency of action potential generation in motoneurons (Burke 1981). The two week period of ES was chosen because the preferential reinnervation of appropriate motor and inappropriate sensory nerve pathways was evident eight weeks after surgical repair but was not apparent at two weeks (Fig. 2E) (Brushart 1993). The ES effect was dramatic: preferential motor reinnervation of appropriate motor pathways was evident at three weeks rather than ~6 weeks *and* all the motoneurons had regenerated their axons into the motor nerve branch within the three weeks (Fig. 3A) as compared to the eight to ten week period observed when the proximal nerve stump of the transected and repaired femoral nerve was sham stimulated immediately after the surgery (Fig. 2E) (Al-Majed et al 2000b). By backlabeling regenerating axons that had just crossed the femoral nerve repair site, it was demonstrated that the ES accelerated the outgrowth of axons and their crossing of the repair site (Brushart et al 2002). The dramatic effect of the ES in accelerating axon growth into the distal nerve stump is well illustrated by the obvious increase in the numbers of regenerating YFP-positive axons in the nerve graft and their progression through the graft after ES as compared to the staged axon regeneration in the unstimulated common peroneal nerve where relatively few regenerating axons entered into the nerve graft (Fig. 3C).

The increased outgrowth of axons in response to the ES results in earlier muscle reinnervation (Fig. 4) and functional recovery after both mixed and sensory nerve injury and surgical repair in animal models and in humans (Ahlborn et al 2007; Gordon et al 2010; Wong et al 2015). In rats, the earlier recovery is seen as earlier evoked compound

muscle and sensory potentials after sciatic nerve repair and one hour 20Hz electrical stimulation (Singh et al, 2010), earlier return of normal angles of the hindlimbs during locomotion (Ahlborn et al 2007;Gordon et al 2010;Wong et al 2015), return of the plantar extensor reflex in response to a sudden drop (Pockett and Gavin 1985) and of motor and sensory evoked potentials (Ahlborn et al 2007;Gordon et al 2010;Wong et al 2015). An earlier recovery of evoked muscle contractions was reported in the early study of the effect of 4 Hz continuous electrical stimulation to the soleus nerve on reinnervation of muscle in rabbits (Nix and Hopf 1983). Finally, in humans, the one hour paradigm of 20 Hz electrical stimulation of the median nerve after carpal tunnel release surgery accelerated the reinnervation of the muscles of the thenar eminence with an earlier return of sensory compound action potentials (Ahlborn et al 2007;Gordon et al 2010;Wong et al 2015).

The finding that one hour of ES was effective in promoting motor axon regeneration was serendipitous because the same period of ES was equally effective in promoting sensory axon regeneration (Brushart et al 2005;Geremia et al 2007). When the time period of ES was prolonged from one hour to two weeks in the reverse order to which the time period of ES had been manipulated for motor axon regeneration (Al-Majed et al 2000b), the ES was ineffective in accelerating sensory nerve outgrowth (Geremia et al 2007). In marked contrast to the finding that one hour of ES applied at the time of nerve repair resulted in increased numbers of sensory neurons that expressed BDNF and its receptor trkB, this failure of periods of greater than one hour to accelerate sensory nerve regeneration was associated with downregulation of trkB receptor expression in sensory neurons (Geremia et al 2007). It is important to note, however, that sensory nerve regeneration is frequently inferior to motor nerve regeneration both in the absence and the presence of ES. This was observed in the recovery of sensation during locomotion as compared to the recovery of motor activity after surgical repair of a motor nerve to the extensor muscles in cats (Gordon et al 1980). When the regeneration of motor and sensory nerves was determined by backlabeling motor and sensory neurons that had regenerated, there were always fewer sensory neurons that regenerated their axons as compared to motoneurons (Elzinga et al 2015;Udina et al 2010). This was the case when axon regeneration was enhanced by rolipram administration or by ES (see Fig. 11). One contributing factor may be the greater susceptibility of injured sensory neurons to cell death, up to ~50% of sensory neurons being susceptible in contrast to injured motoneurons that all survive nerve injuries even after ligation of transected nerves to prevent nerve regeneration (Fu and Gordon, 1997;Gordon et al 1991;Vanden Noven et al 1993;Welin et al 2009).

The ES did *not* increase the *rate* of regeneration, as demonstrated using radioactive protein labelling in the motoneurons (Brushart et al 2002). This effect of the ES contrasts with the effect of a conditioning lesion that increases regeneration rate (Bisby and Pollock 1983). The conditioning lesion requires a prior nerve injury, a method not clinically applicable however, in contrast to the ES paradigm. In the experiments of Al-Majed et al, the duration of the ES was decreased progressively down to one hour, aiming for a more clinically viable period of ES that could be adopted in the operating theatre for human nerve surgical repair (Al-Majed et al 2000b). It was also established in that study that the antidromic action potentials elicited by the ES of the proximal femoral nerve stump were essential for the

effect of accelerating the regeneration of the axons, the effect being blocked by proximal, but not distal application of tetrodotoxin during the one hour of ES (Al-Majed et al 2000b).

Molecular basis of ES accelerated axon outgrowth

Schwann cells—ES upregulates the expression of several neurotrophic factors in Schwann cells *in vitro* (Huang et al 2010; Koppes et al 2014) and *in vivo* (Wenjin et al 2011). These include NGF and BDNF, the effect of ES being mediated via the entry of calcium ions via voltage-gated channels and downstream activation of erk pathways that mediate synthesis of BDNF (Wenjin et al 2011). Neurotrophic factors that are differentially expressed by denervated distal motor and sensory nerve stumps, most of the expression being attributed to the Schwann cells that they contain (Brushart et al 2013; Hoke et al 2006), are likely to play an essential role in the choices that are taken by regenerating motor and sensory nerves to enter into the appropriate motor and sensory pathways, respectively. As outlined by Gordon (Gordon 2014), the elevated expression of the neurotrophic factors reaches peak levels at ~2 weeks after nerve transection that coincides with the time when the preferential ingrowth of appropriate motor and sensory axons begins (Fig. 2E and Fig. 5A). Examples of the selective and delayed expression of neurotrophic factors is shown in Fig. 5B and C. Note for example the selective expression of pleiotrophin in motor and of BDNF in sensory Schwann cells. This selective expression was resolved with several innovative surgical paradigms that included the elimination of motor nerves from mixed nerves by section of ventral roots (Hoke et al 2006).

Neurons—The expression of neuronal regeneration associated genes after injury is not only greatly elevated but is accelerated by the one hour period of 20Hz ES: BDNF and the trkB receptor are upregulated more quickly and significantly more when the motoneurons are subjected to ES (Al-Majed et al 2000a). Thereafter, the cytoskeletal proteins tubulin and actin and the growth associated protein, GAP-43, were elevated to higher levels and more quickly after ES (Fig. 6A–D) (Al-Majed et al 2000a; Al-Majed et al 2004; Wong et al 2015). As shown in Fig. 6A–D, this effect was transient, the highest expression of the neurotrophic factors and the cytoskeletal proteins declining after the peak expression levels were reached.

Using mouse genetics to eliminate either BDNF or NT-4/5 expression in allografts used to repair cut nerves, or repairing cut nerves with allografts depleted of cells after repeated freezing and thawing, these neurotrophins were shown to be important for successful axon regeneration in unstimulated nerves but their presence in the pathway of the regenerating axons within the allografts was not required for the enhancing effect of the one hour of ES (Fig. 6E) (English et al 2007). As shown also for the effects of treadmill exercise (see below), the effectiveness of one hour of ES at 20Hz in promoting accelerated axon outgrowth is independent of the neurotrophin and/or Schwann cell content of the allograft through which the regenerating axons grew. In agreement with the explanation first put forth by Al-Majed et al (Al-Majed et al 2000b), English and colleagues hypothesized that the effect of ES was mediated by an autocrine or paracrine action of neurotrophins released by the neurons (English et al 2007).

The gene for BDNF contains multiple 5' non-coding exons that are alternatively spliced to form as many as 22 different mRNAs, all coding for the same BDNF protein (Aid et al 2007). These different mRNAs are found in different proportions in different types of neurons (Aid et al 2007) but their production in response to different cellular signaling events may offer insights as to the cellular basis for the effects of ES in promoting axon regeneration. Exon IV that contains three distinct calcium response elements upstream from the transcription initiation site (Zheng et al 2011) is implicated in the activity-dependent synthesis of BDNF, the mRNAs containing the exon IV acting through one or more of the response elements. Activity-dependent expression of BDNF is reduced by mutating the calcium response element that contains a cyclic AMP (cAMP) response element binding protein (CREB) motif (Hong et al 2008), and because cAMP is elevated by ES (Udina et al 2008; Udina et al 2010) in conjunction with increased axon outgrowth (Udina et al 2010), synthesis of CREB likely is involved in the activity-dependent BDNF upregulation. Similarly, activity-dependent BDNF may also involve the SRY-box containing gene 11 (Sox11). This gene induces BDNF gene transcription via activity of mRNAs containing exon I of the BDNF gene (Salerno et al 2012) and axon regeneration is reduced by blocking Sox11 expression using siRNA (Jankowski et al 2009).

Exercise with and without electrical stimulation promotes nerve regeneration

Udina and colleagues, reviewing the conflicting data on the effects of exercise regimens on nerve regeneration, went on to 1) examine the effects four weeks of two hours of daily treadmill exercise beginning on the fifth day after nerve injury and 2) compare these with the effects of 20Hz ES administered acutely for one hour or chronically for one hour per day (Asensio-Pinilla et al 2009). The rationale for studying the effects of exercise on nerve regeneration was the findings of Gomez-Pinilla et al that BDNF expression rises after exercise (Gomez-Pinilla et al 2002; Vaynman and Gomez-Pinilla 2005; Vaynman et al 2004). The acute ES or the exercise promoted muscle reinnervation but that the combination of ES and exercise had a more beneficial effect during the early phase of regeneration (Asensio-Pinilla et al 2009). English and colleagues also pursued the efficacy of exercise on nerve regeneration demonstrating that two weeks of moderate daily treadmill exercise begun three days after surgical repair of transected rat nerves, enhanced axon regeneration (Wood et al 2012). Indeed, exercise effect was even more robust than the enhanced regeneration after ES (Fig. 7) (Sabatier et al 2008; Wood et al 2012). Similar outcomes had been implicated previously from several other laboratories using different exercise protocols (Hutchinson et al 2004; Marqueste et al 2004; Molteni et al 2004; van Meeteren et al 1997; van Meeteren et al 1998) that included swimming (Gutmann and Jakoubek 1963; Herbison et al 1973; Teodori et al 2011) and even rhythmic limb movements in anesthetized animals (Udina et al 2011). The efficacy of the exercise regimens that enhanced nerve regeneration required the upregulation of neuronal neurotrophic factors (Wilhelm et al 2012).

The findings of Jones and her colleagues that testosterone administration combined with 30 minutes of 20Hz ES just prior to facial nerve crush, was more effective than either exogenous testosterone or ES alone at accelerating nerve regeneration and recovery of the

blink reflex and vibrissae orientation in castrated male rats (Sharma et al 2009; Sharma et al 2010b) was the basis for the follow up of English and colleagues of a possible role of androgens in the exercise effect of enhancing nerve regeneration after injury. Moreover, that the continuous treatment of the injured rat facial nerve with testosterone resulted in a more sustained though slower onset in the upregulation of neurotrophic factors including BDNF, with the combination of ES and testosterone providing a more rapid *and* sustained upregulation of these genes (Sharma et al 2010a), provided further rationale to explore a role of androgens in the exercise effect in promoting nerve regeneration.

Two weeks of moderate daily exercise that was initiated three days after nerve transection and repair was effective in promoting axon growth, but the effective patterns of exercise were different in male and female mice: one hour of continuous treadmill walking per day, at a slow speed, was effective in promoting axon regeneration in males but not females whilst, in females an interval training regimen (two minutes of fast running followed by a five minute rest period, repeated four times) resulted in enhanced regeneration. Interval training was ineffective in males (Fig. 8A) (Sabatier et al 2008; Wood et al 2012). That androgens are critical to efficacy of the exercise regimens was demonstrated in two ways. The first was by eliminating the exercise effect in male rats by castration that localized the androgens to the gonads. The second was by promoting axon regeneration in unexercised female rats when testosterone conversion to estradiol was inhibited by inhibiting the aromatase responsible for the conversion with anastrozole (Wood et al 2012). In the females, the androgens are likely to be neuronal and/or glial because the aromatase inhibition did not raise serum testosterone in the treated female rats. The third was by blocking the gender-appropriate exercise effect of increasing nerve regeneration in both male and female mice by sustained treatment with flutamide, a competitive androgen receptor blocker (Thompson et al 2014). Finally, that androgens are *also* critical for the efficacy of the one hour 20 Hz ES in promoting axon regeneration was demonstrated using flutamide in male and female mice (Thompson et al 2014). The expression of BDNF mRNAs containing exon VI is regulated by androgens (Ottem et al 2010), consistent with the requirement of androgen receptor signaling for the effects of both one hour of ES and exercise

Of considerable interest are the recent findings that the gender-specific exercise programs prevented the withdrawal of synaptic contacts from the somata of the neurons that normally occurs after nerve injury (Alvarez et al 2010; Alvarez et al 2011; Blinzinger and Kreutzberg 1968; Bullinger et al 2011; Liu et al 2014) (Fig. 9B). The withdrawal is accompanied by reduced amplitude and increased latency of the evoked excitatory postsynaptic potentials recorded from the motoneurons (Bullinger et al 2011; Mendell et al 1976; Rotterman et al 2014; Vanden Noven and Pinter 1989) that accounts for the month long decline in motor nerve activity along with the expected decline in sensory nerve activity, as determined by cross-correlation techniques applied to the neural activity recorded during cat locomotion (Gordon et al 1980). The (VGLUT1+) synaptic inputs emanating from group Ia afferent neurons are normally withdrawn permanently and the inhibitory (GAD67+) inputs are normally withdrawn transiently following peripheral transection (Alvarez et al 2011; Rotterman et al 2014). These synaptic inputs were retained and/or restored onto the motoneurons ten weeks later by the gender-specific exercise programs of continuous and

interval training for two weeks in male and female rats, respectively (Brandt et al 2015;Liu et al 2014) (Fig. 9). The effect of the exercise is likely BDNF-dependent because the synaptic contacts on intact motoneurons were reduced in conditional BDNF knock-out transgenic mice (Krakowiak et al 2015). These findings indicate that the BDNF upregulation in response to exercise and/or ES effectively sustain the synaptic input into the motoneurons during activity such that the accelerated axon regeneration is likely to be accompanied by central activation of the neurons.

Misdirection of regenerating axons and functional consequences

Regenerating axons frequently regenerate towards and reinnervate targets that they did not innervate formerly. The cases of misdirection include 1) the regeneration of motor nerves to innervate muscle fibers within a muscle that they did not formerly supply resulting in loss of the classical mosaic distribution of 3–4 muscle fiber types in muscle cross-sections that is replaced by the clumping of the same fiber type (Dubowitz and Brooke 1973), 2) the random reinnervation of two or more muscles (Gillespie et al 1986;Thomas et al 1987), and the initial random reinnervation by motor and sensory axons of appropriate and inappropriate motor or sensory pathways (Brushart and Mesulam 1980) (Brushart 1978;Brushart 1988;Brushart 1993;Brushart et al 2005). In the case of the misdirection of motor axons to two or more muscles whose activation is normally antagonistic, as for example, the reinnervation of ankle extensor and flexor muscles (de Ruiter et al 2008;English 2005), enhancing axon regeneration with ES or/and exercise impacts these two situations differently. In experiments in which retrogradely labelled motoneurons of the tibial and common peroneal nerves were located along the caudo-rostral axis of the ventral horn in the lumbosacral spinal cord, the normal rostral position of the common peroneal motoneurons (English 2005;Kobbert and Thanos 2000;McHanwell and Biscoe 1981;Swett et al 1986) was shifted to a more caudal position after sciatic nerve transection and repair. This indicated the reinnervation of some ankle flexor muscles by tibial motoneurons that had previously innervated ankle extensor muscles. The caudal shift in the position of motoneurons innervating common peroneal nerve targets was even greater when the sciatic nerve was stimulated for one hour at 20Hz (ES) immediately after the nerve was transected and surgically repaired but not if the rats were subjected to gender-appropriate exercise regimens (Fig. 10A). Most of the enhanced motor axon regeneration by ES was the result of regeneration of motor axons into the common peroneal nerve from the topographically inappropriate region of the spinal cord (Fig. 10A) (English 2005). Yet, despite the striking appropriate reinnervation of muscles by the regenerating axons in exercised mice, the normal reciprocal activation of flexor tibialis anterior and extensor soleus muscles was lost after sciatic nerve transection and repair irrespective of whether the sciatic nerve was subjected to ES after the repair (C) (English et al 2009;Sabatier et al 2011a;Sabatier et al 2011b).

To investigate the functional significance of this loss of topographic specificity, namely the degree to which regenerating motor axons reinnervate the same or functionally similar targets as they did before injury, the timing of EMG activity of reinnervated flexor and extensor muscles was studied in rats in which the amount of the misdirection of regenerating common peroneal and tibial axons was manipulated and the timing of the EMG compared to

that in contralateral normally innervated muscles (Fig. 10C). In contrast to the normal reciprocal EMG activity in the flexor and extensor muscles during treadmill locomotion, the antagonistic muscles were abnormally coactivated after sciatic nerve transection and surgical repair whether or not the nerve regeneration was accelerated by ES or gender-specific exercise [Fig. 10C (Hamilton et al 2011)]. When axon regeneration was enhanced by gender-appropriate exercise without loss of topographical specificity, there was a small reduction in the inappropriate soleus activation during foot lift but the normal reciprocal activation was still lost (Boeltz et al 2013). Even when the misdirection of regenerating axons was reduced by transection and repair of the common peroneal and tibial branches of the sciatic nerve, innervating the flexor and extensor muscles, respectively, the co-activation of these antagonist muscles was abnormal (Sabatier et al 2011a; Sabatier et al 2011b). In the same manner that co-activation of flexor and extensor muscles was noted when these muscles were purposely cross-reinnervated by inappropriate nerve in cat hindlimbs (Gordon, Thomas et al, 1988), the co-activation of the muscles likely stiffens the ankle with little or no obvious compensation.

ES promotes nerve regeneration after delayed nerve repair in humans and rats

Experiments in which the effects of ES on nerve regeneration and target reinnervation were investigated in human patients who underwent carpal tunnel release surgery, demonstrated first that just an hour of 20Hz ES was effective in promoting the regeneration of all the median nerves that were disrupted (with disconnection of the distal axons from the cell bodies) and the reinnervation of the muscles in the thenar eminence (Fig. 11A–C) (Gordon et al 2010). Second, these experiments showed that the ES was effective in promoting the regeneration of axons after chronic injuries because the patients had suffered severe symptoms for up to five years. The axotomized median neurons that must regenerate their axons over a relatively short distance of ~100 mm to reach denervated muscle fibers in the thenar eminence, demonstrate poor muscle reinnervation when the nerves were not electrically stimulated (Fig. 11A), evidence consistent with the chronic axotomy of the neurons and chronic denervation of the Schwann cells in the distal nerve stumps as a consequence of the delayed carpal tunnel release surgery. Surgical repair of injured nerves is frequently delayed especially under conditions where the nature and the severity of the injury are not clear, as in the case of carpal tunnel syndrome and for nerve injuries that are complicated by multisystem injuries (Kline and Hudson 1995; Lundborg 2004; Sulaiman et al 2005; Sulaiman et al 2011; Sulaiman and Kline 2006). Proximal nerve injuries such as brachial plexus injuries are examples but surgeons, now more cognizant of the poorer outcomes of delayed as compared to immediate surgical repair of injured nerves, are now performing surgical repair as soon as possible.

Regenerating nerves that remain without target connections (chronic axotomy) as they grow over long distances, progressively fail to express regeneration associated genes, the expression declining exponentially over a 6 month time course in rats (Gordon et al 2014). There is a concomitant decline in the regenerative capacity of these chronically axotomized neurons (Fu and Gordon 1995a). Chronic denervation of Schwann cells during these

protracted periods of nerve regeneration has an even more profound effect in reducing regenerative capacity of neurons; nevertheless these atrophic Schwann cells myelinate the few axons that do manage to regenerate (Fu and Gordon 1995a;Fu and Gordon 1995b;Sulaiman and Gordon 2000). The reduced regenerative capacity is accompanied by a rapid decline in expression of neurotrophic factors (Brushart et al 2013;Hoke et al 2006). When the axons of chronically injured neurons are subjected to a refreshment injury as is performed prior to surgical repair either by direct coaptation or via a nerve or artificial conduit, there is a second but less vigorous upregulation of the regeneration associated genes. If the proximal and distal stumps of chronically injured nerves are refreshed and surgically reunited, ES is also effective, increasing axon regeneration beyond the normal limitations of the chronic injury (Fig. 3D–F) (Elzinga et al 2015). The explanation for the effect may be that the ES elevates the neurotrophic factors and their receptors, including trkB, in the chronically axotomized neurons as in the acutely axotomized neurons (Al-Majed et al 2000a;Al-Majed et al 2004;Geremia et al 2007) with concomitant elevation of the growth potential of these neurons and increased sensitivity of the axons to BDNF from Schwann cells and/or the neurons themselves. In turn, the regenerating axons will release mitogens which may promote the proliferation of the dormant Schwann cells in the chronically denervated nerve stumps. The dividing Schwann cells likely re-express regeneration associated genes and enter the growth permissive state to support the axon regeneration. These mechanisms remain to be determined, the possibilities suggested, awaiting experimental exploration.

Conclusions

The efficacy of brief ES of the proximal stump of an injured nerve in promoting nerve regeneration in animal models has been verified in several independent studies (Alrashdan et al 2011;Brushart et al 2002;Brushart et al 2005;Eberhardt et al 2006;Foecking et al 2012;Franz et al 2008;Geremia et al 2007;Gordon 2015;Gordon et al 2010;Gordon et al 2007;Gordon et al 2008;Hetzler et al 2008;Lal et al 2008;Monaco et al 2013;Nix and Hopf 1983;Sharma et al 2009;Sharma et al 2010a;Sharma et al 2010b;Singh et al 2012). The application to human surgical repair has been successful in a proof of principle study of brief ES after carpal tunnel release surgery (Gordon et al 2010) as well as a study demonstrating accelerated regeneration of sensory nerves and functional recovery after digital nerve transection and surgical repair (Wong et al 2015). Further studies of the efficacy of ES in humans are ongoing. Daily ES of denervated skeletal muscle for one hour has also been shown recently to accelerate muscle reinnervation in a rat model of nerve injury and surgical repair and should be explored further (Willand et al 2014). The efficacy of daily exercise after nerve surgery on the other hand, remains to be determined in human patients. However postsurgical treatment normally restricts movement of the surgically repaired nerves to avoid disruption of the surgical repair so that protocols for exercise as a means to improve nerve regeneration remain uncertain. Whilst the mechanisms by which ES and/or exercise enhance nerve regeneration remain relatively poorly understood, the efficacy of ES and/or exercise in accelerating nerve regeneration is very encouraging. The misdirection of regenerating axons after surgical repair remains a problem for the appropriate activation of reinnervated muscles but lessons learnt from surgical procedures such as nerve and tendon

transfers to allow for movement of specific muscles to restore function are likely to contribute to our understanding of the central plasticity that may underlie these adaptations (Addas and Midha 2009; Anastakis et al 2008; Malesy et al 1998).

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Figure 1. The conversion of neurons and Schwann cells to a growth mode

A. Motoneurons undergo chromatolytic changes including the movement of the nucleus to an eccentric position reflecting B. the increased gene expression of several growth associated genes including neurotrophic factors such as glial and brain derived neurotrophic factors and their receptors, cytoskeletal proteins including tubulin and (in A) the reduced expression of transmitter associated genes and neurofilament. C. Schwann cells in the denervated Schwann cells participate in Wallerian degeneration of the axon and myelin debris and upregulate neurotrophic factors and the p75 receptors for neurotrophic factors at the same time as downregulating myelination associated genes that include P₀.

Figure 2. Staggered nerve regeneration

A; Just proximal to the site of surgical repair, regenerating axons visualized with silver staining may turn back and even loop (as described earlier by Cajal (1928)). B. Silver stained regenerating axons cross the surgical site (not shown on the left) into the distal nerve stump at different rates due to C. the staggering of the regenerating axons at the suture site as shown by GFP+ fluorescent axons from the proximal nerve stump (left) crossing into the distal nerve stump on the right. D. Femoral nerves that had crossed a site of nerve transection and surgical repair and had just entered the denervated distal nerve stump were backlabelled with a fluorescent dye to reveal the staggered axon regeneration where motoneurons send their axons into the distal nerve stump over a protected period of a month. E. Backlabelling of rat femoral motoneurons that regenerated their axons 25 mm into either appropriate motor or inappropriate sensory nerve branches with two different dyes demonstrated that the numbers of the motoneurons send their axons equally into both branches initially but, after two weeks progressively more motoneurons regenerate their axons and these axons enter into *only* the appropriate motor branch. Motoneurons that are colabeled are those that regenerated axons into both branches, the numbers of these remaining constant throughout the 8 to 10 period in which all the motoneurons regenerate axons.

Figure 3. Electrical stimulation (ES) accelerates axon outgrowth across a site of nerve transection and surgical repair

A. Immediately following the transection and surgical repair of the rat femoral nerve, the nerve proximal to the suture site, was electrically stimulated at 20Hz for one hour. B. All femoral motoneurons regenerated their axons a distance of 25 mm into motor and sensory nerve branches within three weeks after the repair surgery and the electrical stimulation. This accelerated growth compares with the 8–10 week period when the femoral nerve was subjected to sham stimulation shown in Fig. 2F. C. Confocal images of whole mounts of the cut common peroneal nerve of a *thy-1-YFP-H* transgenic mouse repaired with a nerve graft from a wild type littermate contain profiles of regenerating axons. The subset of ~35 motor and sensory axons in this nerve that express yellow fluorescent protein (YFP) in these mice are highly visible against the dark background of the graft. Each image is a reconstruction the entire repaired nerve made by stitching together single 10 μ m thick optical sections through the nerve. There was an obvious accelerated outgrowth of axons across the suture site and into the distal nerve stump when the common peroneal nerve was subjected to 20Hz 1 hour ES as compared to sham ES (Unstimulated).

Figure 4. Electrical Stimulation (ES) accelerates target reinnervation

Electrical stimulation at a frequency of 20Hz for one hour accelerates the outgrowth of axons across the site of surgical repair of transected nerve stumps to result in accelerated target reinnervation.

Figure 5. Preferential and delayed expression of neurotrophic factors in the denervated distal stump

A. Figurative illustration of the time course of femoral neurons regenerating their axons into the cutaneous and muscle branches with all the axons entering into the appropriate pathway at times later than three weeks coincides with B. the delayed upregulation of neurotrophic factors in the dorsal (sensory) and ventral (motor) roots. Heparin growth factor (HGF), brain derived neurotrophic factor (BDNF), glial derived neurotrophic factor (GDNF), nerve growth factor (NGF), insulin growth factors 1 and 2 (IGF-1 and IGF-2) and pleiotrophin (PTN).

Figure 6. Electrical stimulation accelerates the upregulation of regeneration associated genes that are essential for the effect

A. Following a period of one hour 20Hz electrical stimulation (ES), upregulation of neurotrophic factors and their receptors followed by upregulation of cytoskeletal proteins is increased and accelerated in the neurons. B. ES increases the mean ($\pm$ SE) axon outgrowth into distal nerve stumps after suture of common peroneal nerve in a *thy-1-YFP-H* transgenic mouse to a distal nerve stump from either a wild type litter mate, from NT4/5^{-/-} transgenic mice, or acellular grafts. Hence, it is the neuronal neurotrophic factors and not those of the Schwann cells that account for the accelerated axon outgrowth after ES.

Figure 7. Daily exercise is more effective than electrical stimulation in promoting axon regeneration

The cumulative distributions lengths of axon profiles that regenerate from the proximal nerve stump of the common peroneal nerve of a *YFP*-transgenic mouse into a common peroneal isografts from a wild type litter mate two weeks after the surgical repair of the cut nerve. The curve was shifted to the right when the CP proximal nerve stump was subjected to electrical stimulation for 1 hour at 20 Hz. This shift was much greater when the mice were subjected to daily treadmill exercise. In this example, male mice were subjected to continuous training in which the mice were placed on a level, motor-driven treadmill at a speed of 10 meters/min for an hour daily. Each symbol is the average of six mice and the size of the symbol is proportional to the SEM. The horizontal dashed line at the 50th percentile shows the location of the median axon profile length for each group.

Figure 8. The efficacy of the specific exercise regimens for male and female mice in promoting nerve regeneration are mediated by androgen receptors

A. Confocal images of the common peroneal nerves in male and female *thy-1-YFP* transgenic mice which express yellow fluorescent protein in the axons of ~35 motor and sensory neurons, two weeks after either continuous or interval training. Continuous training (slow walking at 10 meters/min for one hour per day) was effective in promoting nerve regeneration in males but not females and interval training (four repetitions of short sprints at 20 meters/min for 2 minutes following by 5 minutes of rest) was effective in females and not males. B. Flutamide, an androgen receptor antagonist released from Silastic capsules implanted three days prior to onset of treadmill training or electrical stimulation (ES) for one hour at 20 Hz eliminated the effects of both ES and gender-appropriate exercise (*) in both males and females. Median regenerating axon profile lengths, expressed as mean fold changes ($\pm 95\%$ confidence limits) relative to untreated controls (vertical dashed line).

Figure 9. Gender-specific exercise sustains synaptic contacts on axotomized motoneurons
 A. In this 1 μ m thick confocal image of a single retrogradely labeled motoneuron (CTB-AF545), excitatory synapses containing the vesicular glutamate transporter 1 (VGLUT1), and arising mainly from primary afferent neurons, and inhibitory synapses containing glutamic acid decarboxylase 67 (GAD67) were identified using immunofluorescence, and used to measure the percentage of the perimeter of the neuron in contact with the different immunoreactive structures. B. The mean ($\pm$ SEM) percent synaptic coverage by VGLUT1 (top) and GAD67 (bottom) is reduced on motoneurons after sciatic nerve transection (untrained) but is sustained in male and female rats when ‘continuous’ and ‘interval’ treadmill exercise, respectively, is initiated three days after transection and continued for two weeks.

Figure 10. Misdirection of regenerating motor nerves is increased after brief electrical stimulation

A. The relative caudo-rostral distribution of motoneurons with axons in the mouse common peroneal (CP) nerve is shown for mice in which the sciatic nerve is intact and for mice four weeks after sciatic transection and repair. The normal caudo-rostral distribution of CP motoneurons in the intact mice (Intact) was shifted to a more caudal position in the spinal cord after sciatic nerve repair in 'Untreated' mice. This shift was exacerbated when the transected sciatic nerve was subjected to ES at 20 Hz for one hour but not when the mice were subjected to daily exercise, continuous training (slow walking at 10 meters/minute for one hour per day) in male mice and interval training (four repetitions of short sprints at 20 meters/min for 2 minutes following by five minutes of rest) in female mice. B. Pie charts showing that 94% of the compound muscle action potentials recorded from soleus muscles were elicited by stimulation of the tibial nerve but that after sciatic nerve transection and surgical repair, this was reduced to 72%. Selective electrical stimulation of the CP nerve branch at 20Hz for one hour (ES) reduced this further to 27%. Hence a one hour period of selective ES enhanced regeneration of cut CP axons so that only 27% of the axons regenerated within the appropriate CP nerve pathway with the remaining being misdirected to antagonist muscles that they did not formerly supply. C. The normal alternation of rectified and integrated electromyographic (EMG) activity in the flexor tibialis anterior (TA) muscle and the extensor soleus (Sol) muscle was lost after reinnervation, irrespective of whether the CP nerve was subjected to selective ES. The arrows below the EMG traces indicate lift and fall of the foot during locomotion.

Figure 11. Brief low frequency electrical stimulation accelerates nerve regeneration and muscle reinnervation after delayed repair

A. The median nerve in humans was subjected to one hour electrical stimulation (ES) at 20Hz following carpal tunnel release surgery after chronic injury. In the patients in which B. the median nerve was not stimulated and the number of median nerves with intact target muscle connections (MUNE) was ~50% of normal numbers (shown by the dotted line), the release surgery resulted in a small but insignificant increase in MUNE whilst in C. where the median nerve *was* subjected to ES, there was already a significant increase above the ~180 intact motor units with the numbers increasing to normal within 6–12 months. D. In rats, the common peroneal and tibial nerves were cut and ligated prior to their cross-suture after two months delayed nerve repair. E. The same ES paradigm as used in the patients resulted in a significant increase in the numbers of E. common peroneal (CP) motoneurons and F. common peroneal sensory neurons that regenerated their axons into the tibial nerve stump.