Brief Electrical Stimulation Accelerates Axon Regeneration in the Peripheral Nervous System and Promotes Sensory Axon Regeneration in the Central Nervous System

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Injured peripheral but not central nerves regenerate their axons but functional recovery is often poor. We demonstrate that prolonged periods of axon separation from targets and Schwann cell denervation eliminate regenerative capacity in the peripheral nervous system (PNS). A substantial delay of 4 weeks for all regenerating axons to cross a site of repair of sectioned nerve contributes to the long period of separation. Findings that 1h 20Hz bipolar electrical stimulation accelerates axon outgrowth across the repair site and the downstream reinnervation of denervated muscles in rats and human patients, provides a new and exciting method to improve functional recovery after nerve injuries. Drugs that elevate neuronal cAMP and activate PKA promote axon outgrowth in vivo and in vitro, mimicking the electrical stimulation effect. Rapid expression of neurotrophic factors and their receptors and then of growth associated proteins thereafter via cAMP, is the likely mechanism by which electrical stimulation accelerates axon outgrowth from the site of injury in both peripheral and central nervous systems.

Keywords: neurophysiology, neuroscience, muscle function, neuromuscular disease

The capacity of the nervous system for repair is a poignant issue for those suffering central and/or peripheral nerve injuries, stroke, and diseases that include demyelinating diseases and neuropathies. For those paralyzed by spinal cord injuries for example, experimental demonstration of some functional recovery have not as yet translated into substantial mobility despite worldwide research efforts. Challenges toward achieving long-distance regrowth of axons are particularly formidable in the injured central nervous system (CNS) despite promising findings.

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with combinational approaches in animal models of spinal cord injury (Moon & Bunge, 2005; Fouad & Pearson, 2004; Bunge & Pearse, 2003; Verma, Garcia-Alias, & Fawcett, 2008). These approaches include strategies to digest extracellular matrix components of the scar tissue such as chondroitin sulfate proteoglycans (CSPGs) with the enzyme chondroitinase ABC, and to overcome the inhibitory environment of the central myelin produced by the oligodendrocytes. The latter approaches have used combinations of neurotrophic factors, inhibitors of Rho, which is the small GTPase activated downstream of the p75 and NgR receptor complex of central myelin, chondroitinase ABC, and rolipram to pharmacologically elevate intracellular cAMP to bypass the receptor complex and Rho-associated inhibition of axon growth in the CNS (Grimpe et al., 2005; Pearse et al., 2004; Lehmann et al., 1999; Fenrich & Gordon, 2004; Fitch & Silver, 2008; Gonzenbach & Schwab, 2008; Lu & Tuszynski, 2008; Fawcett, 2006).

Central myelin contains several myelin associated proteins including Nogo-66 and Nogo-A, myelin associated glycoprotein (MAB), and oligodendrocyte myelin glycoprotein (OMgp). These proteins are currently believed to act as growth-inhibitory molecules that, via the common receptor subunit, NgR on the neuronal membranes, activate Rho to mediate collapse of growth cones (Fitch & Silver, 2008; Cao et al., 2007; Zheng et al., 2005; Ferraro, Alabed, & Fournier, 2004; Schwab, 2004; Bandtlow, 2003; Grados-Munro & Fournier, 2003; Yiu & He, 2006; Fihbin, 2006; Hannila, Siddiq, & Filbin, 2007; Hunt, Coffin, & Anderson, 2002; Liu, Fournier, GrandPre, & Strittmatter, 2002). In contrast Schwann cells of the peripheral nervous system (PNS) do not express the NgR receptors although the macrophages that infiltrate during Wallerian degeneration do (Fry, Ho, & David, 2007). Schwann cells proliferate and co-operate with infiltrating macrophages to phagocytose and remove myelin debris. They line the endoneurial tubes of the distal nerve stumps to form the bands of Bungner that guide and support the regenerating axons (Fu & Gordon, 1997; Sulaiman, Midha, & Gordon, 2009). The presence of the growth supportive Schwann cells in the growth pathways of the distal nerve stumps and the regenerative capacity of the motor and sensory neurons in the PNS have been highlighted and contrasted with the inability of axons to regenerate in the CNS (Fu & Gordon, 1997). These have, however, masked the clinically recognized frequency of poor functional outcomes of surgical repair of injured peripheral nerves in human subjects (Sulaiman & Gordon, 2008; Fenrich & Gordon, 2004; Sulaiman, Midha, & Gordon, 2009). The poor outcomes have too often been attributed, incorrectly, to irreversible atrophy and replacement of denervated skeletal muscles and sense organs by fat (Kline & Hudson, 1995; Sulaiman & Gordon, 2009; Gordon et al., 2009; Sulaiman, Midha, & Gordon, 2009; Gordon, Sulaiman, & Boyd, 2003; Gordon, Boyd, & Sulaiman, 2005; Sunderland, 1968).

In this review, we first briefly consider the factors that account for poor functional recovery after nerve injury and repair in the PNS of human patents. We describe how we modeled nerve injury in animals to replicate delays in axons regenerating and reaching their denervated targets—chronic axotomy—and the chronic denervation of Schwann cells in the growth pathway during the long periods of axon regeneration at rates of 1–3mm/day. These experiments demonstrate the relatively short time window of opportunity when axon regeneration is optimal after which regenerative capacity declines progressively with time and
distance. Second, we review experiments that demonstrate the effectiveness of brief electrical stimulation in accelerating axon outgrowth from the proximal nerve stumps of injured neurons and thereby, axon regeneration and target reinnervation. The electrical stimulation also promotes axon outgrowth in the CNS. The mechanisms of the effectiveness of the electrical stimulation are explored and the data to date are reviewed.

**Motor and Sensory Axon Regeneration in the Peripheral and Central Nervous Systems: Problems**

### Axons in the Peripheral but not the Central Nervous Systems Regenerate

Peripheral nerves regenerate their axons after crush or transection injuries outside of, but not within the spinal cord and brainstem. Crush injuries leave the endothelium surrounding each axon intact. Axons regenerate within the endothelial tubes where the denervated Schwann cells support and guide the regenerating axons to their original targets (Fu and Gordon, 1997; Zochodne, 2008). Injuries that disrupt the endothelium, such as those that transect the nerve, necessitate that Schwann cells enter the injury site and that extracellular matrix proteins which include the growth-promoting molecules of laminin and fibronectin, reorganize before the regenerating axons navigate through to enter the endoneurial tubes of the distal nerve stump (Witzel, Rohde, & Brushart, 2005). However, in contrast to crush injuries, transected motor and sensory axons that regenerate are frequently misdirected into inappropriate distal nerve pathways to randomly reinnervate denervated targets (Mesulam & Brushart, 1979; Thomas, Stein, Gordon, Lee, & Elleker, 1987).

Peripheral nerves include the axons of motoneurons of the spinal cord and lower brainstem that supply the musculature of the body and head, and sensory axons of dorsal root ganglion (DRG) neurons that transmit sensory information from the periphery to the CNS. Injury to the central process of the DRG neurons also leads to axon regeneration but the rate of regeneration is slower and the regenerating axons proceed only as far as the white matter of the spinal cord (Richardson & Verge, 1987; Zhang et al., 2007; Fenrich & Gordon, 2004). Myelination of axons within the white matter of the CNS is produced by the envelopment of several axons by the oligodendrocytes as opposed to the envelopment of single axons in peripheral nerves by Schwann cells (Elder, Friedrich, & Lazzarini, 2001; Weinberg & Spencer, 1978). Oligodendrocytes express many inhibitory myelin-associated proteins (Yiu & He, 2003; Mukhopadhyay, Doherty, Walsh, Crocker, & Filbin, 1994; Filbin, 2003; DeBellard, Tang, Mukhopadhyay, Shen, & Filbin, 1996; Shen, DeBellard, Salzer, Roder, & Filbin, 1998) which, together with the components of the glial scar, constitute the CNS growth environment that is nonpermissive to the regeneration of the central axons from the DRG neurons and injured central axons (Grados-Munro & Fournier, 2003; Tang, 1997; Busch & Silver, 2007; Silver & Miller, 2004).
Poor Functional Recovery After Peripheral Nerve Injuries

Clinical experience of poor functional recovery in human patients after surgical repair of peripheral nerve injuries, particularly those of brachial and lumbosacral plexi, has too often been interpreted as failure of regenerating axons to reinnervate target tissues which have undergone irreversible atrophy (Kline, D. G. & Hudson, A. R, 1995; Noble, Munro, Prasad, & Midha, 1998). Slow rates of regeneration of ~1 and ~3mm/day in human and animal species (Sunderland, 1947a; Sunderland, 1947c; Sunderland, 1947b; Gutmann, Guttmann, Medawar, & Young, 1942), respectively, necessitate months and even years for axons to regenerate over the long distances. The regeneration rate of ~3mm per day was originally determined for rabbit sensory and motor axons. The distance at which the “pinch” of the regenerated nerve elicited a reflex and the rate of return of response to skin nociceptive stimuli were used to calculate sensory nerve regeneration rates. Motor nerve regeneration rates were calculated from the time for recovery of a given motor function such as the reflex placing of the paw after crush injuries at different distances along the nerve (Gutmann et al., 1942). This slow rate of regeneration corresponds with the rate of slow axonal transport of cytoskeletal proteins such as actin and tubulin (McLean, 1985; Watson, Hoffman, Fittro, & Griffin, 1989; Kline & Hudson, 1995; Sulaiman & Gordon, 2003).

Gutmann and Young used a cross-suture surgical paradigm to examine the capacity of regenerating nerve to reinnervate chronically denervated peroneus muscles in the rabbit (Gutmann & Young, 1944). The muscles were chronically denervated for 17 months by section of the common peroneal nerve. Tibial nerve was cut and cross-sutured to distal stump of the cut peroneal nerve and the morphology of the muscle examined 9m later. The authors described the presence of axons that were and were not successful in making neuromuscular connections, concluding that highly atrophic muscles become progressively resistant to reinnervation by regenerating axons. We used the same experimental paradigm in a rat model of nerve injury to systematically reinvestigate the effects of chronic denervation as well as the effects of chronic disconnection of neurons from targets by nerve section–chronic axotomy (Fu & Gordon, 1995a; Fu & Gordon, 1995b; Sulaiman et al., 2002; Sulaiman, Voda, Gold, & Gordon, 2002; Sulaiman & Gordon, 2002; Gordon, Sulaiman, & Boyd, 2003; Gordon, Sulaiman, & Boyd, 2003). To examine the effects of chronic axotomy, we cut the tibial nerve to chronically axotomize tibial neurons for up to 12m after which a cross-suture of the tibial and freshly cut common peroneal nerves encouraged regeneration of the axons and reinnervation of the denervated muscles (Fu & Gordon, 1995a). We also chronically denervated the common peroneal nerve stump for periods of time of up to 12m before cross-suturing the freshly axotomized tibial nerves to the common peroneal nerve stumps. The cross-sutured nerves were allowed at least 4 months to regenerate and reinnervate the tibialis anterior muscle when the number of reinnervated motor units, their isometric contractile force and the number of muscle fibers reinnervated by each motor unit was determined (Fu & Gordon, 1995b). Consistent with the results of Gutmann and Young, the number of reinnervated motor units declined dramatically. However the decline to less than
10% within 6 months of the cross-suture of the chronic denervated nerve could not be accounted for by the failure of regenerating axons to reinnervate the atrophic muscles that Gutmann and Young had described. This is because those few motoneurons that regenerated their axons reinnervated up to 5 times their normal number of muscle fibers to generate increased motor unit contractile forces (Fu & Gordon, 1995b). Later counts of motoneurons that regenerated their axons into chronically denervated common peroneal distal nerve stumps revealed that indeed, the chronic denervation of the Schwann cells and not the muscle fibers themselves, had progressively reduced the regenerative capacity of the axons within the nerve sheaths (Sulaiman & Gordon, 2000). The reduction in regenerative capacity through chronically denervated nerve sheaths was associated with a parallel decline in the growth supportive state of the chronically denervated Schwann cells within the sheaths (Gordon, Sulaiman, & Boyd, 2003; You, Petrov, Chung, & Gordon, 1997; Hall, 1999; Li, Terenghi, & Hall, 1997). Importantly, the few axons that did regenerate successfully became fully remyelinated, demonstrating sustained capacity of the surviving Schwann cells to remyelinate axons (Sulaiman & Gordon, 2000).

In summary, the effects of the long-term chronic axotomy and Schwann cell denervation account for progressive and complete failure of axon regeneration and reinnervation of the more distal musculature and sense organs after repair of nerve injuries such as those of the brachial plexus.

**Staggered Axon Regeneration**

An initial latent period of a few days before axons regenerate within the endoneurial tubes of the distal nerve stumps has been determined by the “pinch test” that evaluates rate of sensory axon regeneration and by determining the time for recovery of a given motor function such as toe spreading after lesions at different distances from the muscles (Holmquist, Kanje, Kerns, & Danielsen, 1993; Bisby & Keen, 1984; Sunderland, 1947a; Gutmann, Guttmann, Medawar, & Young, 1942). The latent period was first defined in 1942 as the period of “retrograde degeneration, branching, and the relatively slow process of outgrowth across the suture scar” although this “scar delay” or period of time before arrive of new axon tips in the distal nerve stump was only slightly shorter after crush injury than after transection and suture repair (Gutmann, Guttmann, Medawar, & Young, 1942). This period is actually much more protracted than has been appreciated. Counting dye-labeled motoneurons that had regenerated their axons into the distal nerve stump at progressively longer periods after nerve section and surgical repair revealed a slow progressive regeneration of axons where only half of the motoneurons had regenerated their axons over a distance of 25 mm in 2 weeks (Figure 1A). This is different from the prediction of all the motoneurons regenerating their axons at 3mm/day after a short latent period of days. We coined the term *staggered axonal regeneration* for the progressive axon regeneration over an 8–10 week period for all the motoneurons to regenerate their axons over a 25mm distance (Figure 1A; Al-Majed, Neumann, Brushart, & Gordon, 2000).

Interestingly, a report of a protracted time course of slow axonal transport of several proteins including cytoskeletal proteins, had actually anticipated but not recognized staggered axonal regeneration (Forman, & Berenberg, 1978) and the early studies of Gutmann and colleagues had recognized that the regeneration
Figure 1 — Low frequency (20Hz) bipolar electrical stimulation, irrespective of duration, accelerates outgrowth of regenerating motor axons into appropriate motor pathways after nerve injuries. A) The femoral nerve in Sprague Dawley rats was cut and the stumps resutured with microsurgical technique. At 2 weeks and up to 10 weeks after nerve repair, the regenerated axons in the motor nerve to the quadriceps muscle and the sensory nerve to the skin 25 mm from the suture repair site were exposed to fluorescent dyes fluororuby and fluorogold that were taken up by the motoneurons. The mean (±SE) of the number of dye-labeled motoneurons that regenerated their axons in the motor (M), sensory (S) and both branches (B) of the femoral nerve are plotted as histograms as a function of the time after nerve repair. At 2 and 3 weeks, there were as many motoneurons regenerating their axons into motor and sensory branches, the quadriceps and the saphenous nerves, respectively. Thereafter there was no change in the number of backlabeled motoneurons and therefore the number that regenerated inappropriately into the saphenous nerve but the progressive increase in numbers of backlabeled motoneurons from the quadriceps nerve branch demonstrated the preferential motor reinnervation originally described by Brushart in 1993 (Brushart, 1993) and the staggered axon regeneration described by Al-Majed et al. (2000). B) Counts of motoneurons that regenerated their axons 25 mm into motor and sensory pathways after 2 weeks 20Hz electrical stimulation, proximal to the site of nerve section and surgical repair. The stimulation mediated a very rapid onset of preferential motor reinnervation at 2 weeks after surgical repair of the transected femoral nerve. In addition, all the motoneurons had regenerated their axons by 3 rather than 8 weeks after surgical repair. C) Counts of motoneurons that regenerated their axons into motor and sensory pathways as in A and B at 3 weeks after surgical repair of the transected femoral nerve with and without 1h electrical stimulation immediately after nerve section and repair. Application of a blocking dose (60mg/ml) of tetrodotoxin (TTX) proximal to the site of electrical stimulation effectively prevented the stimulation-induced increase in the number of motoneurons that regenerated their axons into the motor branch. D) Effect of 1hr 20Hz electrical stimulation on the number of sensory neurons in the L4–6 dorsal root ganglia that regenerated their axons into the motor (M), sensory (S) and both (B) branches of the femoral nerve after 3 weeks. The electrical stimulation accelerated appropriate regeneration of sensory axons in the appropriate saphenous nerve branch of the femoral nerve.
rates that they had reported for rabbit hindlimb nerves represented the fastest regenerating axons (Gutmann, Guttmann, Medawar, & Young, 1942).

The technique of retrograde labeling for counting neurons that regenerate their axons into the distal nerve stump after nerve injury has been used extensively and has been validated in several laboratories including our own, has been validated (Harvey, Grochmal, Tetzlaff, Gordon, & Bennett, 2005; Obouhova, Clowry, & Vrbova, 1994; Richmond et al., 1994; Swett, Wikholm, Blanks, Swett, & Conley, 1986; Eberhardt et al., 2006; Brushart et al., 2002; Brushart, Gerber, Kessens, Chen, & Royall, 1998). These neuronal counts are always much less than the counts of regenerated axons in the distal nerve stumps from histological examination of nerve cross-sections. The latter include multiple regenerated axons from each axotomized neuron as each axon proximal to the nerve injury emits many regenerated axons, the reported ratio of regenerating axons to proximal axons being as high as 20:1 (Aitken, Sharman, & Young, 1947) and as low as 5:1 (Mackinnon, Dellon, & O’Brien, 1991). Therefore, simple counts of regenerated axons in the distal nerve stumps may be misleading. For example, the effects of exogenous neurotrophic factors on axon regeneration can be misleading if these are based solely on axon counts: brain derived neurotrophic factor (BDNF) and glial cell derived neurotrophic factor (GDNF) both stimulate sprouting of new axons but they do not increase the number of motoneurons that regenerate their axons (Boyd, & Gordon, 2002; Boyd & Gordon, 2003a; Boyd & Gordon, 2003b).

We predicted that the ‘staggered axon regeneration’ occurred at the suture site with a more constant rate of regeneration of 3mm/day within the distal nerve stumps. To test this prediction, we backlabeled femoral motoneurons that had regenerated their axons across the surgical site of resutured proximal and distal stumps. We injected a fluorescent dye at a crush site close to the surgical site (Figure 2). The dye is transported back to the cell bodies from the injection site. Findings that the number of motoneurons that regenerated their axons across the suture site increased progressively over a protracted period of 4 weeks, a surprisingly long period of time, confirmed our hypothesis (Brushart et al., 2002). Similarly, the regeneration of sensory axons was ‘staggered’ with only a proportion of the DRG neurons regenerating their axons initially and the number increasing with time (Brushart, Jari, Verge, Rohde, & Gordon, 2005). Hence the backlabeling of axons that had crossed the suture site at different times after suture elucidated the ‘staggering’ of the axon as they emerged from the proximal nerve stump to regenerate across the site of the suture.

In the same manner that elaboration of the cross-bridge theory of muscle contraction had its origins in the original morphological studies in the 19th century (Ford, Huxley, & Simmons, 1977), the findings of staggered axon regeneration across the suture site had actually been made at the turn of the 20th century, by Ramon Y Cajal (Cajal, 1928). Cajal had used silver staining to visualize the progress of nerve fibers regenerating after a peripheral nerve section with or without a sequential surgical apposition of proximal and distal nerve stumps. He described the outgrowth of axons from the proximal nerve stump that crossed the surgical site in a varied manner. Despite apposition of nerve stumps, growing
axons often took tortuous routes to navigate the gap and, in some cases, axons formed unusual spirals that even grew in the opposite direction within the proximal nerve stump (Cajal, 1928). In a later study, Brushart and his colleagues used transgenic methodology to express a yellow fluorescent protein in neurons of the mouse to visualize the regenerating axons after a femoral nerve transection and surgical repair (Redett et al., 2005; Brushart & Mesulam, 1980). This study not only confirmed Cajal’s findings but placed the progressive axon outgrowth that we had discovered in the context of the initially disorganized extracellular matrix at the suture site and the randomly placed Schwann cells that had migrated from both proximal and distal nerve stumps. Similarly, Rafuse and colleagues described the staggered outgrowth in their study of rat nerves regenerating across a suture line (Franz et al., 2008). Staggered outgrowth explains the prolonged period of crossing of regenerating axons across the site of suture of proximal and distal nerve stumps.

Figure 2 — Low frequency (20Hz) electrical stimulation for 1h accelerates, by 4 days the sensory and motor axon outgrowth across the surgical gap between transected and surgically repaired union of the proximal and distal nerve stumps of the rat femoral nerve and 1.5mm into the distal nerve stump. Sensory and motor neurons that regenerated their axons across the suture site to enter the distal nerve stumps were backlabeled by microinjecting 0.5µl of fluororuby 1.5mm into the stump after a brief crush. The numbers (mean ± SE) of backlabeled sensory and motor neurons that regenerate their axons within 4 days were significantly higher in the stimulated group.
Solutions: Motor and Sensory Axon Regeneration Is Accelerated by Electrical Stimulation

Low Frequency Electrical Stimulation, Irrespective of Duration, Accelerates Outgrowth of Regenerating Motor Axons Into Appropriate Motor Pathways After Nerve Injuries in the PNS

In the 1980s it was reported that low frequency electrical stimulation of peripheral nerves after crush injury accelerated return of reflex foot withdrawal and contractile force in the reinnervated leg muscles (Nix & Hopf, 1983; Pickett & Gavin, 1985). These formed the basis for our hypothesis that the electrical stimulation accelerated axon regeneration and in turn muscle reinnervation after section and microsurgical repair of a peripheral nerve. We chose to electrically stimulate the proximal nerve stump of a cut and repaired rat femoral nerve at the low frequency of 20Hz continuously for 2 weeks (Al-Majed, Neumann, Brushart, & Gordon, 2000). This stimulation regimen was based on the average firing frequency of motoneurons and that motoneurons regenerate randomly into appropriate and inappropriate distal nerve stumps 2 weeks after nerve section and repair (Brushart, 1988; Brushart, 1993; Brushart, 1993). At progressively longer intervals after the surgical repair, two different dyes were applied to the motor nerve branch to quadriceps muscle and to the saphenous nerve that conveys only sensory nerves from the skin (Figure 1A). Thereby we counted dye-labeled motoneurons that had regenerated appropriately and inappropriately into motor and sensory nerve branches. We found that all the motoneurons that regenerated their axons into the distal nerve stumps, eventually regenerated into the appropriate endoneurial tubes. These directed their axons appropriately into the muscle nerve branch and into the denervated muscle (Figure 1A). Stimulated and nonstimulated nerve regeneration was compared over the same 8 week period. Remarkably, the low frequency stimulation promoted the axon regeneration of all the motoneurons over a 25mm distance within 3 weeks rather than the protracted period of 8–10 weeks (Al-Majed, Neumann, Brushart, & Gordon, 2000; Figure 1B). Again, motoneurons had likely regenerated their axons randomly into motor and sensory nerve pathways with axons arriving more quickly at the distal nerve stump to select the appropriate pathways.

To establish a more clinically feasible method to accelerate axon regeneration, we progressively shortened the period of electrical stimulation to 1h just after surgical repair of the transected femoral nerve. This short 1h period did not diminish the effectiveness of the stimulation in accelerating motor regeneration (Figure 1C; Al-Majed, Neumann, Brushart, & Gordon, 2000). The 1h electrical stimulation accelerated axon regeneration when the proximal stump was stimulated before or after the suture of the proximal and distal nerve stumps in rats and mice (Al-Majed, Neumann, Brushart, & Gordon, 2000; Franz, Rutishauser, & Rafuse, 2008). The accelerating effect is mediated via conduction of action potentials to the cell body since the effect was eliminated by pharmacological conduction block with application of the sodium channel blocker, tetrodotoxin (Figure 1C).
Axonal outgrowth from the proximal nerve stump and/or regeneration rate may be accelerated to account for the stimulation effect of accelerated axon regeneration. To decide between these possibilities, we a) used a new method of applying retrograde dyes just distal to the repair site to evaluate how many neurons regenerated their axons across the suture site (Figure 2) and b) determined regeneration rate in radiolabeling experiments (Brushart et al., 2002; Brushart et al., 2002). Counts of both sensory neurons and motoneurons that regenerated their axons across the suture site demonstrated that the 1h electrical stimulation significantly increased the numbers, i.e., the stimulation accelerated axon outgrowth across the suture site (Figure 2; Brushart et al., 2002). Evaluation of the regeneration rate showed that the stimulation-induced accelerated axon regeneration was not due to increased rate of regeneration of the axons within the distal nerve stumps (Brushart et al., 2002). The latter accounts for accelerated regeneration after a nerve lesion in the PNS and/or the CNS that is preceded by a conditioning lesion of the peripheral nerve (McQuarrie, Grafstein, & Gershon, 1977; Bisby & Pollock, 1983; Udina et al., 2008; Lankford, Waxman, & Kocsis, 1998).

By observing a subpopulation of motor and sensory neurons that express green fluorescent protein in transgenic mice, Rafuse and colleagues reported that each parent axon emitted more regenerating axon sprouts in response to electrical stimulation, possibly increasing the chances of encountering the Schwann cells within the endoneurial tube that guide and support the regenerating axons down the distal nerve stump (Franz, Rutishauser, & Rafuse, 2008). This is consistent with there being longer and more regenerating axons after 1h 20Hz electrical stimulation (English, Schwartz, Meador, Sabatier, & Mulligan, 2007; English, Meador, & Carrasco, 2005).

Insights gained from the evidence for accelerated axon outgrowth by the electrical stimulation now permits full appreciation of the early work that revealed a more rapid reinnervation of denervated targets after electrical stimulation of crushed nerves in rabbits and rats (Nix, & Hopf, 1983; Pockett & Gavin, 1985) (see Gordon et al., 2009). We have now extended these early findings to experiments in carpal tunnel patients who suffered a loss of more than 50% of their functional motor units in the median eminence (Gordon et al., 2009; Gordon, Brushart, Amirjani, & Chan, 2007). We electrically stimulated the median nerve proximal to and immediately following carpal tunnel release surgery. We placed stainless steel electrodes on the nerve during the surgery for the 1h 20Hz electrical stimulation. The electrodes were removed thereafter by pulling them through the skin. We recorded electromyographic signals from the muscles of the thenar eminence in response to maximal electrical stimulation of the median nerve: the compound muscle action potential, and single motor unit action potentials in response to all-or-none stimulation of single motor axons. The ratio of the compound action potential and the mean size of the unit action potentials provided an estimate of the number of innervated motor units in the thenar muscles before the carpal tunnel release surgery and at 3 month intervals after the surgery. The 1h 20Hz electrical stimulation was effective in promoting reinnervation of muscles by all the motoneurons within 9 months as opposed to the nonsignificant rise in numbers of functional motor units in the patients whose median nerve was not stimulated after the surgical release (Gordon et al., 2009; Gordon, Brushart, Amirjani, & Chan, 2007). These studies not only demonstrate that electrical stimulation that
we had demonstrated to accelerate axon outgrowth in rats, was also effective in accelerating muscle reinnervation after nerve injury in human patients.

**Brief but Not Prolonged Low Frequency Electrical Stimulation Accelerates Sensory Axon Regeneration Into Appropriate Sensory Pathways After Peripheral Nerve Injury**

The same brief 1h period of 20Hz electrical stimulation to the proximal nerve stump immediately after nerve repair also accelerates sensory axon outgrowth and directs their axons specifically into correct sensory nerve pathways (Figure 1D) (Brushart, Jari, Verge, Rohde, & Gordon, 2005). This effect is also mediated via action potential conduction to the cell body since the effect was eliminated by a tetrodotoxin blockade of the centrally conducted action potentials (Geremia, Gordon, Brushart, Al-Majed, & Verge, 2007). The effect of the electrical stimulation on sensory neurons differed from that on the motoneurons. Motor axon outgrowth was accelerated by 20Hz electrical stimulation irrespective of the duration of the stimulation, 1h stimulation being as effective as 1d or even 2w periods of stimulation (Al-Majed, Neumann, Brushart, & Gordon, 2000). In contrast, a 1h period of 20Hz stimulation but not longer, was effective in accelerating sensory axon outgrowth (Geremia, Gordon, Brushart, Al-Majed, & Verge, 2007). The stimulation increased the number of sensory neurons that regenerated their axons across the suture site by a factor of 1.7, as compared with the >threefold increase in the number of motoneurons that regenerated axons (Figure 2). It remains to be determined whether the reduced effectiveness of the electrical stimulation in accelerating sensory axon regeneration is linked to a more rapid outgrowth of sensory than motor axons (Suzuki, Ochi, Shu, Uchio, & Matsuura, 1998). We found that 27% of the sensory neurons regenerated their axons across the suture site within 4 days as compared with 3% of the motoneurons (Figure 2). By 3 weeks, significantly more sensory neurons had regenerated their axons into the distal nerve stumps as compared with the motoneurons (Figure 1). This may be linked to the greater heterogeneity in sensory versus motor neuronal populations, with a subpopulation of ~45% sensory neurons expressing NGF receptors and the plasticity associated gene GAP-43 (Verge, Tetzlaff, Richardson, & Bisby, 1990). The neurons appear to be primed for rapid neurite outgrowth as for example, they emit collateral sprouts in response to exogenous NGF (Mearow, Kril, Gloster, & Diamond, 1994; Diamond, Coughlin, Macintyre, Holmes, & Visheau, 1987).

**Brief Electrical Stimulation of the Peripheral Nerve Accelerates Axon Outgrowth in the CNS**

Injury to peripheral but not central axons elicits a strong cell body response in DRG sensory neurons, including upregulation of both growth associated proteins (Richardson, & Issa, 1984) and the mammalian transient receptor potential calcium-permeable cation channels (canonical TRPC subfamily of 7 members). The latter are linked to neurite outgrowth in vitro (Wu, Huang, Richardson, Priestley, & Liu, 2008). This cell body response to a conditioning crush or transection lesion of the peripheral nerve is sufficient to promote regeneration of cut central axons in
the dorsal columns (Figure 3 and 4) in association with elevation of cellular cAMP and upregulation of growth associated genes (Neumann, Skinner, & Basbaum, 2005; Neumann, Bradke, Tessier-Lavigne, & Basbaum, 2002; Neumann & Woolf, 1999; Udina et al., 2008; Richardson & Verge, 1986; Richardson, Issa, & Aguayo, 1984; Wu et al., 2007; Mason, Lieberman, Grenningloh, & Anderson, 2002; Kenney & Kocsis, 1998; Chong et al., 1994; Wong & Oblinger, 1990).

The conditioned lesion not being clinically feasible, we explored whether a 1h period of 20Hz electrical stimulation that also upregulates cAMP, promotes axon outgrowth into the same CNS lesion site as does the conditioning lesion. We found that electrical stimulation of the intact peripheral nerve promotes axon outgrowth in the CNS concomitant with cAMP elevation but does not accelerate axon elongation through the CNS lesion in the same manner as the conditioning lesion (Figure 4; Udina et al., 2008). These findings are in accord with findings

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**Figure 3** — An in vivo method to compare the effect of 1) a conditioning lesion (CL), 2) 20Hz 1hr and 3) 200Hz 1h electrical stimulation of the sciatic nerve on the regeneration of dorsal root ganglion (DRG) sensory axons that were transected in the dorsal columns at the T8 level of the spinal cord at the time of the CL or electrical stimulation. At 14 weeks, cholertoxin B (CTB) was injected central to the site of the lesion or electrical stimulation for anterograde labeling of the sensory axons at the lesion site in the CNS.
that elevated cAMP promotes neurite and axon outgrowth of DRG sensory neurons on nonpermissive substrates of central myelin (Hannila & Filbin, 2007; Pearse et al., 2004; Qiu, Cai, & Filbin, 2002).

Mechanisms of Accelerated Axon Outgrowth

The Cell Body Response to Axotomy

The Cell Body Response in Axotomized Neurons. While all neurons mount a cell body response with upregulation of regeneration associated genes, the response is more robust in neurons regenerating in the PNS (Bisby, 1980; Bulsara, Iskandar, Villavicencio, & Skene, 2002; Verge, Tetzlaff, Richardson, & Bisby, 1990; Tetzlaff & Bisby, 1990; Miller, Tetzlaff, Bisby, Fawcett, & Milner, 1989; Fu & Gordon, 1997; McPhail, Fernandes, Chan, Vanderluit, & Tetzlaff, 2004). Transcriptional upregulation of growth associated proteins such as GAP-43, CAP-23 and SCG10, is directly correlated with regenerative capacity of neurons in the PNS and CNS (Mason, Lieberman, Grenningloh, & Anderson, 2002). These proteins interact with second messenger systems in growth cones to affect the cortical cytoskeleton and in turn, promote axonal growth (Caroni, 1997; Mason, Lieberman, Grenningloh, & Anderson, 2002; Korshunova et al., 2008; Strittmatter, Igarashi, & Fishman, 1994). In situ hybridization was used effectively to demonstrate the upregulation of growth associated and cytoskeletal proteins, including Tα1-tubulin and actin, in axotomized sensory neurons and motoneurons after peripheral nerve transection with or without nerve repair (Gordon, 1983; Al-Majed, Tam, & Gordon, 2004; Fu & Gordon, 1997; McQuarrie & Lasek, 1989; Tetzlaff, Lederis, Cassar, & Bisby, 1989; Verge, Tetzlaff, Richardson, & Bisby, 1990; Bisby & Tetzlaff, 1992; Fenrich & Gordon, 2004; Strittmatter, Igarashi, & Fishman, 1994; Chong et al., 1992). Increased intracellular levels of tubulin and actin meet the requirements for growth cone advancement while the concomitant reduction of neurofilament protein accounts for the reduced caliber of the axons proximal to the lesion from whence the axons sprout and regenerate into the distal nerve stump (Meiri, Pfenninger, & Willard, 1986; Fenrich & Gordon, 2004; Strittmatter, Igarashi, & Fishman, 1994; Gordon, Gillespie, Orozco, & Davis, 1991; Fu & Gordon, 1997).

Figure 4 — Electrical stimulation (ES) promotes axon outgrowth into CNS lesion site but not regeneration over long distances, in contrast to the conditioning lesion (CL) that promotes both outgrowth and elongation of axons into the site. A,C,E,G) Camara lucida drawings of 25µm spinal cord sagittal sections of cholera toxin B-labeled DRG sensory axons that regenerated into the lesion site (shown in gray), 14 weeks after the lesion with and without the CL or ES. These drawings indicate that the ES promotes axon outgrowth into the lesion site but that this effect was not as dramatic as that of the CL. B,D,F,H) Comparisons of the cumulative sum of the number (±SE) of regenerating axons that advanced into the lesion site expressed as a percentage of all the labeled axons proximal to the lesion are plotted as a function of the distance cranial from the T8 lesion site in the spinal cord. Axon outgrowth was significantly elevated by 20Hz but slightly but not significantly increased by 200Hz (A-C). Electrical stimulation accelerated axon outgrowth but not regeneration rate in contrast to the CL that accelerates both to promote regeneration of axons up to 1800µm into the lesion (D). Statistical significance vs Sham when p < .05.
Upregulation of Neurotrophic Factors After Axotomy. Expression of the growth associated proteins mediating axon outgrowth in axotomized motoneurons is preceded by upregulation of neurotrophic factors and their receptors, the most prominent of which are brain derived neurotrophic factor (BDNF) and its receptors trkB and p75 (Figure 5), and glial derived neurotrophic factor (GDNF) and its receptors. These appear to be essential for axon regeneration; trkB antibodies reducing regenerative capacity and exogenous BDNF and/or GDNF increasing axon regeneration after prolonged axotomy (Boyd, & Gordon, 2003b; Boyd & Gordon, 2003a; Boyd & Gordon, 2002; Boyd & Gordon, 2001). The intrinsic neuronal and Schwann cell supply of neurotrophic factors is sufficient to support axon regeneration but their progressive depletion during prolonged axotomy and/or Schwann cell denervation requires exogenous sources of growth factors to sustain axonal regeneration (Gordon, Sulaiman, & Boyd, 2003; Furey, Midha, Xu, Belkas, & Gordon, 2007). Differential expression of several growth factors by Schwann

![Graphs](image-url)

Figure 5 — Brief (1h) 20Hz electrical stimulation accelerates upregulation of neurotrophic factors and their receptors that precedes accelerated upregulation of growth associated genes in axotomized motoneurons after femoral nerve transection and surgical repair. Semiquantitative in situ hybridization with synthetic oligonucleotide probes end-labeled with $^{135}$S-ATP was used to measure expression of mRNA encoding BDNF (A), its receptor, trkB (B) and the regeneration associated genes, tubulin (C) and GAP-43 (D) for details see (Verge et al., 1992; Al-Majed, Brushart, & Gordon, 2000). The 12µm sections of fresh-frozen lumbar spinal cord were hybridized overnight and then exposed to emulsion for adequate resolution of the silver grains that were activated by the $^{135}$S-ATP per probe (Kobayashi, Bedard, Hincke, & Tetzlaff, 1996). The fraction of the area occupied by the autoradiographic silver grains was multiplied by the total cell area and expressed as a percentage of the signal ($\pm SE$).
cells in denervated endoneurial sheaths of motor and sensory axons has been suggested to play a key role in the preferential selection of regenerating motor and sensory axons for their appropriate pathways (Hoke et al., 2006; Brushart, Jari, Verge, Rohde, & Gordon, 2005; Brushart, 1993).

**Brief Electrical Stimulation Upregulates Expression of Neurotrophic Factors and Growth-Associated Proteins.** Findings that tetrodotoxin blockade of action potential propagation to the cell body prevented stimulation-induced acceleration of axon regeneration (Al-Majed, Neumann, Brushart, & Gordon, 2000; Geremia, Gordon, Brushart, Al-Majed, & Verge, 2007), indicated that the cell body response to axotomy was likely to be the locale of the stimulation-induced effect. The stimulation promotes a rapid and marked upregulation of BDNF and trkB receptors that precedes the pronounced upregulation of growth associated proteins including tubulin and GAP-43 (Figure 5)(Al-Majed, Brushtart, & Gordon, 2000; Gere mia, Gordon, Brushart, Al-Majed, & Verge, 2007). However, the correlation of accelerated axon outgrowth and upregulation of mRNA for growth associated proteins suggests but does not prove a causal relationship. Findings that the stimulation failed to enhance axon regeneration in NT4/5 knockout mice (English, Schwartz, Meador, Sabatier, & Mulligan, 2007) and that a selective functional blocking antibody against BDNF blocked the enhanced axonal outgrowth by 1h 20Hz electrical stimulation (Tyreman et al., 2008) provided more direct evidence. Hence, the expression, synthesis, release and binding of neurotrophins to trkB receptors on stimulated neurons, mediate the accelerated axon outgrowth response to electrical stimulation. A likely scenario is that the NT4/5 and BDNF synthesized by the neurons, act via auto- and paracrine routes to promote expression of growth associated genes in response to trkB activation.

**Role of cAMP in Accelerated Axon Outgrowth From Motoneurons in Response to Brief Electrical Stimulation**

It was the conditioning lesion experiments that first drew attention to cAMP as a mediator of accelerated axon regeneration, the conditioning lesion promoting sensory axon growth in the PNS and CNS in association with increased intracellular cAMP and exogenous cAMP mimicking the effect (Udina et al., 2008; McQuarrie, Grafstein, & Gershon, 1977; Carlsen, 1982; Kilmer & Carlsen, 1984; Neumann, Bradke, Tessier-Lavigne, & Basbaum, 2002; Neumann & Woolf, 1999). The conditioning effect of the peripheral nerve lesion can be blocked by pharmacological block of the endogenous cAMP (Qiu et al., 2002). Given that there are basal levels of cAMP in motoneurons (Aglah, Gordon, & Posse De Chaves, 2008) we examined whether infusing rolipram, a specific inhibitor of the neural phosphodiesterase type IV, would accelerate axon outgrowth. This was indeed the case, the rolipram accelerating outgrowth of axons with increased numbers of motoneurons that regenerated their axons across the suture site and through the distal nerve stump, akin to the brief electrical stimulation effect. Furthermore, the effect of the rolipram treatment was sufficient to also accelerate the reinnervation of denervated skeletal muscles (Gordon et al., 2009).

In vitro, pharmacological elevation of cAMP promoted neurite outgrowth from embryonic motoneurons on a growth permissive substrate (Figure 6). Forskolin which stimulates adenylyl cyclase to increase endogenous cAMP, dibutyryl cAMP
Figure 6 — Pharmacological elevation of cAMP stimulates neurite outgrowth and in motoneurons plated on a growth permissive polyornithine/laminin-coated culture dish. Motoneurons plated at 200 cells per well of a 96-well dish or 30,000 per 24-well dish, were treated at the time of plating with 3µM forskolin or 1mM of dbcAMP (A-C) or with 10µM Rolipram (D-E). The mean (±SE) percent of motoneurons with neurites and the mean (±SE) length of the longest neurites were determined after 24h in tissue culture. The experiments were done in triplicate and repeated three times. The mean (±SE) number of motoneurons that extended neurites (A,D) and the mean (±SE) length of the longest neuritis (B,E) were significantly increased in medium containing 3µM forskolin, 1mM dbcAMP, or 10µM rolipram. The increase with rolipram was accompanied by a significant increase in cAMP levels (F). Representative phase-contrast micrographs of the motoneurons with and without Forskolin or dbcAMP are shown (C). The differences between treated and untreated controls were determined by the Student t test. Data were analyzed by One Way ANOVA with Newman-Keuls Multiple range test. Significance is indicated: *** p < .001 (with respect to the untreated control).
(dbcAMP), a soluble cAMP analog, and rolipram each increased the number of the motoneurons that extend neurites and neurite length (Figure 6; Aglah, Gordon, & Posse De Chaves, 2008). SQ22356, a soluble and potent adenylyl cyclase inhibitor that reduces endogenous cAMP, evokes a dose-dependent decline in neurons that extend neurite and their length (Figure 7).

**Figure 7** — Pharmacological reduction in intracellular cAMP causes decreased neurite outgrowth and extension in motoneurons in vitro. Motoneurons cultured at a density of 200 cells per 96-well dish or 30,000 per 24-well dish, were treated with SQ22536 at different concentrations at the time of plating and the mean ± SE of (A) intracellular cAMP levels and (B) percent of motoneurons which extended neurites determined 18h after treatment. Data were analyzed by One Way ANOVA with Newman-Keuls Multiple range test. Significance is indicated with respect to the untreated control *** p < .001.
In line with the common downstream intracellular target of cAMP being protein kinase A (PKA), we found that forskolin and dbc-AMP phosphorylated the PKA RIIB subunit in cultured motoneurons (Figure 8A) in parallel with the increase in numbers of neurons with neurites and the length of the neurites (see Figure 6AB). PKA phosphorylation and increased neurite length in response to the elevated cAMP were both reduced by the PKA inhibitor, H89 (Figure 8). These experimental findings indicate that cAMP acts via PKA to accelerate axon outgrowth in response to electrical stimulation in vivo. However, unlike DRG sensory neurons where cAMP activates ERK downstream of PKA in vitro (Cai et al., 2001), elevated motoneuronal cAMP in response to forskolin or dbcAMP did not phosphorylate ERK2 above basal levels or increase the pERK/Total ERK ratio. Agents that reduced cAMP including SQ22356 and H89, did not change ERK2 phosphorylation, consistent with basal ERK2 activation in motoneurons being independent of cAMP (Figure 9). As a control, BDNF induced a sustained and significant Erk activation that, taken together with the data from the Western blot analysis in Figures 8 and 9, demonstrates that ERK activation is not an essential component of the effectiveness of cAMP in promoting motor axon growth.

**Figure 8** — PKA downstream of cAMP mediates neurite outgrowth in motoneurons in vitro. A) Motoneurons cultured at a density of 30,000 cells per 24-well dish were treated at the time of plating with forskolin (3µM), dbcAMP (1mM), H89 (20µM), Rp-cAMP (500µM), as well as forskolin or dbcAMP together with H89 or Rp-cAMP. (A) PKA activation was examined by detecting the phosphorylated form of the PKA regulatory subunit RII. (B) Motoneurons cultured at a density of 2000 cells per 96-well dish were treated at the time of plating with H89 (20µM) or Rp-cAMP (500 µM) in the absence or presence of forskolin (3µM) or dbcAMP (1mM) and neurite length was measured after 24h. (A) Treatment of motoneurons with forskolin and dbcAMP induced PKA activation by phosphorylation. Conversely H89, a PKA inhibitor, and the cAMP antagonist Rp-cAMP inhibited PKA basal activity and reduced forskolin- and dbcAMP-induced PKA activation. (B) There were corresponding reductions in the mean (± SE) length of neurites with H89 and Rp-cAMP in the presence of forskolin that increases cAMP and with H89 in the presence of dbcAMP. Data were analyzed by One Way ANOVA with Newman-Keuls Multiple range test. Significance is indicated with respect to the untreated control *** p < .001.
Conclusions

There is a limited window of opportunity for functional recovery after peripheral nerve injury and surgical repair because the growth state of the axotomized neurons and the growth-support of the Schwann cells in the denervated distal nerve stumps deteriorate over time. Hence, it is imperative to explore methods to sustain these states and thereby, prevent their time-dependent deterioration. The staggering of regenerating axons that proceeds at surprisingly slow rates across the surgical repair site is a key component of delayed axon regeneration. The marked acceleration of axon outgrowth that we observed in response to a 1h period of 20Hz electrical stimulation of axotomized neurons, has considerable potential to promote earlier reinnervation of denervated targets. The accelerated axon crossing of the repair site could, at least in part, effectively counteract the time constraints of the growth-permissive environment of the PNS and the capacity of the neurons to regenerate their axons.

Transection of a sensory nerve evokes rapid depolarization at the injury site, followed by a burst of action potentials referred to as an “injury discharge” lasting up to minutes (Wall & Gutnick, 1974; Berdan, Easaw, & Wang, 1993; Chung, 1993).
Findings that TTX-blockade of the discharge just before and during the stimulation period reduces the sensory cell body response 3 days later, indicates that the discharge in of itself, is important for axon outgrowth after injury (Pettersson, Gordon, Tireman, & Verge, 2006). If this is so, the brief period of 1h 20Hz electrical stimulation would further this promotion because the electrical stimulation accelerates outgrowth of both motor and sensory axons. As shown in Figure 10, electrical stimulation is effective, irrespective of the duration of stimulation, in raising cAMP to upregulate BDNF and trkB via PKA activation and, in turn, upregulates regeneration associated genes to promote axon outgrowth. In motoneurons, rolipram by elevating cAMP effectively increases axon outgrowth in vitro. That electrical stimulation is likely to act via cAMP to promote BDNF and trkB expression and in turn, promotes synthesis of growth associated proteins, is supported by in vitro evidence that elevated cAMP acts via PKA to promote neurite outgrowth. However,

**Figure 10** — Schematic representation of the mechanism by which a brief period of low frequency electrical stimulation promotes axon outgrowth in motor and sensory neurons. Electrical stimulation increases intracellular cAMP that, via PKA leads to the transcription and translation of neurotrophic factors. These in turn act via adenylase cyclase to sustain the levels of intracellular cAMP that act downstream of PKA to upregulate the regeneration associated genes to synthesize sufficient cytoskeletal proteins such as tubulin and actin and growth associated proteins such as GAP-43 to promote the outgrowth of axons from the proximal stump of a transected nerve.
unlike sensory neurons, cAMP affects on neurite outgrowth in motoneurons are not mediated via the ERK pathway.

In sensory neurons, electrical stimulation is effective in accelerating axon outgrowth only when it is brief in duration, longer durations of stimulation down-regulating trkB expression in the neurons. The electrical stimulation increases cAMP as it does in motoneurons. The cAMP in turn, is likely to exert its effects via PKA and downstream targets to promote the synthesis of neurotrophins. These in turn, may be released to act in an autocrine and/or paracrine manner to promote expression of regeneration associated genes, and in turn to promote axon outgrowth (Figure 10).

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