Experimental Neurology 302 (2018) 75–84

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research paper

Electrical stimulation as a conditioning strategy for promoting and accelerating peripheral nerve regeneration

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ARTICLE INFO

Keywords:
Nerve regeneration
Electrical stimulation
Conditioning lesion
Peripheral nerve
Axon regeneration
Microsurgical repair
Regeneration-associated genes
Growth associated protein-43
Brain derived neurotrophic factor
Glial fibrillary acidic protein

ABSTRACT

The delivery of a nerve insult (a "conditioning lesion") prior to a subsequent test lesion increases the number of regenerating axons and accelerates the speed of regeneration from the test site. A major barrier to clinical translation is the lack of an ethically acceptable and clinically feasible method of conditioning that does not further damage the nerve. Conditioning electrical stimulation (CES), a non-injurious intervention, has previously been shown to improve neurite outgrowth in vitro. In this study, we examined whether CES upregulates regeneration-associated gene (RAG) expression and promotes nerve regeneration in vivo, similar to a traditional nerve crush conditioning lesion (CCL). Adult rats were divided into four cohorts based on conditioning treatment to the common peroneal (fibular) nerve: i) CES (1 h, 20 Hz); ii) CCL (10 s crush); iii) sham CES (1 h, 0 Hz); or iv) naïve (unconditioned). Immunofluorescence and qRT-PCR revealed significant RAG upregulation in the dorsal root ganglia of both CES and CCL animals, evident at 3–14 days post-conditioning. To mimic a clinical microsurgical nerve repair, all cohorts underwent a common peroneal nerve cut and coaptation one week following conditioning. Both CES and CCL animals increased the length of nerve regeneration (3.8-fold) as well as the total number of regenerating axons (2.2-fold), compared to the sham and naïve-conditioned animals ($p < 0.001$). These data support CES as a non-injurious conditioning paradigm that is comparable to a traditional CCL and is therefore a novel means to potentially enhance peripheral nerve repair in the clinical setting.

1. Introduction

Peripheral nerve injury is common, identified in 3% of all clinical presentations to major emergency centers (Noble et al., 1998). Despite surgical advancements and dedicated rehabilitation, clinical outcomes have not significantly improved in recent decades (Lundborg, 2000). The slow rate of regeneration (1–3 mm/day) is insufficient to reinnervate distal targets. Extended denervation results in a local environment at the distal stump and motor/sensory targets that is not conducive to regeneration. Therefore, an intervention to accelerate peripheral nerve regeneration is of utmost clinical importance.

Forty years of animal studies have revealed that conditioning lesions, in which the peripheral nerve is damaged by a crush or cut prior to a test nerve injury, promotes nerve regeneration. The conditioning lesion instructs the neuronal cell bodies to change their gene expression pattern to an 'injured/repair' state. Regeneration-associated gene (RAG) expression is upregulated along with proteins Tα1-tubulin, β-actin, and other axonal structural proteins necessary for nerve regeneration (Lund, 2002; Richardson et al., 2009). Following nerve transection, a conditioned nerve will accelerate its regenerative capacity by up to 5-times that of unconditioned nerve (Gordon et al., 2008).

Unfortunately, the therapeutic potential of the conditioning lesion cannot be translated to the clinic because of two fundamental obstacles: a) the timing of a nerve injury is usually unpredictable, and b) one cannot justify intentionally damaging intact nerves in patients. Distal nerve transfer surgery, however is one scenario in which, at a prescheduled time, a healthy intact nerve is intentionally cut. In this elective procedure, the proximal stump of an intact nerve (innervating a nonessential motor or sensory target) is cut and coapted to the denervated stump of an injured nerve, to reinnervate its target. These patients are, therefore, ideal candidates for conditioning, which could be delivered to the donor nerve prior to the nerve transfer procedure. How to deliver this nerve conditioning effect at the bedside without injuring the donor nerve remains a more elusive challenge.

Abbreviations: CES, conditioning electrical stimulation; CCL, crush conditioning lesion; RAG, regeneration-associated genes; CP, common peroneal; SGC, satellite glial cells; DRG, dorsal root ganglion

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https://doi.org/10.1016/j.expneurol.2017.12.013
Received 15 September 2017; Received in revised form 23 December 2017; Accepted 26 December 2017
Available online 29 December 2017
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Electrical nerve stimulation is a non-invasive technique for improving nerve regeneration that, similar to the crush conditioning lesion (CCL), upregulates RAG expression in regenerating neurons (Al-Majed et al., 2000b; Al-Majed et al., 2004; Geremia et al., 2007). To date, electrical stimulation has only been used post-operatively. Although post-surgical electrical stimulation reduces the delay of regeneration across the injury gap (Al-Majed et al., 2000), it does not improve the rate of axonal extension beyond this site (Brushart et al., 2002). Accelerated axon regeneration beyond the cut site, as observed following a CCL, is required for timely distal target reinnervation. Udina et al. (2008) proposed electrical stimulation as a conditioning strategy (Udina et al., 2008). After stimulation of the intact sciatic nerve (1 h, 20 Hz), it significantly increased neurite outgrowth of the affected dorsal root ganglia (DRG) neurons when assayed in vitro one week later. However, the use of conditioning electrical stimulation (CES) in an in vivo model of peripheral nerve regeneration has not been investigated.

The goal of this study is to test the hypothesis that CES of intact nerves prior to transection and microsurgical repair upregulates RAGs and accelerates peripheral nerve regeneration in a manner similar to a CCL.

2. Methods

2.1. Animals

Adult Sprague Dawley rats (175 g; Charles River laboratory), were placed under the care of the Health Sciences Laboratory Animal Services (HSLAS) at the University of Alberta. A total of 60 animals were used in these experiments: 24 animals for RAG analysis at the DRG (3 days after conditioning; n = 3/group), 12 animals for DRG qRT-PCR analysis (n = 3/group), and 24 animals for nerve regeneration studies and delayed DRG RAG expression (n = 6/group).

2.2. Conditioning surgery

All surgical procedures were approved by the University of Alberta Animal Research Ethics Board. Each cohort of animals was equally divided into four groups based on the type of treatment to the common peroneal (CP) nerve (also called the fibular nerve): CES, CCL, sham electrical stimulation or naïve (no conditioning). In preparation for surgery, the animals were given a dose of subcutaneous buprenorphine (0.01 mg/kg/animal) and anesthetized by oral inhalation of isoflurane (2% titrated and delivered at 1–2 L/min). A longitudinal incision was made over the right lateral lower limb from knee to mid-tibia and the CP nerve was identified at the level of the fibular neck. The nerve was carefully isolated and conditioning was performed as described below. All incisions were sutured with a two-layer closure using 4–0 Vicryl (Ethicon Inc., Sommerville NJ). Surgical details specific to the type of conditioning are as follows: i) CES: stainless steel wires bared of insulation at the ends were connected to a SD-9 stimulator (Grass Instruments Co., Quincy, MA). The cathode wire was wrapped around the CP nerve at the level of the fibular neck, and the anode wire was placed into the tibialis anterior muscle. CES was performed by delivering continuous 20 Hz of 0.1 ms duration of balanced biphasic pulses, with voltage titrated to maintain a visible twitch in the lower limb extensors, for 1 h (Udina et al., 2008). ii) CCL: a non-toothed fine hemostat (5 mm tip) was used to crush the CP nerve at the level of the fibular head for 10 s. Complete axotomy was confirmed visually by examination of the nerve. iii) Sham electrical stimulation: for 1 h, the electrodes were placed at the same locations as the CES group, but no current was delivered. iv) Naïve: no surgical intervention was performed. Three days following conditioning, animals were euthanized to harvest the L4 and L5 DRGs for RAG qRT-PCR and immunocytochemistry respectively. The examiner was blinded to the conditioning state of each animal.

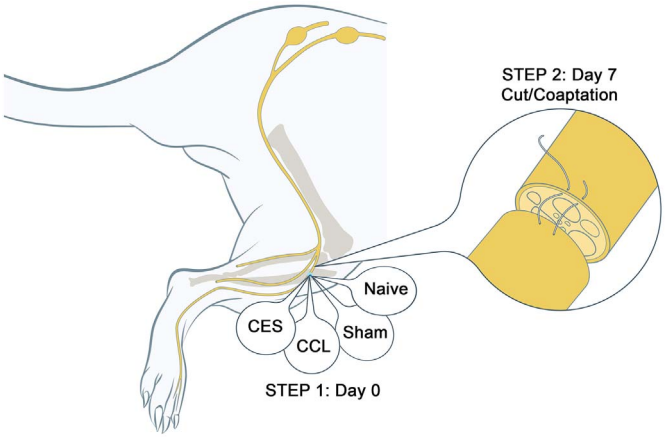

Fig. 1. Experimental design of conditioning paradigm and cut and microsurgical repair. Step 1 (Day 0): conditioning of the common peroneal (CP) nerve with CCL, CES, or sham CES 20 mm distal to the sciatic trifurcation. The naïve group does not undergo surgery at this time. Step 2 (Day 7): the CP nerve is cut 10 mm proximal to the site of conditioning (10 mm distal to the sciatic trifurcation) and an epineurial repair is performed.

2.3. Nerve transection and microsurgical repair surgery

Post-conditioning, the remaining cohort of 24 animals was left in their cages for 7 days prior to CP transection and microsurgical repair. The animals were anesthetized to surgical stage and an incision was made posterior to the lateral middle third of the palpable femur. The biceps femoris muscle was released from the vastus lateralis muscle to facilitate identification of the sciatic nerve. The sciatic nerve was traced distally and the site of trifurcation was dissected to isolate the CP nerve from the tibial and sural nerves. One centimeter distal to the trifurcation, the CP nerve was cut and immediately repaired using 9–0 silk suture under 3.5 × loupe magnification (Fig. 1). The hamstrings were re-suspended with stitches, and the skin was closed in two-layers using a running subcutaneous and horizontal mattress stitch with 4–0 Vicryl suture. Animals received 0.01 mg/kg/animal buprenorphine post-operatively. One week later, the regeneration bridge, and a 1 cm segment of both the proximal and distal nerve were harvested in order to quantify the rate of regeneration. The L4 and L5 DRGs were harvested to analyze RAG expression at the neuronal cell bodies.

2.4. Tissue analysis

Animals were euthanized by carbon dioxide and exsanguinated by opening the thoracic cavity and cutting the left ventricle of the heart. The sciatic nerve was re-exposed, and the site of cut/microsurgical repair was identified. A 3-cm toothpick was placed on the CP nerve to stabilize the proximal nerve, the regeneration bridge and the distal nerve regions for harvesting. The proximal sciatic nerve was then traced back to the vertebral column and the L4 and L5 DRGs were collected. Tissues were fixed in Zamboni's fixative (paraformaldehyde, picric acid and NaOH) (American MasterTech Scientific, Lodi, CA) for 4 h, rinsed with 0.01 M PBS five times, post-fixed in 30% sucrose solution overnight at 4 °C, then embedded and frozen in optimum cutting temperature (OCT) (Sakura Finetek, Torrance, CA) using indirect exposure to liquid nitrogen. Longitudinal sections of the nerves and DRG (12 μm) were thaw-mounted onto Superfrost Plus microscope slides (Thermo Fisher Scientific, Waltham, MA) and stored at − 80 °C until processing.

2.5. Immunofluorescence

Slides were warmed to room temperature for 30 min before antigen retrieval in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0; American MasterTech Scientific, Lodi CA) for 20 min at 80 °C and then cooled to room temperature. Slides were washed three

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