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Electrical stimulation and testosterone differentially enhance expression of regeneration-associated genes

Nijee Sharma [a,*], Sam J. Marzo [b], Kathryn J. Jones [b,c,d], Eileen M. Foecking [b]

[a] Neuroscience Program, Loyola University Chicago, Stritch School of Medicine, Maywood, IL 60153, USA
[b] Department of Otolaryngology – Head and Neck Surgery, Loyola University Chicago, Stritch School of Medicine, Maywood, IL 60153, USA
[c] Department of Cell Biology, Neurobiology, and Anatomy, Loyola University Chicago, Stritch School of Medicine, Maywood, IL 60153, USA
[d] R and D Services, Edward Hines, Jr. VA Hospital, Hines, IL 60141, USA

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ABSTRACT

As functional recovery following peripheral nerve injury is dependent upon successful repair and regeneration, treatments that enhance different regenerative events may be advantageous. Using a rat facial nerve crush axotomy model, our lab has previously investigated the effects of a combinatorial treatment strategy, consisting of electrical stimulation (ES) of the proximal nerve stump and testosterone propionate (TP) administration. Results indicated that the two treatments differentially enhance facial nerve regenerative properties, whereby ES reduced the delay before sprout formation, TP accelerated the overall regeneration rate, and the combinatorial treatment had additive effects. To delineate the molecular mechanisms underlying such treatments, the present study investigated the effects of ES and TP on expression of specific regeneration-associated genes. Following a right facial nerve crush at the stylomastoid foramen, gonadectomized adult male rats were administered only ES, only TP, a combination of both, or left untreated. Real time RT-PCR analysis was used to assess fold changes in mRNA levels in the facial motor nucleus at 0 h, 6 h, 1 d, 2 d, 7 d, and 21 d post-axotomy. The candidate genes analyzed included two tubulin isoforms (α_1-tubulin and β_{II}-tubulin), 43-kiloDalton growth-associated protein (GAP-43), brain derived neurotrophic factor (BDNF), pituitary adenylate cyclase-activating peptide (PACAP), and neuritin (candidate plasticity-related gene 15). The two treatments have differential effects on gene expression, with ES leading to early but transient upregulation and TP producing late but steady increases in mRNA levels. In comparison to individual treatments, the combinatorial treatment strategy has the most enhanced effects on the transcriptional program activated following injury.

Introduction

Despite the robust ability of the peripheral nervous system to regenerate, functional and clinically relevant recovery following injury is often suboptimal. Delays in nerve regeneration may occur due to hindered axonal outgrowth across the site of injury, slow regeneration rate over long distances, inadequate reinnervation, or target atrophy (Valero-Cabre et al., 2004; Lee and Wolfe, 2000; Lundborg, 2000). A crush or transection injury switches a fully differentiated adult neuron into a growth mode, inducing a coordinated pattern of gene expression that underlies the intrinsic regenerative capacity of peripheral nerves. This growth program includes an increase in expression of transcription factors, cell-adhesion molecules, growth factors, cytokines, and structural components needed for axonal elongation (Makwana and Raivich, 2005). Therapeutic strategies that accelerate the onset or enhance the degree of this regenerative response may improve the functional recovery outcome following injury.

Gonadal steroid hormones have significant trophic effects on the nervous system and play a neuroprotective role in conditions of injury or disease (Schumacher et al., 1996; De Nicola, 1993; Jones, 1993; Bialek et al., 2004; Tetzlaff et al., 2006; Fargo et al., 2008b). Over a course of several studies in the rodent facial nerve axotomy model, our lab has shown that testosterone administration at the time of injury accelerates functional recovery and increases the rate of nerve regeneration (Tetzlaff et al., 2006; Kujawa and Jones, 1990; Kujawa et al., 1989; Kujawa et al., 1991). These neurotherapeutic effects of testosterone are mediated by its binding to the androgen receptor, abundantly found in the motor nuclei of brainstem and spinal cord neurons (Yu and McGinnis, 2001; Tetzlaff et al., 2007; Kujawa et al., 1995). Molecular studies further demonstrate that testosterone administration following injury attenuates GFAP expression and increases levels of ribosomal RNA and regeneration-

* Corresponding author. Hines VA Hospital 5000 South 5th Ave. Bldg. 1, Room A241A Hines, IL 60141-3030, USA. Fax: +1 708 202 2327.
E-mail address: nijeesharma@gmail.com (N. Sharma).

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associated genes such as β_{II}-tubulin and GAP-43 (Kinderman and Jones, 1994; Jones and Oblinger, 1994; Jones et al., 1997b; Jones et al., 1997a). However, since receptor-mediated mechanisms require multiple steps for signal transduction, the therapeutic effects of testosterone may be delayed in comparison to therapies that can exert more immediate effects.

Multiple studies have shown that low frequency electrical stimulation (ES) of the proximal nerve stump following injury promotes axonal regeneration. ES in the rat femoral and sciatic nerve injury models results in enhanced regeneration and improved reinnervation specificity (Al-Majed et al., 2000b; Vivo et al., 2008). Studies also report that ES accelerates the onset of functional recovery, increases axonal growth across the injury site, and improves neuronal survival following axotomy (Pockett and Gavin, 1985; Brushart et al., 2002; Morimoto et al., 2002). Although the mechanisms by which ES mediates its effects are not fully known, it has been demonstrated that it influences the neuronal soma response, rapidly increasing expression of neurotrophin-4/5 and BDNF, as well as their receptor tyrosine receptor kinase B, trkB (English et al., 2007; Al-Majed et al., 2000a). ES is also associated with increased expression of α_1-tubulin and GAP-43 in motor and sensory nerve regeneration systems (Al-Majed et al., 2004; Geremia et al., 2007).

We have previously investigated the therapeutic potential of ES and TP in a rat facial nerve axotomy model and found that the combinatorial treatment has additive effects on acceleration of functional recovery (Lal et al., 2008; Hetzler et al., 2008). To investigate the mechanism underlying the additive effects of gonadal steroids and ES, we used a radioisotopic labeling method to measure facial nerve outgrowth and found that ES of the proximal nerve stump reduced the delay before sprout formation begins but failed to accelerate the regeneration rate (unpublished data). TP treatment, on the other hand, augmented the regeneration rate but had no effect on the sprouting delay. While each single treatment had differential effects, their combined treatment enhanced both of the regenerative properties, resulting in an accelerated rate and a reduced delay in sprouting. Therefore, the combinatorial treatment strategy may have a better therapeutic potential since ES may be used to initiate regeneration and testosterone to increase the rate of elongation over time.

Since a successful regenerative response is dependent upon expression of regeneration-associated genes, the objective of the current study was to examine the effects of ES and TP on gene regulation. We hypothesized that treatment with ES or TP alone may upregulate different populations of genes and/or display temporal differences in gene regulation, while their combined treatment may have additive effects. The number of genes that compose the regeneration program is immense and yet incomplete. Therefore, the present study characterized changes in expression of six genes in response to ES and TP. These genes were chosen as they have previously been shown to be regulated in the facial motor nucleus following injury. These include two members of the tubulin family (α_1-tubulin and β_{II}-tubulin) that provide cytoskeletal support for the elongating axon, as well as GAP-43, a well-known regeneration-associated gene that regulates the growth cone during regeneration. The study also includes BDNF and PACAP, known for their multiple neurotrophic effects. Lastly, we analyzed expression of neuritin, originally identified as candidate plasticity-related gene 15, which enhances neurite extension *in vitro* and induces dendritic growth and axonal elaboration *in vitro* (Nedivi et al., 1998, Cantallops et al., 2000, Javaherian and Cline, 2005). Following a facial nerve crush injury in adult rats, the effects of ES and/or TP on gene expression were assessed using real time RT-PCR analysis. The results demonstrate that the two treatments not only target different genes, but also that ES rapidly but transiently upregulates gene expression whereas TP has delayed but sustained effects.

Methods

Animals and nerve injury paradigm

Adult male Sprague–Dawley rats (2 months old) were purchased from Harlan (Indianapolis, IN) and used for all experiments. Animals were housed under a 12 h light/dark cycle and received a standard rodent diet and water ad libitum. Three to five days prior to nerve injury, rats were anesthetized with isofluorane and castrated. All surgical procedures were completed in accordance with the National Institutes of Health guidelines on care and use of laboratory animals for research purposes and approved by the institutional animal care and use committee.

For facial nerve axotomy, rats were anesthetized by intraperitoneal injections of Ketamine (100 mg/ml; 0.1 ml/100 g body weight) and Xylazine (20 mg/ml; 0.025 ml/100 g body weight). The right facial nerve was crushed near its exit from the stylomastoid foramen. The crush axotomy paradigm left the neural sheath intact to provide a route for the regenerating axons. Two successive 30-second crushes, on alternating sides, were done with fine jeweler's forceps to ensure a full crush. Successful crush was verified by complete loss of the eyeblink reflex and loss of vibrissae orientation and movement in rats upon recovery from anesthesia. Animals were divided into 4 experimental groups: [1] no treatment, animals receiving axotomy but no treatment, [2] ES only, animals receiving axotomy and ES treatment, [3] TP only, animals receiving axotomy and TP treatment, and [4] ES + TP, animals receiving axotomy and the combination of ES and TP treatments.

Electrical stimulation

A custom electrode apparatus constructed in our laboratory was implanted in all rats (Lal et al., 2008). Two Teflon-coated wires (Cooner Wire), bared of insulation for 2–3 mm, were soldered to two "male" connector pins in a connector strip (Allied Electronics). The connector assembly was cemented into a syringe base using dental acrylic. At the time of axotomy, the base of the syringe was sutured onto the paraspinal back muscles of rats. Wires were run through subcutaneously and sutured ~2 mm proximal to the injury site (cathode) and ~3–5 mm away from it (anode). The connector pins of rats were attached to leads of an electrical stimulator (W-P Instruments, Inc.), and rats were stimulated at a voltage at which they displayed a right ear flutter. Starting immediately post-axotomy, rats were either stimulated with supramaximal pulses delivered at a frequency of 20 Hz or sham stimulated for 30 min daily until sacrifice.

Hormone administration

Immediately following injury, two Silastic capsules (0.062 in. id × 0.095 in. od; 10-mm length), equilibrated in physiological saline and containing 100% crystalline TP (Sigma), were subcutaneously implanted in rats receiving hormone treatment. The dosage given has previously been shown to establish supraphysiological levels of systemic TP (Kujawa et al., 1989; Hetzler et al., 2008; Tanzer and Jones, 2004).

Real time RT-PCR

Rats were sacrificed by CO_2 asphyxiation at 0 h, 6 h, 1 d, 2 d, 7 d, or 21 d following facial nerve axotomy. Brains were removed and placed in a rat brain matrix (Redding, CA) on ice to provide support during sectioning. Using 2 razor blades spaced 1 mm apart, sections were taken at the border of the pontine band and moving caudally. The precise rostral–caudal location of the facial motor nucleus and symmetry between the control and axotomized