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Reduced expression of regeneration associated genes in chronically axotomized facial motoneurons

T. Gordon *, S. You, S.L. Cassar, W. Tetzlaff **

Department of Neuroscience, University of Alberta, Edmonton, AB T6G 2S2, Canada
ICORD (International Collaboration on Repair Discoveries), Canada
Department Zoology, University of British Columbia, Vancouver, V5Z 1M9, BC, Canada
Department Surgery, University of British Columbia, Vancouver, BC, Canada

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ABSTRACT

Chronically axotomized motoneurons progressively fail to regenerate their axons. Since axonal regeneration is associated with the increased expression of tubulin, actin and GAP-43, we examined whether the regenerative failure is due to failure of chronically axotomized motoneurons to express and sustain the expression of these regeneration associated genes (RAGs). Chronically axotomized facial motoneurons were subjected to a second axotomy to mimic the clinical surgical procedure of refreshing the proximal nerve stump prior to nerve repair. Expression of α1-tubulin, actin and GAP-43 was analyzed in axotomized motoneurons using *in situ* hybridization followed by autoradiography and silver grain quantification. The expression of these RAGs by acutely axotomized motoneurons declined over several months. The chronically injured motoneurons responded to a refreshment axotomy with a re-increase in RAG expression. However, this response to a refreshment axotomy of chronically injured facial motoneurons was less than that seen in acutely axotomized facial motoneurons. These data demonstrate that the neuronal RAG expression can be induced by injury-related signals and does not require acute deprivation of target derived factors. The transient expression is consistent with a transient inflammatory response to the injury. We conclude that transient RAG expression in chronically axotomized motoneurons and the weak response of the chronically axotomized motoneurons to a refreshment axotomy provides a plausible explanation for the progressive decline in regenerative capacity of chronically axotomized motoneurons.

Introduction

Functional recovery after chronic nerve injury is generally poor. This has generally been attributed to deterioration of the peripheral growth environment as well as irreversible atrophy of the denervated targets (Gutmann, 1948); for review see Fu and Gordon, 1997). During axon regeneration especially over long distances, many neurons remain without targets, a state of chronic axotomy (Fu and Gordon, 1997). In experiments in which chronically axotomized lumbar motoneurons were encouraged to regenerate axons into the permissive environment of a freshly denervated nerve stump, a progressive deterioration in the neurons' capacity to regenerate axons demonstrated that prolonged axotomy reduces regenerative capacity of injured neurons (Fu and Gordon, 1995a, 1997). Axotomized motoneurons undergo characteristic changes in gene expression which has been considered to be a switch from "transmitting" to "growth" (Fu and Gordon, 1997; Gordon, 1983). These changes include increased expression of immediate early genes such as c-jun (Arthur-Farraj et al., 2012; Herdegen et al., 1997), neurotrophic factors and their receptors (Al-Majed et al., 2000) cytoskeletal proteins, tubulin and actin (Al-Majed et al., 2004; Bisby and Tetzlaff, 1992), as well as growth associated proteins, most prominently GAP-43 (Skene, 1989) and CAP-23 (for review see Boyd and Gordon, 2003; Caroni, 1997; Fu and Gordon, 1997). In addition, genes associated with neurotransmission are down-regulated including choline-acetyltransferase and acetylcholinesterase (Fernandes et al., 1998; Flumerfelt and Lewis, 1975; Tetzlaff and Kreutzberg, 1984). Collectively the genes of the cytoskeletal proteins, tubulin, actin, and GAP-43 that are upregulated after injury, are frequently referred to as regeneration associated genes (RAGs) (Fu and Gordon, 1997).

The rate of axonal growth correlates directly with the rate of transport of tubulin and actin in slow component b (SCb) of axonal transport during both development and nerve regeneration in the adult (Hoffman and Lasek, 1980). The upregulation of tubulin and actin concurrent with a downregulation of neurofilament expression has been suggested to increase the fraction of tubulin and actin which is transported with the SCb component to the growing axons (Tetzlaff et al., 1996). The

* Correspondence to: T. Gordon, Department of Surgery, Division of Plastic Reconstructive Surgery, 5549A, The Hospital for Sick Children, 555 University Ave, Toronto, ON M5G 1X8, Canada. Fax: +1 416 813 6637, +1 647 678 1314 (cell).
** Correspondence to: W. Tetzlaff, ICORD (International Collaboration on Repair Discoveries), Blusson Spinal Cord Center, 818 W. 10th Avenue, Vancouver, V5Z 1M9, Canada.
E-mail addresses: tessat.gordon@gmail.com (T. Gordon), tetzlaff@icord.org (W. Tetzlaff).

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protein kinase C substrates GAP-43 and CAP-23, are concentrated in the axonal growth cone and regulate the actin based cytoskeletal motility via PI(4,5)P(2) modulation (Frey et al., 2000; Laux et al., 2000). GAP-43 is required for the stimulation of axonal growth on neuronal cell adhesion molecules such as N-CAM (Meiri et al., 1998). The transgenic overexpression of GAP-43 and CAP-23 induces terminal axonal sprouting at neuromuscular endplates (Aigner et al., 1995) and regeneration of the central axons of sensory neurons (Bomze et al., 2001). Hence, RAGs are required for a vigorous regeneration response. This is supported by studies in which electrical stimulation accelerates axon outgrowth *and* accelerates and enhances the expression of neurotrophic factors and their receptors, followed by acceleration and enhancement of RAGs (Al-Majed et al., 2000; Geremia et al., 2007; Gordon et al., 2009). Their expression is an important component of the molecular response of neurons to axotomy referred to as the 'cell body response" (Abe and Cavalli, 2008; Gordon, 1983; Gordon et al., 2009; Grafstein, 1975; Michaelevski et al., 2010; Rishal and Fainzilber, 2010).

Neuronal expression of RAGs has only been studied after acute injury and axonal regeneration after nerve crush with the exception of a study of GAP-43 expression in chronically axotomized dorsal root ganglion neurons (Bisby, 1988). In the latter study GAP-43 expression was found to be elevated after axotomy and sustained over the 6 months period of study. We report here that, in motoneurons, neither the expression of GAP-43 mRNA nor that of cytoskeletal tubulin and actin mRNA is sustained with time after axotomy. In addition, we undertook the present study to determine whether the poor regenerative capacity of chronically axotomized motoneurons is due to their failure to re-express or sustain the expression of these genes when the proximal nerve stump is subjected to a refreshment injury prior to suture of proximal and distal nerve stumps to encourage nerve regeneration. We studied axotomized facial motoneurons and demonstrated that the chronically axotomized facial motoneurons fail to fully re-express the mRNA for the tubulin, actin and GAP-43 after a refreshment injury.

Materials and methods

Surgical procedures

Sprague–Dawley male rats (Charles River) weighing 200–300 g were used for this study and all surgeries were done under deep anesthesia with intraperitoneal injection of a combination of chloral hydrate (150 mg/kg) plus sodium pentobarbital (30 mg/kg). The experimental procedures were in agreement with the guidelines of the Canadian Council for Animal Care and approved by the local animal care committees.

Design A [Chronic time course of expression of regeneration associated genes (RAGs)]

Facial motoneurons were axotomized 3–5 mm distal to the stylomastoid foramen and a 5–8 mm long segment of the facial nerve was resected to prevent reconnection with the distal stump. The rats were allowed to survive 6 or 11 months for *chronic axotomy* of the facial motoneurons and killed by an overdose of sodium pentobarbital (50 mg/kg) followed by transcardial perfusion with 4% paraformaldehyde.

Design B (Chronically axotomized motoneurons respond to a second axotomy)

In order to determine whether chronically axotomized motoneurons respond to a second axotomy (refreshment cut), we cut both the left and right facial nerves 3–5 mm distal to the stylomastoid foramen and resected 5–8 mm on each side to prevent regeneration. We allowed these rats to survive for 6 or 11 months (*chronic axotomy*). Thereafter the neuroma which had formed at the proximal stump of the facial nerve was resected on the right side (*chronic axotomy with refreshment axotomy*) while the left side was left untouched (*chronic axotomy*). Seven days later the rats were killed by an overdose of chloral hydrate followed by perfusion with 4% paraformaldehyde (for histological details, see Fernandes et al., 1999).

Design C (Response of chronically injured motoneuron to refreshment axotomy versus acute axotomy)

To determine whether chronically axotomized motoneurons respond to a refreshment cut in a similar manner and magnitude as to an acute axotomy, we compared the expression of RAGs in chronically axotomized and refreshed vs. acutely axotomized motoneurons in the same animals. We resected the left facial nerve 5 mm distal to its exit at the stylomastoid foramen (as above) and, 6–11 months later, we performed a resection of the neuroma on this left side (*refreshment axotomy after chronic axotomy*). At the same time of the second surgery, the contralateral right facial nerve, which was not previously injured, was resected (*acute axotomy*). Hence, this design allowed us to determine whether the response after refreshment cut is of the same magnitude as after acute axotomy.

In situ hybridization (ISH)

ISH was performed according a modified protocol by Miller et al. (1987) which has been described in extensive detail previously (Tetzlaff et al., 1991). For GAP-43, total α- and ß-tubulins, and actin, cDNA probes were used which were kindly provided by Dr. Pate Skene (GAP-43) (Basi et al., 1987) and Dr. N. Cowan (Mα1-tubulin and Mß2-tubulin) (Lewis and Schmalbruch, 1995) and the neurofilament-M probe was kindly provided by Dr. J.P. Julien (Julien et al., 1986). Both total α- and ß-tubulin cDNAs contain coding regions which are conserved among all other members of the α- or ß-tubulin family, thus they reveal total α- or ß-tubulin mRNA levels, respectively. In addition, for GAP-43, a 50-mer (5′GCATCGGTAGTAGCAGAGCCATCTCCCTCCTTCT TCTCCACACCATCAGCAA-3′) complementary to bases 270-220 (Basi et al., 1987) was used. No homologies were found in BLASTN database searches (Altschul et al., 1990). These oligo-probes were end-labeled using deoxynucleotide terminal transferase and ^{35}S-ATP according to a standard protocol (Ausubel et al., 1987). One hundred and seven cpm/ml of this probe was applied to the proteinase K treated, postfixed, and dehydrated sections (see above) using a hybridization cocktail that contained formamide (50%), 4x sodium chloride sodium citrate (SSC), dextran sulfate (0.1 g/ml), sarcosyl (1%), salmon sperm DNA (250 μg/ml) yeast tRNA (500 μg/ml), DDT (50 mM) and phosphate buffer (0.02 M, pH 7.2). After overnight hybridization at 43°C, the sections were washed four times in 1x SSC for 15 min at 55°C, rinsed in distilled water, dried, dipped in Kodak NTB2 emulsion, and exposed for 2 (T-α1) or 7 days (GAP-43) respectively.

In control experiments we determined that the concentration of the oligonucleotide probes used and the duration of hybridization of >14 h were saturating. We also confirmed in northern blots of RNA from facial nuclei, that the probes recognized a single band of expected size under identical hybridization conditions (Tetzlaff et al., 1991); equivalent data were obtained for the oligonucleotide probes (not shown). RNAse digestions and competitions with cold probe were performed as further specificity controls. These resulted in background signals only (not shown). Since hybridizations with sense probes shows that a sequence of similar GC content (typically around 50% for our probes) gives no signal, but still does not rule out cross-hybridizations, we refrained from using them. We obtained the same results using cDNA probes several hundred base pairs long as with the oligonucleotide probes. Single stranded oligonucleotide probes, however, revealed stronger signals overall and thus required 4–6 times shorter radioautographic exposure times.

Quantification of in situ hybridization and statistics

Darkfield pictures of the silver grains in the facial motoneurons were visualized. The motoneurons were stained for 20 s in diluted cresyl