Neuroscience Letters 471 (2010) 119–124

Contents lists available at ScienceDirect

Neuroscience Letters

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

Evidence for endothelial nitric oxide as a negative regulator of Schwann cell dedifferentiation after peripheral nerve injury

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ARTICLE INFO

Article history:
Received 22 December 2009
Received in revised form 9 January 2010
Accepted 11 January 2010

Keywords:
GAP-43
eNOS
Hypoglossal nerve
Adenoviral vectors
Peripheral nerve injury

ABSTRACT

The loss of intimate contact with axons triggers Schwann cells (SCs) to switch from a myelin-producing phenotype to a dedifferentiated, proliferating non-myelin-forming state after nerve injury. SC dedifferentiation is required for effective nerve regeneration. Negative regulators of SC dedifferentiation are promising targets to accelerate function recovery in acquired peripheral neuropathies. We recently reported that nitric oxide (NO) synthesized by endothelial NO synthase (eNOS) slows down functional recovery and axon regeneration after XIIth nerve crushing. This harmful action could be effected by a NO-delaying action on SC dedifferentiation. Adenoviral vectors directing the expression of a dominant negative mutant for eNOS (AVV-TeNOS) or the enhanced green fluorescent protein (AVV-eGFP) were individually injected into the distal stump just after XIIth nerve crushing. Growth-associated protein 43 (GAP-43), strongly over-expressed in dedifferentiated SCs and regenerating axons, was up-regulated in AVV-TeNOS-transduced nerves relative to AVV-eGFP-treated nerves. AVV-TeNOS increased the number of GAP-43-positive cells and bands of Bungner but did not alter the number of Hoechst-positive nuclei relative to AVV-eGFP. These results signal endothelial NO as a negative regulator of the SC dedifferentiation process, but not of SC proliferation rate, after nerve injury. Vascular-derived factors should be taken into account as feasible extrinsic regulators of SC plasticity.

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Schwann cells (SCs) play a pivotal role in promoting axon regeneration through the distal stump after nerve injury. Wallerian degeneration involves axon degeneration as well as myelin breakdown and clearance in the distal part of the damaged nerve [8]. In this scenario SC dedifferentiation is required for effective nerve regeneration [9]. That is, in response to axon degeneration, SCs switch from a myelin-producing state to a dedifferentiated, proliferating non-myelin-forming state [20]. Dedifferentiated SCs form strands (bands of Bungner) accompanied by the up-regulation of multiple regeneration-associated proteins, such as growth factors, neurotrophins, cytokines, and cell adhesion molecules that promote axon regeneration [1,20]. Among them, the growth-associated protein 43 kDa (GAP-43) is strongly up-regulated in dedifferentiated SCs [4,15–17]. GAP-43 functions as a plasticity protein involved in cell shape change and motility [4,17], which allow SC re-ordering to form bands of Bungner, the cellular substrate for guiding growing axons.

Negative regulators for myelination activating the SC dedifferentiation program involve many extrinsic and intrinsic signals [9]. Cell-extrinsic injury-related signals from neighboring SCs and neurons accelerate and influence SC dedifferentiation *in vivo* [9]. However, evidence of the involvement of vascular signals in regulating SC dedifferentiation remains elusive. Regulators provided by endothelial cells modulating the change to a dedifferentiated phenotype of SCs could be promising targets to accelerate function recovery in acquired peripheral neuropathies.

The highly reactive gas nitric oxide (NO) could be a candidate to regulate SC dedifferentiation. The three major isoforms of NO synthase (NOS) – neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) – are up-regulated at the distal stump of the injured nerve. iNOS is *de novo* expressed in dedifferentiated SCs and in infiltrated macrophages, whereas nNOS is up-regulated in injured ganglionar neurons and *de novo* expressed in damaged motoneurons. However, eNOS is constitutively expressed in blood vessels being up-regulated in *vasa nervorum* after nerve damage [12]. Strikingly, eNOS inhibition accelerated motor function recovery after XIIth nerve crushing by facilitating axon regeneration [21].

We hypothesized that endothelial NO acts as a negative regulator of SC dedifferentiation after nerve injury. To tackle this issue, we analyzed the expression level of the dedifferentiated SC marker GAP-43, as well as the density of SCs and bands of Bungner, after intra-nerve administration of an adenovirus directing the expression of a dominant negative mutant for eNOS after XIIth nerve

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doi:10.1016/j.neulet.2010.01.024

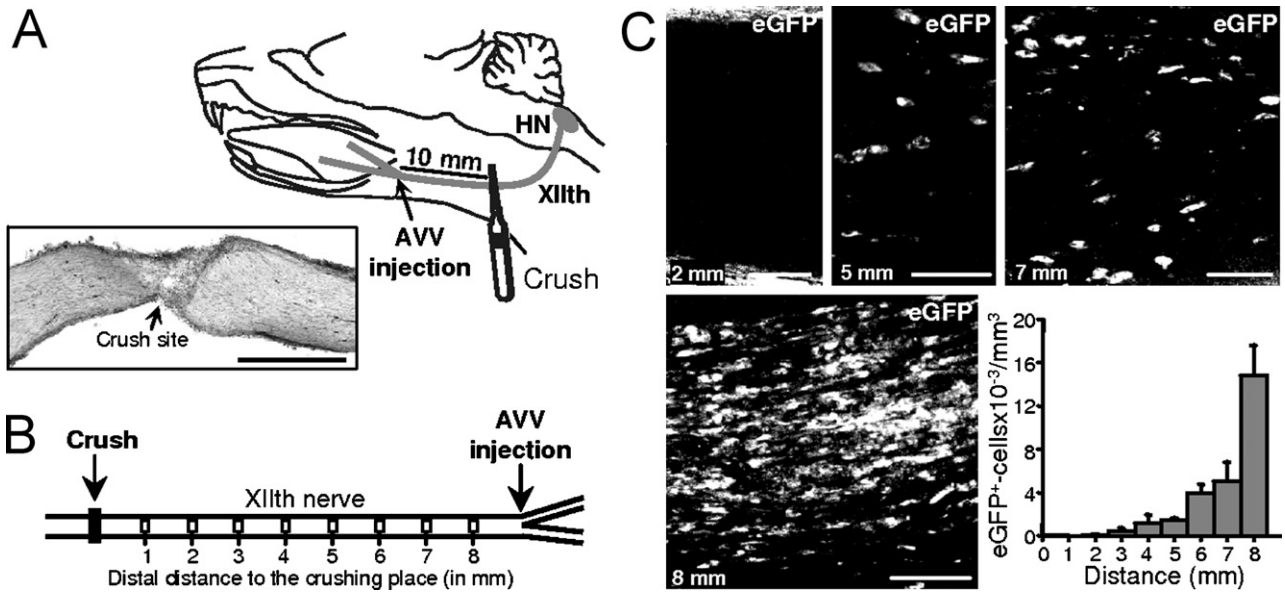

Fig. 1. Experimental design and extent of adenoviral transduction. (A) Crushing of the XIIth nerve 10 mm proximal to its bifurcation and adenoviral intra-nerve microinjection at the bifurcation level were performed in the same surgical session. *Inset*: Photomicrograph of a sagittal section at the level of the crushing site extracted 3 h after injury, stained by neutral red. Note that axons are fully transected but endoneural tube is preserved. (B) Drawing of the XIIth nerve illustrating the anatomical location of the crushing (grey rectangle) and the adenoviral injection site. The areas (250 μm × 250 μm) in which eGFP-transduced cells were counted are indicated. (C) Confocal images of sagittal sections of the XIIth nerve showing eGFP-transduced cells at the indicated distances relative to the site of crushing. The plot represents the average number of eGFP-positive cells (in thousands per cubic millimeter) at the indicated distances (in mm) distal to the injury site. Scale bars: A, inset, 500 μm; C, 75 μm.

crushing. Results support that endothelial NO interferes with SC dedifferentiation.

Experiments were performed in accordance with the guidelines of the European Union Council (86/609/UE) and Spanish regulations (BOE 67/8509-12; BOE 1201/2005) on the use of laboratory animals, and approved by the local Animal Care and Ethics Committee.

Adult male Wistar rats (Animal Supply Services, University of Cádiz, Spain), weighing 250–400 g, were anesthetized (chloral hydrate; 0.5 g/kg; i.p.) and the right XIIth nerve was isolated from surrounding tissue. Subsequently, the common nerve trunk was crushed with microdissecting tweezers, applied for 30 s 10 mm proximal to the nerve bifurcation, as previously described [7] (Fig. 1A). After lesion, the nerve became translucent and it was confirmed that transection did not occur (Fig. 1A, inset).

In the same session that the XIIth nerve injury was inflicted, we applied a replication-deficient adenoviral vector directing the expression of a truncated mutant form of eNOS (AVV-TeNOS; 2.2×10^{10} pfu/ml) under the control of the human cytomegalovirus (hCMV) promoter. The vector AVV-eGFP (4.6×10^{10} pfu/ml), directing the expression of the enhanced green fluorescent protein under control of the hCMV, was used as a control. For intra-nerve injection, glass micropipettes with broken tips (~50 μm) were filled with a solution of 3 μl of a suspension containing one of the two vectors. The micropipettes were advanced with a micromanipulator through the perineurium along the axis of the nerve near the branching point of the XIIth nerve (Fig. 1A). Adenovirus was slowly injected (~5 min), driven by an oil-filled tubing system. More details for intra-nerve injection were previously described by our group [21]. The incision was sutured and cleaned with an antiseptic solution (povidone–iodine).

Two days after crushing and intra-nerve injection of the adenovirus, animals were anesthetized (as above) and perfused transcardially, first with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, at 4 °C. The right XIIth nerves were carefully removed, postfixed for 2 h in the same fixative solution, and cryoprotected by overnight immersion in 30% sucrose in PB at 4 °C. Although length between crushing and injection sites was 10 mm, fixation procedures shortened it by 1 mm. Nerves were cut in 2 mm length segments from the crushing site to the bifurcation. Serial sagittal sections (30 μm thick) from nerve segments were obtained using a cryostat and mounted in gelatinized slides.

GAP-43 immunostaining was used to histologically characterize regeneration, because GAP-43 expression is strongly induced in regenerating fibers and in dedifferentiated SCs in the distal nerve stump after nerve lesion [4,15,17]. For GAP-43-immunofluorescence, sections were rinsed in PBS and immersed in 2.5% (w/v) bovine serum albumin, 0.25% (w/v) sodium azide, and 0.1% (v/v) Triton X-100 in PBS for 30 min, followed by incubation at 4 °C overnight with a polyclonal antibody against GAP-43 developed in rabbit (1:1000; Chemicon, Temecula, CA). Subsequently, the tissue was rinsed in PBS and incubated for 2 h at room temperature with an anti-rabbit IgG conjugated with Cy5 (cyanine 5; 1:400; Jackson ImmunoResearch, West Grove, PA). Omission of the primary antibody resulted in no detectable staining. Furthermore, to differentiate between GAP-43-positive growth cones and cells, sections were counterstained with a nuclear marker. Tissue processed by GAP-43 immunohistochemistry was washed with PBS and immersed for 1 h in a solution containing Hoechst 33342 (10 mg/ml stock from Molecular Probes, Invitrogen; final dilution: 1:500 in PBS) to label nuclei. Finally, sections were washed with PBS and covered with a solution containing propyl gallate (0.1 mM in PBS:glycerol, 1:9). Slices from AVV-eGFP- or AVV-TeNOS-treated rats were processed in parallel. Fluorescent signals were analyzed using either a Leica (Nussloch, Germany) confocal microscope or BX60 Olympus epifluorescence microscope.

Adenovirally transduced cells, detected by eGFP expression, were quantified under a 40× objective under confocal microscopy. Fig. 1B shows a cartoon representing the location of areas where transduced cells were quantified. For low-magnification analysis of GAP-43-immunoreactivity, images at 4 and 5 mm from the crushing site were captured and processed for background subtraction to obtain the maximum dynamic range of intensities (from 0 to 250) and analyzed using the software provided by

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