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Artemin induced functional recovery and reinnervation after partial nerve injury

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ABSTRACT

Systemic artemin promotes regeneration of dorsal roots to the spinal cord after crush injury. However, it is unclear whether systemic artemin can also promote peripheral nerve regeneration, and functional recovery after partial lesions distal to the dorsal root ganglion (DRG) remains unknown. In the present investigation, male Sprague Dawley rats received axotomy, ligation, or crush of the L5 spinal nerve or sham surgery. Starting the day of injury, animals received intermittent subcutaneous artemin or vehicle across 2 weeks. Sensory thresholds to tactile or thermal stimuli were monitored for 6 weeks after injury. Immunohistochemical analyses of the DRG and nerve regeneration were performed at the 6-week time point. Artemin transiently reversed tactile and thermal hypersensitivity after axotomy, ligation, or crush injury. Thermal and tactile hypersensitivity reemerged within 1 week of treatment termination. However, artemin-treated rats with nerve crush, but not axotomy or ligation, subsequently showed gradual return of sensory thresholds to preinjury baseline levels by 6 weeks after injury. Artemin normalized labeling for NF200, IB4, and CGRP in nerve fibers distal to the crush injury, suggesting persistent normalization of nerve crush-induced neurochemical changes. Sciatic and intradermal administration of dextran or cholera toxin B distal to the crush injury site resulted in labeling of neuronal profiles in the L5 DRG, suggesting regeneration functional restoration of nonmyelinated and myelinated fibers across the injury site into cutaneous tissue. Artemin also diminished ATF3 and caspase 3 expression in the L5 DRG, suggesting persistent neuroprotective actions. A limited period of artemin treatment elicits disease modification by promoting sensory reinnervation of distal territories and restoring preinjury sensory thresholds.

1. Introduction

Incomplete recovery from nerve damage often leads to loss of sensory function that may be paradoxically accompanied by severe, intractable neuropathic pain [1,23]. Exogenously administered glial cell-derived neurotrophic factor (GDNF) or artemin has promoted neuronal regeneration in conditions of neuropathic pain [7,11,26] and brachial avulsion [22]. However, clinical trials have demonstrated that systemic GDNF administration produces substantial adverse effects such as pain, weight loss, bowel urgency, and paraesthesias [14,18], which may stem from the relatively broad distribution of GDNF receptors. Artemin, a neurotrophin in the family of GDNF ligands currently under clinical investigation for treatment of sciatica (clinicaltrials.gov), preferentially binds to the GDNF family receptor (GFR) GFRα3, which is expressed primarily on nociceptive sensory neurons within the dorsal root ganglion (DRG) [20]. Such restricted expression may be advantageous in allowing for systemic administration within the clinical setting with diminished side effects.

Previous studies have shown that artemin both prevented and reversed nerve injury-induced thermal and tactile hypersensitivity and blocked multiple nerve injury-induced neurochemical changes during drug administration [9]. In addition, recent studies demonstrated that artemin promotes regeneration of injured primary afferent fibers through the dorsal root entry zone and into the spinal cord after dorsal root crush [11,26]. These changes were accompanied by long-term recovery of synaptic function along with normalization of sensory responses to tactile and thermal stimuli as well as locomotor activity, effects that persisted across

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6 months after a 2-week period of intermittent systemic artemin treatment [26]. In the present investigation, we compared the effects of systemic artemin on nerve injury-induced hypersensitivity (ie, pain) and neurochemical changes, including possible regeneration of peripheral nerves to distal territories, across multiple models of nerve injury-induced pain.

2. Methods

2.1. Animals

Male Sprague Dawley rats (Harlan, Indianapolis, IN), weighing 175 to 250 g at the time of surgery, were housed in a temperature-controlled room on a 12-h light/dark cycle. Food and water were available ad libitum. All testing was performed in accordance with the policies and recommendations of the International Association for the Study of Pain and the National Institutes of Health guidelines for the handling and use of laboratory animals and received approval from the Institutional Animal Care and Use Committee of the University of Arizona.

2.2. Spinal nerve injuries

Anesthesia was induced with 4% isoflurane in air and maintained with 2% isoflurane in air delivered at a rate of 1 L/min. Separate groups of rats underwent (1) transection and removal of a 2- to 3-mm portion of the L5 spinal nerve (axotomy), (2) tight ligation of the L5 spinal nerve with 4-0 silk thread, (3) repetitive crush of the L5 spinal nerve for 60 s with #7 head-bent forceps, or (4) sham surgery. On completion of the surgery, hemostasis was confirmed, the muscles were sutured with silk thread, and the skin was closed with metal clips. Postoperatively, all rats were individually housed, and any rats with motor deficits were humanely killed.

2.3. Artemin administration

Rat artemin (113 amino acids) was refolded from *Escherichia coli* inclusion bodies and purified to >98% homogeneity. Purified artemin migrated as a reducible dimer by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and eluted as a single peak (24 kDa) by size exclusion chromatography and reverse-phase high-performance liquid chromatography. The purified product was confirmed to contain the characteristic cysteine-knot disulfide pattern seen in GDNF and to be fully active in vitro by assaying receptor binding, cell-based c-RET kinase activation and sensory neuronal survival as previously described [9,26]. Artemin (1 mg/kg) was injected subcutaneously on a Monday–Wednesday–Friday schedule across 14 days beginning immediately after surgery (6 total injections) as described previously [9,26]. Vials containing artemin or vehicle were prepared and coded, and the experiments were performed by a different investigator blinded to the codes. The labels were opened at the termination of the study.

2.4. Behavioral observations

Tactile sensory thresholds were determined by the withdrawal threshold of the left paw in response to probing with a series of 8 calibrated von Frey filaments (Stoelting, Wooddale, IL) in logarithmically spaced increments ranging from 0.41 to 15 g (4–150 N) as previously described [9,26]. Thermal sensory thresholds were evaluated by the determination of paw withdrawal latency from an infrared radiant heat source [9,26]. All behavioral assessments were performed in a blinded fashion. Eight rats per treatment group were used by this procedure.

2.5. Neuronal tracing

For tracer injections into the sciatic nerve to evaluate nerve regeneration, rats were anesthetized at 5 weeks after spinal nerve injury with 4% isoflurane, and the sciatic nerves at midthigh region ipsilateral to spinal nerve injury were exposed under aseptic conditions. A 5-μL solution of 0.5% CTB (cholera toxin B subunit, low salt; List Labs) or 10% tetramethylrhodamine–dextran (3000 MW, Molecular Probes) were injected into the sciatic nerve at 3 to 5 sites with a Hamilton syringe. After injection, the muscles and skin were closed with 4-0 silk suture. For tracer injections into the dermis to evaluate possible nerve reinnervation, a 50-μL solution of 0.5% CTB or of 10% tetramethylrhodamine–dextran (3000 MW, Molecular Probes) was injected intradermally into both plantar and dorsum of the ipsilateral hind paw at 5 to 6 sites with a Hamilton syringe. One week after tracer injection into the sciatic nerve or dermis (6 weeks after spinal nerve injury), rats underwent intracardiac perfusions and ipsilateral L5 spinal nerves, and DRGs were collected for immunohistochemical evaluation.

2.6. Immunohistochemistry

Frozen sliced sections of DRG (10 μm) and spinal nerve (10 μm) were serially mounted. Selected sections (at least 120 μm apart from each other if from the same DRG) were incubated with primary reagents 24 h at 4 °C, and with the secondary antibody for 2 h at room temperature. Primary antisera were monoclonal mouse anti-neurofilament 200 (1:5000; Sigma, MO), polyclonal goat anti-CTB IgG (1:5000; List, CA), and polyclonal rabbit anti-CGRP/NPY (1:10,000; Peninsula Laboratories), goat anti-caspase 3 (1:5000; Abcam), polyclonal rabbit anti-ATF3 (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antisera were Cy3-conjugated goat anti-rabbit IgG (1:1000; Jackson Laboratories, West Grove, PA), Alexa Fluor 488-conjugated goat anti-mouse or donkey anti-goat IgG (1:1000; Molecular Probes). For IB4 binding, tissue sections were incubated with a FITC-conjugated IB4 (1:1000; Vector Laboratories, Burlingame, CA) in phosphate-buffered saline (PBS) for 2 h at room temperature. After PBS washes, sections were dried and sealed with fluorescent mounting medium (Vector Laboratories). Fluorescence images were acquired with a Hamamatsu digital camera attached to an Olympus fluorescence microscope and saved as TIFF files. Counting was performed with MetaMorph image analysis software (Molecular Devices, Sunnyvale, CA).

Nickel-enhanced DAB was used for caspase 3 labeling. Sections were washed in PBS for 30 min after primary incubation, then incubated in 1% normal goat serum–PBS with 1:500 biotinylated horse anti-goat IgG secondary antiserum (Vector Labs Inc, Burlingame, CA) for 2 h, then avidin–biotin horseradish peroxidase complex (ABC kit, Vector) for 90 min. The reaction product was developed in diaminobenzidine (SigmaFast, DAB tablets with metal enhancer, Sigma Chemical Co, St Louis, MO) solution for 10 min, then washed repeatedly in PBS, ethanol dehydrated, cleared in xylene, and coverslipped with Permount.

2.7. Image analysis

For counts of the percentage of the labeled neurons in DRG sections, 3 sections (at least 120 μm apart) from each DRG were randomly selected. In each section, a minimum of 150 cells were