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Research report

Modulation of vascular endothelial growth factor (VEGF) expression in motor neurons and its electrophysiological effects

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Abstract

Previous studies have shown that VEGF expression in forebrain increases after experimental manipulations that increase neuronal activity. One question is whether this also occurs in motor neurons. If so, it could be potentially advantageous from a therapeutic perspective, because VEGF prevents motor neuron degeneration. Therefore, we asked whether endogenous VEGF expression in motor neurons could be modulated. We also asked how VEGF exposure would influence motor neurons using electrophysiology.

Immunocytochemistry showed that motor neuron VEGF expression increased after a stimulus that increases neuronal and motor activity, i.e., convulsive seizures. The increase in VEGF immunoreactivity occurred in all motor neuron populations that were examined 24 h later. This effect was unlikely to be due to seizure-induced toxicity, because silver degeneration stain did not show the typical appearance of a dying or dead neuron.

To address the effects of VEGF on motor neuron function, VEGF was applied directly to motor neurons while recording intracellularly, using a brainstem slice preparation. Exposure to exogenous VEGF (200 ng/ml) in normal conditions depressed stimulus-evoked depolarization of hypoglossal motor neurons. There was no detectable effect of VEGF on membrane properties or firing behavior. We suggest that VEGF is upregulated in neurons when they are strongly activated, and VEGF depresses neuronal excitation as a compensatory mechanism. Failure of this mechanism may contribute to diseases that involve a dysregulation of VEGF, excessive excitation of motor neurons, and motor neuron loss, such as amyotrophic lateral sclerosis (ALS).

Keywords: Amyotrophic lateral sclerosis (ALS); Facial nucleus; Hypoglossal nucleus; Seizures; Spinal cord; Status

1. Introduction

The ability to produce endogenous vascular endothelial growth factor (VEGF) may be important for maintaining the health of motor neurons in the brainstem and spinal cord. A decrease in endogenous production of VEGF has been linked to motor neuron degeneration in motor system diseases such as amyotrophic lateral sclerosis (ALS) and Kennedy disease [2,6,9,14,23,24,31]. Also, impairing VEGF production through genetic manipulation results in degeneration of lower motor neurons in adulthood [14,23]. VEGF has also been shown to be neuroprotective in animal models of ischemia or other types of insult or injury [12,25].

To optimize a potential therapeutic approach, it would be advantageous to clarify the ways motor neuron VEGF can be manipulated, and the effects of altered expression on motor neuron function. To address the first issue, we asked whether a robust stimulus that increases neuronal activity and motor behavior, seizures, would increase VEGF in motor neurons. To test this hypothesis, seizures were induced by the muscarinic agonist pilocarpine, a convulsant that is commonly used to induce severe motor seizures that are continuous and prolonged (status epilepticus or "status") [27,28]. During pilocarpine-induced status, there are regular, involuntary motor contractions of all limbs (myoclonic jerks), as well as involuntary facial and oral movements (mastication) [27,28]. Therefore, descending and peripheral inputs would be likely to activate motor neurons more than would occur under normal conditions. Animals were eval-

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uated for changes in VEGF expression 24 h after the onset of status, because this time appeared to be associated with increased VEGF expression in forebrain [12]. It also appears to be the time when activity-dependent changes in other growth factors are maximal [20].

To predict any clinical potential for VEGF therapy, it would be useful to clarify its effects on motor neuron function. Therefore, a second aim of this study was to apply exogenous VEGF to motor neurons and evaluate any physiological effects on membrane properties or synaptic transmission. Physiological effects might be expected given the evidence for direct effects of VEGF on motor neuron survival in recent studies [2,14]. In addition, there could be indirect effects mediated by the influence of VEGF on the vasculature or signal transduction pathways [13]. However, to our knowledge, no studies of motor neuron physiology after exposure to VEGF have been reported to date.

2. Materials and methods

2.1. General methods

Experiments were conducted according to the guidelines set by the New York State Department of Health and the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee of Helen Hayes Hospital.

Male Sprague–Dawley rats (Taconic Farms) were provided food and water *ad libitum* and housed using a 12 h light/dark cycle. All chemicals were purchased from Sigma–Aldrich unless otherwise noted.

2.2. Seizure induction

Animals were housed in clear cages to optimize observation of seizures. Each animal was injected with atropine methylbromide (1 mg/kg, s.c.) followed by pilocarpine hydrochloride (380 mg/kg, s.c.) 30 min later. The dose was chosen to maximize the chance of inducing status epilepticus while minimizing mortality. After pilocarpine injection, animals were then observed continuously for evidence of behavioral seizures using the Racine scale [16]. Most animals developed mild (stages 1–3) and then severe (stages 4 and 5) limbic seizures, and eventually a stage 4 or 5 seizure occurred which did not stop (status epilepticus or "status"). The onset of that stage 4 or 5 seizure was defined as the onset of status, and typically occurred within 60 min of pilocarpine administration. The stage 4 or 5 seizure that signaled the onset of status was followed immediately by loss of postural tone, and animals subsequently displayed rhythmic muscular contractions, typically while lying on their side.

One hour after the onset of status, and animals were injected with diazepam (5 mg/kg i.p.). After this time, animals continued to have subtle motor contractions for several hours. In addition to the motor contractions of the limbs, animals exhibited movements of the facial area, as indicated by movements of the whiskers, the mouth and tongue. This is noted because it could be relevant to the increase in VEGF expression in facial and hypoglossal motor neurons described in the Results. After approximately 5–6 h, animals received 5% dextrose-lactate Ringer's solution (2.5 ml, s.c.).

Control animals were treated identically, i.e., atropine, diazepam, dextrose-lactate Ringer's solution, but were administered 3 ml/kg phosphate-buffered saline (i.p.) instead of pilocarpine. They were administered diazepam approximately 2 h after saline injection, similar to the timing of diazepam injection in rats that had status.

2.3. Anatomical methods

Animals were anesthetized with an overdose of urethane (2.5 mg/ kg, i.p.), and then transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains and spinal cords were left *in situ* and refrigerated overnight. On the following day, brains and spinal cords were removed and postfixed in 4% paraformaldhyde in 0.1 M phosphate buffer for 1 week, and 50-μm thick coronal sections were made with a Vibratome (Ted Pella Inc.). After processing, sections were viewed with an Olympus BX-51 microscope and photographed with a digital camera (Camera: Optronics; Frame grabber: Foresight Imaging; Software: Microbrightfield Inc.)

2.3.1. VEGF immunocytochemistry

Sections from control and treated animals were processed concurrently, using free-floating sections. Sections were placed in one compartment of an eight-compartment plexiglas tray that was rinsed with 0.5% bovine albumin. Each compartment was filled with approximately 6 ml of reagents. In each compartment, five sections were selected from a given animal, so that areas that included motor neuron nuclei were well-represented. In addition, there were at least 12 sections from cervical spinal cord. Similar sections, but from other animals, were placed in separate compartments. Sections were processed in the same order (compartment 1 followed by compartment 2, etc.) so that duration of exposure to reagents was similar for all sections. Sections were first immersed in 0.1 M TRIS buffer (pH 7.4) and placed on a rotator for 5 min. This was repeated twice (i.e., total, 3 min × 5 min). Sections were then transferred to 1% H_2O_2 in 0.1 M TRIS buffer for 30 min, followed by 0.1 M TRIS buffer (3 min × 5 min), and then transferred to 0.1% Triton X-100 in 0.1 M TRIS buffer (TRIS A) for 10 min, followed by Triton X-100 and 0.005% bovine serum albumin (BSA) in 0.1 M TRIS buffer (TRIS B; 10 min). Sections were then incubated in 10% normal goat serum in TRIS B for 1 h, followed by incubation in anti-mouse VEGF antibody, made in goat (1:500; R&D Systems). Incubation was conducted as follows: the compartmentalized tray was placed on a rotator at room temperature overnight. In some cases, incubation was conducted for 2 days in the cold (4 °C). The two procedures led to results that were indistinguishable so they are presented together in Section 3. Sections were then washed in TRIS A for 10 min, TRIS B for 10 min, and incubated in biotinylated horse anti-goat IgG diluted in TRIS B (1:400, Vector Laboratories) for 45 min. Sections were then washed in TRIS A for 10 min followed by 0.1% Triton X-100 and 0.005% bovine serum albumin in 0.5 M TRIS buffer (TRIS D; 10 min). This was followed by incubation in avidin–biotin horseradish peroxidase complex (ABC), made in TRIS D, for 2 h (ABC Standard kit; 1:1000; Vector Laboratories). Sections were then washed in 0.1 M TRIS buffer (3 min × 5 min), and developed in 0.022% 3,3′-diaminobezidine (DAB), and 1 mM $NiCl_2$ in TRIS buffer. The development was stopped by consecutive TRIS washes (3 min × 5 min). Sections were mounted on subbed slides, dehydrated in a graded series of alcohols, cleared in xylene and coverslipped in Permount (Fisher).

2.3.2. Silver stain

Silver stain was used instead of fluorojade B, because fluorojade B is ineffective in staining degenerating motor neurons [1]. The silver stain procedure was modified from one previously reported [22]. Sections were mounted on subbed slides, washed in distilled H_2O (3 min × 5 min), and then pretreated with a solution composed of the following, in equal volumes: 9% aqueous sodium hydroxide and 1.2% aqueous ammonium nitrate. Sections were then exposed to 60 ml of 9% NaOH, 40 ml of 16% ammonium nitrate, and 0.6 ml of 50% silver nitrate (all solutions were diluted in distilled water). Sections were then washed for 10 min 1 ml of 1.2% ammonium nitrate, 300 ml 95% ethyl alcohol, and 0.05% sodium carbonate. Finally, sections were developed in a 1:1 aqueous solution with the following constituents: 1 ml 1.2% ammonium nitrate, 100 ml 95% ethyl alcohol, 15 ml 37% formalin, and 0.005% citric acid monohydrate (pH 5.8). Sections were mounted in 0.1 M TRIS buffer and fingernail polish was used to cement coverslips in place. Slides were stored in the dark.

2.3.3. Electrophysiology

2.3.3.1. Slice preparation. Untreated male Sprague–Dawley rats (19–28 days old, 50–75 g) were anesthetized by inhalation of CO_2 and decapitated after loss of consciousness. The brain was rapidly removed, and was immersed in ice-cold sucrose-based artificial cerebrospinal fluid ("sucrose-ACSF," in mM: 126 sucrose, 5.0 KCl, 2.0 $CaCl_2$, 2.0 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 D-glucose; pH 7.4). The brainstem was severed from the forebrain by a coronal cut at the anterior tip of the cerebellum, and the anterior surface glued to a Teflon-coated tray. The tray was immersed in ice-cold sucrose ACSF and 400 μm-thick slices were cut using a Vibroslice (World Precision Instruments). Slices