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Research Paper

Synaptic loss and firing alterations in Axotomized Motoneurons are restored by vascular endothelial growth factor (VEGF) and VEGF-B

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ABSTRACT

Vascular endothelial growth factor (VEGF), also known as VEGF-A, was discovered due to its vasculogenic and angiogenic activity, but a neuroprotective role for VEGF was later proven for lesions and disorders. In different models of motoneuronal degeneration, VEGF administration leads to a significant reduction of motoneuronal death. However, there is no information about the physiological state of spared motoneurons. We examined the trophic role of VEGF on axotomized motoneurons with recordings in alert animals using the oculomotor system as the experimental model, complemented with a synaptic study at the confocal microscopy level. Axotomy leads to drastic alterations in the discharge characteristics of abducens motoneurons, as well as to a substantial loss of their synaptic inputs. Retrograde delivery of VEGF completely restored the discharge activity and synaptically-driven signals in injured motoneurons, as demonstrated by correlating motoneuronal firing rate with motor performance. Moreover, VEGF-treated motoneurons recovered a normal density of synaptic boutons around motoneuronal somata and in the neuropil, in contrast to the low levels of synaptic terminals found after axotomy. VEGF also reduced the astrogliosis induced by axotomy in the abducens nucleus to control values. The administration of VEGF-B produced results similar to those of VEGF. This is the first work demonstrating that VEGF and VEGF-B restore the normal operating mode and synaptic inputs on injured motoneurons. Altogether these data indicate that these molecules are relevant synaptotrophic factors for motoneurons and support their clinical potential for the treatment of motoneuronal disorders.

1. Introduction

The vascular endothelial growth factor (VEGF) was initially characterized by its vasculogenic, angiogenic properties and capacity to increase the permeability of blood vessels (Ferrara and Henzel, 1989; Senger et al., 1983; Yancopoulos et al., 2000). However, recent evidence indicates that VEGF likely exerts direct effects on neurons so that, at present, VEGF is also considered a neuroprotective factor (Lambrechts and Carmeliet, 2006; Lange et al., 2016; Storkebaum et al., 2004).

Interestingly, a causal link between low levels of VEGF and amyotrophic lateral sclerosis (ALS) has been established (Sathasivam, 2008). In particular, the design and investigation of the $VEGF^{\delta/\delta}$ mutant mice (characterized by low levels of VEGF) have shown that these animals develop an adult-onset motoneuron disease resembling ALS (Oosthuyse et al., 2001). Also in humans, *post-mortem* studies in ALS patients have demonstrated low levels of VEGF and its receptor VEGFR-2 in the spinal cord (Brockington et al., 2006; Sathasivam, 2008). Moreover, when mice that overexpress VEGF ($VEGF^{+/+}$) are crossed with the mutant mice G93A superoxide dismutase 1 ($SOD1^{G93A}$), a classical model of ALS, the double-transgenic mice show delayed motoneuron loss and prolonged survival as compared to the $SOD1^{G93A}$ single-transgenic mice (Wang et al., 2007). It has been also demonstrated that the viral delivery of VEGF to $SOD1^{G93A}$ mice delays the onset of the disease, slows the progression of motoneuronal degeneration and increases life expectancy (Azzouz et al., 2004; Wang et al., 2016). Similarly, the chronic administration of VEGF in the spinal cord prevents paralysis and motoneuronal death in rats exposed to excitotoxic motoneuron degeneration (Tovar-y-Romo et al., 2007). In addition to motoneurons, neuroprotective effects of VEGF have also been described in other neuronal types and following different types of injury, such as ischemia, epileptic stages, or neurological diseases (Carmeliet and Storkebaum, 2002; Lange et al., 2016; Matsuzaki et al., 2001; Nicoletti et al., 2008; Storkebaum et al., 2004).

VEGF is also known as VEGF-A and is the prototypical member of a related group of trophic factors, that also includes VEGF-B. VEGF-B shares a high degree of homology with VEGF, but, in contrast to VEGF, has low angiogenic activity and is not pro-inflammatory (Ruiz de

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Almodovar et al., 2009). The role of VEGF-B remains enigmatic, but recent evidence points to a direct neuroprotective role in degenerating motoneurons, with the advantage, as compared to VEGF, of not producing vascular side effects, such as inflammation and tissue edema (Manoonkitiwongsa et al., 2004; Poesen et al., 2008).

In addition to the survival-promoting effects of VEGF and VEGF-B, behavioral experiments have described motor improvement in different models of motoneuron degeneration (Azzouz et al., 2004; Poesen et al., 2008; Storkebaum et al., 2005; Tovar-y-Romo et al., 2007). However, there are currently no physiological studies regarding the functional state of those neurons that have been rescued from cell death. We have pursued this question using the chronic alert cat preparation to record the discharge activity of motoneurons, an approach that allows the correlation of neuronal firing with motor performance, before and after the administration of the factor (VEGF or VEGF-B) to injured motoneurons. Abducens motoneurons offer several advantages: their discharge pattern is well characterized, and both their afferents and the signals they carry have been described in detail (Büttner-Ennever, 2006; Davis-López de Carrizosa et al., 2011; Delgado-García et al., 1986a; Escudero et al., 1992). In addition to the physiological study, we have also carried out a confocal immunofluorescence analysis of the synaptic boutons impinging on injured and VEGF-treated motoneurons.

2. Materials and methods

2.1. Animals and surgical procedures

Experiments were performed on adult female cats weighing 2.0–2.5 kg that were obtained from authorized suppliers (Universidad de Córdoba, Spain). All procedures were performed in accordance with the guidelines of the European Union (2010/63/EU) and Spanish legislation (R.D. 53/2013, BOE 34/11370–421) for the use and care of laboratory animals, and were approved by the ethics committee. All efforts were made to reduce the number of animals used.

A total of 12 animals was used for the present study. Six animals were prepared for extracellular single-unit recordings of abducens motoneurons (Fig. 1A) and processed following one of two different protocols (Fig. 1B). First, in the immediate administration protocol (IAP), VEGF or VEGF-B treatment began immediately after the axotomy ($n = 2$ per treatment type). Second, in the delayed administration protocol (DAP), the onset of VEGF administration took place 20 days after axotomy ($n = 2$). We also used six additional animals for the morphological study, two animals for each of the groups: control, axotomy (treated only with vehicle), and VEGF-treated animals (according to the delayed administration protocol). None of the six cats used for morphology derived from the electrophysiological experiments.

Animals were prepared for chronic recordings as previously described (Davis-López de Carrizosa et al., 2010). Briefly, after a protective injection of atropine sulfate (0.5 mg/kg, i.m.) to reduce vagal reflexes, animals were anesthetized with ketamine hydrochloride (20 mg/kg, i.m.) mixed with xylazine (0.5 mg/kg, i.m.) and placed in a stereotaxic frame. Surgery was then performed under sterile conditions to implant stimulating electrodes, scleral coils, and the recording chamber (Fig. 1A). Three bipolar stimulating electrodes were implanted intracranially, in the left (Fig. 1A, St. 1) and right VIth nerves (at their exit from the brainstem) and in the right medial longitudinal fasciculus (MLF) just caudal to the oculomotor nucleus (Fig. 1A, St. 2). These were used to antidromically activate abducens motoneurons and internuclear neurons, respectively. Recordings were performed in the left abducens nucleus (Fig. 1A, Rec.). Coils, made up of two turns of Teflon-insulated stainless-steel wire, were implanted in the sclera of both eyes for the recording of eye movements. A square window (5 × 5 mm) was drilled in the occipital bone to allow transcerebellar access to the brainstem for recordings. A restraining system was also constructed to immobilize the head during the recordings. Postoperative care was provided daily, as needed.

2.2. Chronic extracellular recordings

After 10 days of postoperative recovery, recording sessions started. The animal was gently restrained with elastic bandages and placed in a Perspex box, which was located inside the magnetic field for eye movement recordings (Fuchs and Robinson, 1966). Extracellular recordings were carried out with glass micropipettes, filled with 2 M sodium chloride, attached to a three-axis micromanipulator and advanced through the intact cerebellum to reach the brainstem. The abducens nucleus was identified by the antidromic field potential recorded after electrically stimulating the ipsilateral VIth nerve, which produced the activation of the motoneurons (Fig. 1C; VIn), in some cases aided by the

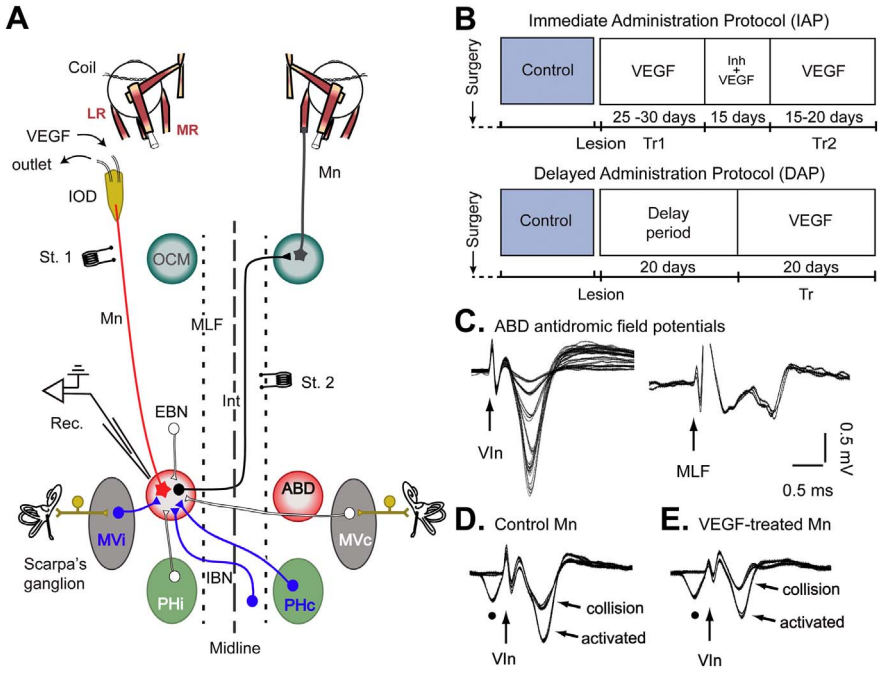

Fig. 1. Experimental design, administration protocols and cell identification. (A) Diagram illustrating the recordings (Rec.) of abducens (ABD) motoneurons (Mn) identified by their antidromic activation from the nerve electrode (St.1). ABD Mn were axotomized and the proximal nerve stump inserted into the intraorbital device (IOD) for VEGF (or VEGF-B) delivery. The major afferents to ABD are illustrated: PH, prepositus hypoglossi nucleus; EBN and IBN, excitatory and inhibitory reticular burst neurons; MV, medial vestibular nucleus. The subscripts i and c indicate ipsilateral or contralateral projection, respectively. White and blue neurons are excitatory and inhibitory afferents, respectively. Other abbreviations: Int, ABD internuclear neurons; LR and MR, lateral and medial rectus muscle; MLF, medial longitudinal fascicle; OCM, oculomotor nucleus. (B) Protocols used for VEGF administration. The time line indicates the different steps carried out for the immediate administration protocol (IAP) or the delayed administration protocol (DAP). Inh, the inhibitor of VEGFR-2, SU1489; Tr, treatment. (C) Antidromic ABD field potentials induced by stimulating the VIth nerve (VIn; depth profile) or the MLF. Arrows point to the stimulus artefact. (D, E) Collision tests used for the identification of ABD Mn in the control (D) and experimental (E) condition. The dots in (D) and (E) point to the orthodromic spontaneous spike. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)