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IGF-1 and BDNF promote chick bulbospinal neurite outgrowth in vitro

Rishard Salie *, John D. Steeves

ICORD (International Collaboration on Repair Discoveries), The University of British Columbia, Vancouver, BC, Canada

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Abstract

Injured neurons in the CNS do not experience significant functional regeneration and so spinal cord insult often results in permanently compromised locomotor ability. The capability of a severed axon to re-grow is thought to depend on numerous factors, one of which is the decreased availability of neurotrophic factors. Application of trophic factors to axotomized neurons has been shown to enhance survival and neurite outgrowth. Although brainstem–spinal connections play a pivotal role in motor dysfunction after spinal cord injury, relatively little is known about the trophic sensitivity of these populations. This study explores the response of bulbospinal populations to various trophic factors. Several growth factors were initially examined for potential trophic effects on the projection neurons of the brainstem. Brain derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF-1) significantly enhance mean process length in both the vestibulospinal neurons and spinal projection neurons from the raphe nuclei. Nerve growth factor (NGF), neurotrophin-4 (NT-4) and glial derived neurotrophic factor (GDNF) did not effect process outgrowth in vestibulospinal neurons. At the developmental stages used in this study, it was determined that receptors for BDNF and IGF-1 were present both on bulbospinal neurons and on surrounding cells with a non-neuronal morphology.
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Keywords: Brainstem; Chick; BDNF; IGF-1; Axonal elongation

While the majority of tissues in the human body are capable of significant repair when damaged, it was long believed that the vertebrate central nervous system (CNS) was completely incapable of regeneration following injury (Cajal, 1928; Feigin et al., 1951; Windle, 1956; Bjorklund et al., 1971; Kwon et al., 2002; Ramer et al., 2005). This observation is counterintuitive, as the vertebrate peripheral nervous system is capable of reinnervating target tissues unassisted following axonal injury (Cajal, 1928). Indeed, although CNS regeneration is not observed in mammals or even in the majority of adult vertebrates, it is not foreign to all organisms. Salamanders are capable of recovering locomotor function after spinal cord transection in the adult organism (Butler and Ward, 1967; Simpson, 1983; Davis et al., 1990), and axonal regeneration in an embryonic environment has also been demonstrated in higher organisms. Chick spinal cord transected prior to the onset of myelination shows significant functional regeneration (Keirstead et al., 1992; Hasan et al., 1993). Furthermore, complete crush or transection injuries to the spinal cord of the marsupial opossum (*Monodelphis domestica*) performed before postnatal day 7 are followed by a high level of functional recovery (Saunders et al., 1998).

In adult mammals, neurons exhibit signs of terminal sprouting upon injury (Bjorklund et al., 1971), however this outgrowth is transient. Following this abortive sprouting, axotomized CNS neurons undergo degeneration of the distal axon, retraction of the proximal axon and atrophy of the soma (Fishman and Mattu, 1993; Kobayashi et al., 1997). Regenerative failure in the vertebrate CNS is likely due to a combination of various obstacles (reviewed in Kwon et al., 2002; Ramer et al., 2005), one of these being changes in the CNS environment as the organism matures. Neurons

* Corresponding author. Present address: Labor 2.81, Dept. of Zellbiologie, Biozentrum, University of Basel, CH-4056 Basel, Switzerland. Tel.: +41 61 267 2064; fax: +41 61 267 2078.

E-mail address: rishard.salie@unibas.ch (R. Salie).

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attempting to regenerate through the adult CNS face an environment rich in neurite outgrowth inhibitors including Nogo-A (Chen et al., 2000; Li et al., 2005), MAG (Tang et al., 1997; Li et al., 2004) and members of the proteoglycan family (Asher et al., 2000; Wilson and Snow, 2000; Borisoff et al., 2003). In addition, the adult CNS lacks appropriate trophic support both to prevent neuronal atrophy and to promote axon growth.

The necessity for appropriate trophic support during embryonic neurodevelopment is well known. Neurotrophins have been found to impact on neuronal survival (Piontek et al., 1999; Gibbons et al., 2005), and atrophy (Kobayashi et al., 1997; Ruitenberg et al., 2004) as well as increasing neurite outgrowth (Ebadi et al., 1997; Ye and Houle, 1997; Davies, 2000; Lu et al., 2005). Other trophic factors such as IGF-1, CNTF and FGF-2 have also been found to promote axonal regeneration in various neuronal populations (Hansson et al., 1986; Nachemson et al., 1990; Houle and Ye, 1997; Pataky et al., 2000). The expression of these trophic factors and their receptors are highly regulated both spatially and temporally. It is for this reason that different neuronal populations may have varied requirements of trophic factors to encourage an optimal growth response.

The evidence for trophic factor involvement in neuronal survival and regenerative behaviour (i.e. sprouting, axon elongation) suggests that trophic factor replacement therapy may, in the future, prove to be part of an effective, multifaceted therapy for SCI. Many of the upper motor neuron populations involved in locomotor control, such as the vestibular, raphe and red nuclei as well as the reticular formation lie within the brainstem (Valenzuela et al., 1990). Presently, however, little is known about the trophic requirements of these bulbospinal neuron populations partly due to a lack of specific molecular markers. Raphespinal neurons are primarily serotonergic, however not all neurons from the various raphe nuclei project to the spinal cord, making identification of the neurons by staining for serotonin somewhat ambiguous. Other neuronal groups of interest such as the vestibular and reticular neurons are presently indistinguishable from other neurons in the brainstem. To allow study of these projection neurons a novel, in vivo cell labelling method was devised in our laboratory (Pataky et al., 2000). Using this powerful labelling technique along with traditional explant culture we have begun to examine these requirements in an effort to identify factors that improve axonal elongation in bulbospinal neuron populations. In this study, various trophic factors were screened for effectiveness in increasing neurite outgrowth of DiI labelled vestibulospinal neurons from cultured explants. We assayed six factors: NGF, BDNF, NT-3, NT-4, IGF-1 and GDNF for positive effects on neurite outgrowth. Selection of each of these factors was based on two criteria. The first was the ability to stimulate the MAP kinase pathway, believed to influence axonal elongation. The second was that receptors for the chosen factor are expressed in the brainstem. Brain derived neurotrophic factor (BDNF) and insulin-like growth factor 1 (IGF-1), were further examined for effects on raphespinal neurons, dose-dependency and presence of receptors on cell bodies and axons.

1. Experimental procedures

1.1. In ovo retrograde tracing

Fertilized White Leghorn Chicken eggs (*Gallus domesticus*) were purchased from University of Alberta, Poultry Research Facility and incubated in a forced-draft incubator (38 °C, saturating humidity). All animals were staged according to the method of Hamburger and Hamilton (Hamburger and Hamilton, 1951). In ovo retrograde tracing of descending axons was performed as in (Pataky et al., 2000). Briefly, on embryonic day 4.5 (E4.5, stages 25–26), a circular hole was cut in the top of the egg to provided access to the embryo inside. A small hole in the air sac membrane was made with watchmakers tweezers. The amniotic membrane was peeled away from the dorsal side of the embryo, between the cervical and thoracic levels. The tip of an insect pin was coated with crystals of DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, Molecular Probes). The pin was inserted at mid-cervical level between the spinal cord and developing vertebrae, making contact with the ventral edge of the cord. The crystal was held in place with the watchmakers tweezers and the pin removed. The eggs were sealed and returned to the incubator until E8 allowing for the retrograde transport of the DiI to the cell bodies of bulbospinal neurons.

1.2. Dissection and cryosectioning

In a sterile field, the embryos (E8) were removed from the eggs into Hanks balanced saline solution (HBSS, 4 °C, 0.5% glucose, $Mg^{2+}$and Ca^{2+} free, Gibco-BRL). The embryos were decapitated, and the brainstem dissected out using fine forceps. The meningial layer and associated vasculature were carefully removed from the brainstems. The brainstems were immersion fixed in 4% paraformaldehyde (BDH Chemicals, Toronto) in 0.1 M PBS from 4 to 12 h and briefly rinsed in 0.1 M PBS. The brainstems were cryoprotected in 30% sucrose (EM Science), embedded in OCT compound and frozen over liquid nitrogen. Tissue was sectioned on a Zeiss Microm cryostat at −20 °C (10–20 μm sections), and mounted on warmed (50 °C) Superfrost Plus slides (Fisher Scientific). Sections were photographed using a Zeiss epifluorescence microscope attached to a SPOT digital camera (Diagnostic Instruments, Inc.). Images were processed using SPOT software.

1.3. Explant culture

Three hundred micrometer thick vestibular and raphe explants were prepared as in Pataky et al., 2000. Explants were initially grown for 14–16 h in Dubecco's Modified

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