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# NEUROPROTECTIVE EFFECTS OF NGF, BDNF, NT-3 AND GDNF ON AXOTOMIZED EXTRAOCULAR MOTONEURONS IN NEONATAL RATS

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**Abstract—Neurotrophic factors delivered from target muscles are essential for motoneuronal survival, mainly during development and early postnatal maturation. It has been shown that the disconnection between motoneurons and their innervated muscle by means of axotomy produces a vast neuronal death in neonatal animals. In the present work, we have evaluated the effects of different neurotrophic factors on motoneuronal survival after neonatal axotomy, using as a model the motoneurons innervating the extraocular eye muscles. With this purpose, neonatal rats were monocularly enucleated at the day of birth (postnatal day 0) and different neurotrophic treatments (NGF, BDNF, NT-3, GDNF and the mixture of BDNF + GDNF) were applied intraorbitally by means of a Gelfoam implant (a single dose of 5 μg of each factor). We first demonstrated that extraocular eye muscles of neonatal rats expressed these neurotrophic factors and therefore constituted a natural source of retrograde delivery for their innervating motoneurons. By histological and immunocytochemical methods we determined that all treatments significantly rescued extraocular motoneurons from axotomy-induced cell death. For the dose used, NGF and GDNF were the most potent survival factors for these motoneurons, followed by BDNF and lastly by NT-3. The simultaneous administration of BDNF and GDNF did not increase the survival-promoting effects above those obtained by GDNF alone. Interestingly, the rescue effects of all neurotrophic treatments persisted even 30 days after lesion. The administration of these neurotrophic factors, with the exception of NT-3, also prevented the loss of the cholinergic phenotype observed by 10 days after axotomy. At the dosage applied, NGF and GDNF were revealed again as the most effective neuroprotective agents against the axotomy-induced decrease in ChAT. Two remarkable findings highlighted in the present work that contrasted with other motoneuronal types after neonatal axotomy: first, the extremely high efficacy of NGF as a neuroprotective agent and, second, the long-lasting effects of neurotrophic administration on cell survival and ChAT expression in extraocular motoneurons.** 

**Key words: neurotrophic factor, ChAT down-regulation, oculomotor, abducens, lesion-induced cell death, postnatal development.**

## INTRODUCTION

As occurs with central nervous system (CNS) neurons, motoneurons are critically dependent on neurotrophic factors derived from their target muscle for survival and for the maintenance of their morphophysiological, synaptic and molecular characteristics (Levi-Montalcini, 1982; Purves, 1986; Lewin and Barde, 1996; Oppenheim, 1996; Gould and Enomoto, 2009). During development, any experimental manipulation that interrupts the flow of these neurotrophic factors from target muscle to motoneurons, such as axotomy, leads to the massive death of the affected motoneuronal pool. As motoneurons mature, they lose dependence on target-derived factors for survival. Nevertheless, axotomized adult motoneurons exhibit numerous alterations in their electrophysiological properties, synaptic inputs and protein expression which are restored when they are allowed to reinnervate their original muscle (Titmus and Faber, 1990; de la Cruz et al., 1996; Navarro et al., 2007). The exogenous administration of different neurotrophic factors to motoneurons has been shown to prevent, in variable degrees, the axotomy-induced cell death in neonates (Sendtner et al., 1992; Yan et al., 1992; Koliatsos et al., 1993; Clatterbuck et al., 1994; Vejsada et al., 1995; Yuan et al., 2000), and the alteration in multiple functional, structural and biochemical properties in the adult (Davis-López de Carrizosa et al., 2009, 2010; Gordon et al., 2003; Terenghi, 1999).

Neurotrophins were the first neurotrophic molecules characterized. They comprise the nerve growth factor (NGF; Levi-Montalcini, 1982), brain-derived neurotrophic factor (BDNF; Leibrock et al., 1989), neurotrophin-3 (NT-3; Jones and Reichardt, 1990) and neurotrophin-4/5 (NT-4/5; Ip et al., 1992). Neurotrophins bind to the tyrosine kinase receptors trkA (NGF), trkB (BDNF and NT-4/5) and trkC (NT-3) (Barbacid, 1994; Chao, 2003). Spinal and cranial motoneurons in the adult rat have

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*Abbreviations:* ALS, amyotrophic lateral sclerosis; ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; ChAT, choline acetyltransferase; CNS, central nervous system; DAB, 3,3′-diaminobenzidine tetrahydrochloride; GDNF, glial cell-line-derived neurotrophic factor; HRP, horseradish peroxidase; NGF, nerve growth factor; NRS, normal rabbit serum; NT-3, neurotrophin-3; NT-4/5, neurotrophin-4/5; P, postnatal day; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline with 0.1% Triton X-100; SEM, standard error of the mean.

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been shown to express the trkB and trkC receptors, but they lack the trkA receptor (Henderson et al., 1993; Koliatsos et al., 1991, 1993; Merlio et al., 1992; Piehl et al., 1994). In contrast, extraocular motoneurons express the three types of trk receptors in very high percentages, both in cats (Benítez-Temiño et al., 2004) and rats (Morcuende et al., 2011). In addition to neurotrophins, other neurotrophic factors have also been demonstrated to act on different types of motoneurons with variable degrees of effectiveness. Among these, glial cell-line-derived neurotrophic factor (GDNF) has been identified as the most potent survival factor for motoneurons (Henderson et al., 1994; Li et al., 1995; Oppenheim et al., 1995; Zhao et al., 2004), especially in neonatal models (Oppenheim et al., 1995; Yan et al., 1995).

It is well known that, after axotomy, motoneurons experience a transient down-regulation in the expression of choline acetyltransferase (ChAT; the enzyme of synthesis of acetylcholine), as part of a more general modification in the pattern of expression of specific molecules. Due to these molecular changes, axotomized motoneurons have been suggested to switch their phenotype from a neurotransmissive role to a regenerative mode (Navarro et al., 2007). In several models, the lesion-induced decrease in ChAT has been proven to be prevented by the exogenous application of neurotrophic factors to the damaged axons (Tuszynski et al., 1996; Wang et al., 1997; Fernandes et al., 1998).

Extraocular motoneurons depend on their target muscles for survival during postnatal development, but they lose this dependence with maturation, as has been described in rats (Morcuende et al., 2005) and cats (Pásaro et al., 1985). Although adult extraocular motoneurons survive axotomy, they suffer severe changes in morphology and electrophysiology, like synaptic stripping and decreased firing rate (Davis-López de Carrizosa et al., 2009, 2010). We have recently reported that the discharge and synaptic alterations induced by axotomy in cat abducens motoneurons recover, in a complementary way, by the exogenous administration of BDNF and NT-3 (Davis-López de Carrizosa et al., 2009). Basically, BDNF restores the tonic component of firing while NT-3 re-establishes the phasic discharge. Axotomized abducens motoneurons also respond to the administration of NGF by regulating their discharge pattern and synaptic composition (Davis-López de Carrizosa et al., 2010).

Interestingly, motoneurons of the oculomotor system are more resistant to neurodegeneration in amyotrophic lateral sclerosis (ALS) disease, when compared to other cranial (for example, facial, trigeminal or hypoglossal) or spinal motoneurons (Ohki et al., 1994; Haenggeli and Kato, 2002). The resistance of extraocular motoneurons to neuromuscular diseases has been related to differences in the expression levels of proteins that presumably would protect motoneurons from degeneration, like a higher level of parvalbumin (Reiner et al., 1995; Obál et al., 2006) or lack of calcitonin gene-related peptide expression (Ringer et al., 2012). However, there are also differences regarding neurotrophic dependence between extraocular motoneurons and other motoneuronal types, which could play an important role mediating the lesser vulnerability of these motoneurons not only in ALS, but also after injury (Gould and Oppenheim, 2011).

Consequently, we were interested in uncovering the degree of dependence of extraocular motoneurons on different neurotrophic factors. With this purpose, newborn rats were monocularly enucleated as a procedure to axotomize extraocular motoneurons, and different neurotrophic factors applied to the orbit immediately after lesion. Survival of extraocular motoneurons and ChAT expression were evaluated at short and long time periods after lesion.

## EXPERIMENTAL PROCEDURES

### Animals and surgical procedures

Wistar rats obtained from an authorized supplier (University of Seville) were used for this study. All experimental procedures were performed in accordance with the guidelines of the European Union (2010/63/EU) and the Spanish legislation (R.D. 53/2013) for the use and care of laboratory animals and approved by the local committee for research. All efforts were made to minimize the number of animals used and their suffering in this study.

Neonatal rats at postnatal day 0 (P0, between 6 and 24 h after birth; $n = 130$) were anesthetized by halothane inhalation and monocularly enucleated as a method to axotomize extraocular motoneurons, leaving them also deprived of target muscles. After an incision made in the junction between both eyelids, the right eye was extirpated and all intraorbital tissues removed (Morcuende et al., 2005). The orbit was then filled with a Gelfoam implant soaked in either sterile saline as a control or NGF, BDNF, NT-3, GDNF or a mixture of BDNF and GDNF as a procedure to deliver the different factors. We applied a dose of 5 μg of each factor, which was similar to that used in previous studies of motoneuronal lesion in neonatal animals (Oppenheim et al., 1995; Sendtner et al., 1992; Vejsada et al., 1995, 1998; Yan et al., 1992, 1993, 1995; Yuan et al., 2000). The eyelid edges were re-opposed and sealed with a cyanoacrylic-based veterinary glue (Vetbond, 3 M, St. Paul, MN, USA) and animals returned to their cages in a procedure that lasted less than 1 h. To analyze the fate of extraocular motoneurons in response to several neurotrophin treatments, each experimental group comprised between five and seven animals. Animals were randomly assigned to a survival time of either 10 (P10) or 30 (P30) days and then deeply anesthetized with sodium pentobarbital (50 mg/kg; i.p.) and transcardially perfused with 100 ml of physiological saline followed by 250 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The brainstem was removed, postfixed for 2 h in the same fixative, and cryoprotected by immersion in a solution of 30% sucrose in 0.1 M sodium phosphate buffer. The tissue