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Effects of different neurotrophic factors on the survival of retinal ganglion cells after a complete intraorbital nerve crush injury: A quantitative *in vivo* study

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ABSTRACT

We examined in adult Sprague Dawley rats the loss of retinal ganglion cells (RGCs) induced by complete intraorbital optic nerve crush (IONC) as well as the effects of several neurotrophic factors to prevent IONC-induced RGC loss. Completeness of the IONC lesion was assessed by investigating the orthograde and retrograde transport of neuronal tracers applied to the origin and termination of the retinotectal pathway. RGC survival after IONC alone or combined with intraocular injection of the neurotrophic factors NT-4, BDNF or CNTF was quantified at survival intervals ranging from 5 to 12 days post-lesion (dpl) by identifying RGCs that had been pre-labelled with fluorogold (FG). RGC loss first appeared at 7 dpl and by 12 dpl only 32% of the RGC population remained in the retina. Intraocular administration of NT-4, BDNF or CNTF resulted in almost a complete protection against IONC-induced RGC loss by 7 dpl, and the protection remained significant by 12 dpl only for NT-4 and BDNF. We have analyzed these results taking into account our previous studies on the loss of RGCs induced by intraorbital optic nerve transection (IONT) and concluded that RGC loss induced by IONC is slower and less severe than that following IONT. Moreover, as for IONT-induced RGC loss, IONC-induced RGC loss may also be prevented with administration of NT-4, BDNF or CNTF, though for NT-4 and CNTF their neuroprotective effects differ depending on the injury type. Overall this data underscore the importance of the type of ON injury on the pattern of RGC degeneration as well as in their response to neuroprotective treatments.

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1. Introduction

Axonal injury to mammalian central nervous system (CNS) neurons results in a loss of function and in retrograde axonal degeneration that may lead to the death of the parent injured neurons. For instance, in the rodent primary visual pathway, a frequently used model to study the effects of axonal injury is to transect the optic nerve (Ramón y Cajal, 1914; Leoz y Arcuate, 1914; Vidal-Sanz et al., 1987; Villegas-Perez et al., 1988, 1993), whereas in other areas of the CNS, such as the spinal cord, the crush injury is a more frequent type of lesion used to study the events associated to neuronal degeneration (David and Lacroix, 2003; Kerr et al., 2008, for review see Silver (2008))

Intraorbital optic nerve transection (IONT) results in the loss of the injured neurons, the retinal ganglion cells (RGCs) (Grafstein and Ingoglia, 1982; Misantone et al., 1984; Villegas-Perez et al., 1993) and within the following two weeks post-lesion, approximately 80% of the RGC population is lost (Villegas-Perez et al., 1993; Mansour-Robaey et al., 1994; Peinado-Ramon et al., 1996; Vidal-Sanz et al., 2000). Moreover, this model has allowed quantitative studies on the capacity of axotomized RGCs for axonal regeneration by several independent laboratories with comparable findings (Vidal-Sanz et al., 1987, 1988; Thanos and Vanselow, 1989; Cho and So, 1989; Vidal-Sanz et al., 1991; Watanabe et al., 1991; Bahr et al., 1992; Robinson, 1994; Sasaki et al., 1996; Aviles-Trigueros et al., 2000). The available data concerning RGC degeneration, cell survival and axonal regeneration after optic nerve crush is inconsistent however, and differs among reports. This is mainly due to the variability of the methodology used to crush the nerve [e.g., inflatable balloon (Burke et al., 1986); watchmaker forceps (Berkelaar et al., 1994; Chierzi et al., 1999) microcrush (Selles-Navarro et al., 2001) or dynamometer (Klocker et al., 2001)], the distance from the eye where the lesion is inflicted (Berkelaar et al., 1994)

Abbreviations: IONC, intraorbital optic nerve crush; IONT, intraorbital optic nerve transection; SCi, superior colliculi; FG, fluorogold; RGCs, retinal ganglion cells; BDNF, brain derived neurotrophic factor; NT-4, neurotrophin 4; CNTF, ciliary neurotrophic factor; SD, standard deviation.

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and the quantity of axons affected, that may range from a partial lesion of the nerve (Yoles and Schwartz, 1998) to a more severe and complete lesion (Burke et al., 1986).

Under experimental conditions involving injury to the entire retinal output, two important variables influencing the severity and the speed of axotomy-induced RGC loss are the distance from the optic nerve head at which the injury is performed and the type of injury, whether axotomy is performed by optic nerve crush or transection (Villegas-Perez et al., 1993; Berkelaar et al., 1994). The importance of one or another type of injury to the retinofugal pathway, i.e. transection versus crush, has been underscored in a recent study of the retinal transcriptome profile comparing mRNA expression from IONC- and IONT-retinas to naïve-retinas using Affymetrix RAE230.2 arrays (Agudo et al., 2008). This study revealed that out of the 3219 sequences regulated following IONT and the 1996 regulated following IONC, only 1078 were commonly regulated by both injuries (Agudo et al., 2008), thus stressing the importance of the type of lesion on the retinal response elicited. Because little is known about the pattern and temporal course of RGC degeneration induced by complete ON-crush injury, in the present studies we have crushed the optic nerve at approximately 3 mm of distance from the eye and determined the magnitude and temporal course of RGC loss.

Administration of neurotrophins, i.e. NT-4, BDNF and CNTF, has been shown to prevent RGC death induced by ON transection (Mey and Thanos, 1993; Mansour-Robaey et al., 1994; Peinado-Ramon et al., 1996; Di Polo et al., 1998; Chen and Weber, 2002). There is less information however, regarding their effects to prevent RGC loss induced by ON crush. In young rats, BDNF or CNTF administration did not result in increased RGC survival after ON crush (Weibel et al., 1995). However, CNTF administration to adult ON-crushed retinas alone (Leaver et al., 2006b) or in combination with either anti-Nogo neutralizing antibody (Cui et al., 2004) or the anti-apoptotic protein bcl-2 (Leaver et al., 2006a) has been shown to increase the survival of RGCs (Leaver et al., 2006a,b) and to induce the regeneration of the injured axons (Cui et al., 2004; Harvey et al., 2006; Leaver et al., 2006a,b). As for NT-4 there are no studies analyzing its effects on RGC survival after this type of axonal lesion. Thus, it was of interest to find out whether the administration of the neurotrophins NT-4, BDNF or of the neurotrophic factor CNTF could prevent or diminish the RGC degeneration associated to IONC.

Here we show that axonal injury inflicted by IONC triggers the death of RGCs which is first significant at 7 days post-lesion (dpl), progresses with time and by 12 dpl only 32% of the RGCs survive. In IONC-injured retinas treated with a single dose of NT-4 or BDNF there are significantly more RGCs than in vehicle treated ones at 7 and 12 days post-lesion. CNTF neuroprotection however, only lasts until 7 dpl decreasing at 12 pl to the values of vehicle-injected retinas. The results of our present study have been compared to previous data from this laboratory (Peinado-Ramon et al., 1996; Vidal-Sanz et al., 2000) on the survival of RGCs after intraorbital optic nerve transection (IONT) with or without administration of neurotrophins. These results are discussed in relation with recent observations from this laboratory about the molecular correlates of these two types of optic nerve injuries (Agudo et al., 2008, 2009).

2. Material and methods

Animal handling and surgery. Sprague Dawley female rats (180–220 g body weight) were obtained from the University of Murcia breeding colony. For anaesthesia, a mixture of xylazine (10 mg/kg body weight; Rompun^{R}; Bayer, Kiel, Germany) and ketamine (60 mg/kg body weight; Ketolar^{R}; Pfizer, Alcobendas, Madrid, Spain) was used. All experimental procedures were carried out in accordance with European Union regulations, our institutional guidelines and the Association for Research in Vision and Ophthalmology guidelines for the use of animals in research. Animals were divided into a control group, which did not undergo any experimental manipulation and several experimental groups according to the analysis performed (see below).

Intraorbital nerve crush (IONC) injury. All experimental animals underwent surgery in the left eye following previously described methods that are standard in our Laboratory (Vidal-Sanz et al., 1987, 1988; Agudo et al., 2008, 2009). In brief, to perform IONC an incision was made in the superior orbital rim, the superoexternal orbital contents were dissected, and the superior and external rectus muscles were removed, then the optic nerve (ON) was crushed during 10 s at 3 mm from the optic disc using watchmaker's forceps. Before and after the procedure, the eye fundus was observed through the operating microscope to assess the integrity of the retinal blood flow. To assess for the effect of IONC on RGC survival ($n = 41$), one week before carrying out the injury, RGCs were retrogradely labelled with fluorogold (see below). Animals were sacrificed at increasing survival intervals after IONC (5, 7, 9 or 12 days), the retinas processed and analyzed. The untouched right eye was used as control.

Validation of the IONC technique. To investigate whether our intraorbital optic nerve crush injury resulted in a complete lesion to the retinofugal projection, the retinotectal retrograde and orthograde axonal transport was analyzed after injury. The idea of these two sets of experiments was to demonstrate unequivocally that our IONC technique resulted in the lesion of the entire retinofugal projection. Moreover, that there was no functional recovery even long time after the lesion. A partial crush may result in an initial lack of axonal transport within the early time periods after injury, but this may be reinstituted some time after when the initial inflammatory component of the lesion is diminished thus allowing surviving axons to recover their axonal transport.

Orthograde transport. The persistence of viable axons after injury was investigated by analysing their capacity for orthograde transport of the β subunit of the cholera toxin (CTB) following standard procedures in our Laboratory (Whiteley et al., 1998; Aviles-Trigueros et al., 2000, 2003; Mayor-Torroglosa et al., 2005). In brief, a single intravitreal injection (5 μl per eye) of CTB (CTB, List biological Laboratories, Campbell, USA) diluted at 1% in saline, was injected in the left eye. CTB, when injected intravitreally, is taken up by RGC somas and transported along their axons towards their principal retinorecipient target region in the brain, the superior colliculi (SCi) (Angelucci et al., 1996). Two groups were prepared, a naïve control ($n = 6$) and an experimental IONC ($n = 6$). In the latter, the left optic nerve was crushed one week before the injection of CTB. Four days after CTB administration, animals were sacrificed, perfused (see below) and their brains dissected. Brains were soaked in 30% sucrose for 48 h. Tissue was embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA), frozen on liquid nitrogen, and kept at −70 °C. Serial cryostat coronal sections of the mid-brain (40 μm) spanning the entire SCi were collected and the presence of CTB was detected as previously described (Whiteley et al., 1998; Aviles-Trigueros et al., 2000, 2003; Mayor-Torroglosa et al., 2005).

Retrograde transport. To investigate the possibility that our IONC injury spared some RGC axons, we have also examined the retrograde transport of Fluorogold (FG). FG was applied to both SCi following procedures that are standard in our Laboratory (see below) (Vidal-Sanz et al., 2001; Salinas-Navarro et al., 2009, in press). Briefly, animals were anesthetized, each mid-brain was exposed and, after removing the piamater overlying the SCi, a small piece of gelatine sponge (Spongostan Film, Ferronsan, Denmark) soaked in FG, diluted at 3% in 10% dimethyl sulfoxide-saline (DMSO,

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