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RETINAL GANGLION CELL NEUROTROPHIN RECEPTOR LEVELS AND TROPHIC REQUIREMENTS FOLLOWING TARGET ABLATION IN THE NEONATAL RAT

K. L. SPALDING,[1]* Q. CUI AND A. R. HARVEY

School of Anatomy and Human Biology, The University of Western Australia, Crawley, Western Australia 6009, Australia

Abstract—Superior colliculus (SC) ablation in neonatal rats results in a rapid increase in retinal ganglion cell (RGC) death. This injury-induced death is reduced by exogenous brain-derived neurotrophic factor or neurotrophin-4/5 (NT-4/5), but the protective effect of these molecules is transient, delaying but not preventing neuronal loss. We sought to discover why neurotrophins only temporarily reduce RGC death after target ablation, focusing on changes in neurotrophin receptor expression and possible changes in growth factor dependency. In unlesioned rats, receptor tyrosine kinase B (trkB) immunohistochemistry revealed no change in the number of trkB positive cells in the RGC layer 24 h after intraocular NT-4/5 injection. However, after SC lesions there were significantly less immunoreactive cells and, surprisingly, even fewer immunoreactive cells in NT-4/5 injected eyes. Semi-quantitative confocal analysis of immunofluorescence intensity revealed an increase in trkB staining in the RGC layer in unlesioned rats 24 h after NT-4/5 injection, whereas in SC-lesioned animals exposed to NT-4/5 there was a significant decrease in staining. To determine whether injured neonatal RGCs can switch their trophic requirements, different doses of ciliary neurotrophic factor were given intraocularly, either alone or combined with NT-4/5. We also tested an SC-derived chondroitin sulfate proteoglycan that has been reported to promote neonatal RGC survival. None of these interventions reduced lesion-induced RGC death 24 or 36 h after SC ablation. In summary, we show that developing RGCs do not shift their trophic dependence to other survival factors following injury; rather, the application of neurotrophins causes a down-regulation of the cognate trkB receptor, presumably altering the long-term responsiveness of neonatal RGCs to exogenous neurotrophins. These data highlight the difficulty in promoting long-term neuronal survival when using one-off administration of recombinant growth factors.
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Developing retinal ganglion cells (RGCs) are believed to compete for limited amounts of trophic factors derived from central target structures; those that receive adequate trophic support survive whereas those that do not undergo programmed cell death (PCD; Carpenter et al., 1986; Sefton et al., 1987; Oppenheim, 1991; Clarke et al., 1998; Ma et al., 1998). Consistent with this proposal, target removal in neonatal rats results in a large increase in RGC death. Six hours following superior colliculus (SC) ablation in the postnatal day 4 (P4) rat, RGC death is already twice normal PCD levels, and by 24 h post-lesion (PL) RGC death has increased about 10-fold (Harvey and Robertson, 1992; Harvey et al., 1994; Cui and Harvey, 1995). PCD and injury-induced RGC death are decreased by exogenous application of brain-derived neurotrophic factor (BDNF) or neurotrophin-4/5 (NT-4/5), applied locally to the cell body (Cui and Harvey, 1994, 1995) or distally to RGC terminals/axons (Ma et al., 1998; Spalding et al., 1998). Importantly however, the protective effect of exogenously applied neurotrophins on RGC survival is not permanent (Clarke et al., 1998; DiPolo et al., 1998), in the neonatal rat serving only to delay RGC death after SC ablation (Cui and Harvey, 1995).

Neurotrophins mediate many of their effects via receptor tyrosine kinases (trkA, trkB and trkC), BDNF and NT-4/5 signaling primarily through the trkB receptor (Huang and Reichardt, 2003). Consistent with their sensitivity to BDNF and NT-4/5, neonatal RGCs express trkB (Jelsma et al., 1993; Koide et al., 1995; Perez and Caminos, 1995; Ugolini et al., 1995; Vecino et al., 2002). Importantly it has been shown that exogenously applied BDNF can regulate trkB protein and mRNA levels, resulting in an attenuation of neurotrophin responsiveness (Frank et al., 1996, 1997). Given that intraocular injection of neurotrophins only temporarily rescues injured neonatal RGCs, in the first part of this study we examine whether there are changes in neonatal RGC trkB receptor levels after SC removal and following neurotrophin application.

There are postnatal changes in trkB expression, and maturing RGCs also become responsive to other types of trophic factor (Jelsma et al., 1993; Meyer-Franke et al., 1995). The cytokine ciliary neurotrophic factor (CNTF) is expressed in the rat retina and influences the differentiation, survival and axonal regeneration of distinct retinal cell populations (Meyer-Franke et al., 1995; Wen et al., 1995; Kirsch et al., 1997; Cui et al., 1999, 2003; Cui and Harvey, 2000; Ji et al., 2004). CNTF receptor α mRNA is expressed predominantly by neurons in the normal developing and mature brain and retina (MacLennan et al., 1996; Kirsch et

[1] Present address: Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institute, SE-171 77, Stockholm, Sweden.
*Correspondence to: K. Spalding, Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institute, SE-171 77, Stockholm, Sweden. Tel: +46-8-728-7464; fax: +46-8-32-4927.
E-mail address: kirspa@mbox.ki.se (K. Spalding).
Abbreviations: BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; DY, Diamidino Yellow; GCL, ganglion cell layer; NGS, normal goat serum; NT-4/5, neurotrophin-4/5; ON, optic nerve; P, postnatal day; PBS, phosphate-buffered saline; PCD, programmed cell death; PL, post-lesion; RGC, retinal ganglion cell; SC, superior colliculus; SCCP, superior colliculus chondroitin sulfate proteoglycan; trk, Trk receptor tyrosine kinases.

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al., 1997) and both CNTF (Wen et al., 1995; Chun et al., 2000) and its receptor (Ju et al., 2000) are up-regulated in adult rat retina after injury. To examine whether injured neonatal rat RGCs switch their trophic requirements from neurotrophins to other growth factors in the hours following SC ablation, different doses of CNTF were applied intraocularly at the time of SC removal. The survival and growth-promoting properties of neurotrophins and CNTF on young RGCs *in vitro* (Meyer-Franke et al., 1995) and *in vivo* (Loh et al., 2001) are additive, perhaps because these factors can act via different intracellular signaling pathways (Stahl and Yancopoulos, 1994; Ip and Yancopoulos, 1996; Alonzi et al., 2001; Huang and Reichardt, 2003). To test whether CNTF prolongs the survival-promoting effect of the neurotrophin NT-4/5, both factors were co-injected into eyes at the time of SC ablation and RGC death was assessed 24 or 36 h later.

A chondroitin sulfate proteoglycan (SCCP) derived from the neonatal rat SC has been reported to promote neonatal RGC survival *in vitro* and *in vivo* (Schulz et al., 1990; Huxlin et al., 1993, 1995a; Nichol et al., 1995). Postulated as being a target-derived factor, developing RGCs may also be supported by this molecule, perhaps explaining why exogenously applied neurotrophins only transiently sustain RGCs after target ablation. In a final series of studies, we therefore tested the ability of SCCP to reduce RGC death 24 h following SC removal.

EXPERIMENTAL PROCEDURES

Retrograde RGC labeling and SC lesions

The methods used to identify and count the number of normal and dying tectally projecting RGCs have been documented (Harvey and Robertson, 1992; Cui and Harvey, 1995). In brief, P2 Wistar rats (day of birth=P0) were anesthetized with ether and a bone flap made to expose the left SC. Using a glass micropipette, 0.1–0.2 μl of a 2% aqueous suspension of the nucleophilic retrograde fluorescent tracer Diamidino Yellow (DY; Sigma, St. Louis, MO, USA) was injected into the superficial layers of the left SC. Excess dye was removed, and after wound closure rats were warmed and returned to their mothers. At P4, rats were re-anesthetized with ether, the DY injection site was removed by gentle suction and gelfoam (UpJohn, Kalamazoo, MI, USA) was placed in the resultant cavity. Surgical procedures were approved by the UWA Animal Ethics Committee and conformed to NHMRC Guidelines. All efforts were made to minimize suffering and to keep the number of animals used in each experiment to a minimum, yet allowing statistical power for quantitative comparisons.

Intravitreal injections

At the time of the SC lesions the contralateral (right) eye was injected with various factors. Normal, non-lesioned rats also received intraocular injections at P4.

NT-4/5 injections for trkB experiments. TrkB receptor expression in the RGC layer of the P5 rat was assessed under four different conditions: (1) normal (no treatment), (2) 24 h following intravitreal injection of NT-4/5 (1 μl at 0.94 μg/μl; Genentech, San Francisco, CA, USA) in normal rats, (3) 24 h following SC ablation, and (4) 24 h following SC ablation and simultaneous intravitreal injection of NT-4/5 (1 μl at 0.94 μg/μl). All groups consisted of a minimum of three animals.

CNTF and NT-4/5. One microliter of CNTF (Peprotech, London, UK), NT-4/5 or a cocktail of CNTF and NT-4/5, was injected into the vitreous of the right eye. CNTF-only-treated animals consisted of three groups: one group of animals received an intravitreal injection of 50 ng CNTF (n=5), a second group received an intravitreal injection of 200 ng CNTF (n=3), and a third group received an intravitreal injection of 500 ng CNTF (n=7). All injections were given at the time of SC ablation and RGC death was assessed 24 h later. Five P4 rats received an intraocular injection of NT-4/5 (0.47 μg) at the time of SC ablation and were perfused 24 h later. An additional group of animals received an intravitreal injection of CNTF (50 ng) and NT-4/5 (0.47 μg) at the time of SC ablation and were perfused 24 (n=4) or 36 (n=7) h later. Sham-injected animals received an eye injection of saline at the time of SC ablation (n=10). Animals were perfused 24 h later.

SCCP. SCCP derived from neonatal rat SC (Schulz et al., 1990) was a kind gift from Dr. Alan Everett (Department of Physiology, The University of Western Australia). One microliter of SCCP (120 μg/μl in Tris-buffered saline, pH 7.4) was injected into the vitreous of the right eye of SC-lesioned P4 rats (n=6). This concentration has been reported to have the greatest effect on neonatal rat RGC survival *in situ* (Huxlin et al., 1995b). A further group of animals (n=13) received only an SC lesion and another group (n=10) received a sham injection of saline into the right eye (n=10). All animals were perfused on P5, 24 h after SC lesions.

Tissue processing and counts of normal and pyknotic DY-labeled RGCs

Rats were anesthetized with an overdose of sodium pentobarbitone (i.p.), and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Perfusion times were as described above. Right eyes were removed immediately after fixation and wholemounts of retinae prepared (Harvey and Robertson, 1992). Regions of the retina containing the brightest DY labeling, usually toward the center of the retina, were chosen for photographic analysis. The ganglion cell layer (GCL) of each retina was photographed through a 40× oil immersion objective. In most retinae 10 fields, a total area of 0.476 mm^2, was photographed and counted. Harvey and Robertson (1992) found that following DY injection into the SC approximately 20–25% (5–7 mm^2) of the total retinal area contained retrogradely labeled cells; thus about 7–10% of the DY-labeled regions were quantitatively analyzed. Slides were numbered and randomly sorted so that the counter did not know the origin of each retinal photograph. Counts of normal and frankly pyknotic RGCs were made (Harvey and Robertson, 1992; Cui and Harvey, 1995). The mean proportion of pyknotic RGCs (number of pyknotic cells divided by the total number of DY-labeled RGCs×100), and the density of normal DY-labeled RGCs were determined.

Immunohistochemistry

P5 rats were anesthetized and perfused as described above. Right eyes were removed immediately after fixation and retinas were dissected out of the eye cup in phosphate-buffered saline (PBS). Retinas were snap frozen in liquid nitrogen and embedded in cryoembedding medium (Jung, Leica Microsystems, Nussloch, Germany). Parasagittal cryostat sections (10 μm) were cut and mounted in series on gelatin-subbed glass slides. Retinal sections from the four different experimental groups (no lesion control, no lesion plus NT-4/5 injection, SC ablation, and SC ablation plus NT-4/5 injection) were mounted on to the same slides; this was done to ensure that sections from different groups were exposed to exactly the same conditions during immunohistochemical processing. Sections were air-dried and stored at −20 °C.

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