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Interactive effects of C3, cyclic AMP and ciliary neurotrophic factor on adult retinal ganglion cell survival and axonal regeneration

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We tested whether combined therapy involving Rho inactivation, elevation of cAMP and supply of ciliary neurotrophic factor (CNTF) (i) increased axotomized adult retinal ganglion cell (RGC) survival and (ii) promoted axonal regeneration into peripheral nerve (PN) autografted onto the cut optic nerve. PN-grafted eyes were injected with combinations of a Rho-inactivating enzyme C3 transferase (C3-11), CNTF and a cell-permeant analogue of cAMP (CPT-cAMP). Four weeks after PN transplantation, RGC survival was quantified using β-III tubulin immunohistochemistry. Regeneration was assessed using retrograde fluorogold tracing and pan-neurofilament immunostaining of grafts. Treatment with C3-11 increased RGC survival but co-injection with CPT-cAMP, CNTF or combined CNTF/CPT-cAMP did not further enhance RGC viability. There were greater numbers of regenerating RGCs after multiple C3-11 injections and regeneration was further and significantly increased after intravitreal injections of all three factors. In the combined C3-11/CNTF/CPT-cAMP treatment group about 15% of RGCs remained viable of which more than half regenerated an axon. These data emphasize the power of combinatorial pharmacotherapeutic and transplant strategies in the treatment of neurotrauma.

Keywords: Optic nerve injury; Neurotrauma; Cyclic nucleotides; Cytokines; C3 transferase

Introduction

The visual system is widely used as a model in which to study neuroprotection and regeneration of adult CNS neurons (Harvey et al., 2006). Adult retinal ganglion cells (RGCs) exhibit little or no spontaneous regenerative responses after injury, most dying by about 14 days after axotomy (Berkelaar et al., 1994; Nickells, 2004). Survival of injured RGCs *in vitro* and *in vivo* can be increased for a period of time by application of recombinant or virally expressed neurotrophic factors such as brain-derived neurotrophic factor (BDNF), neurotrophin 4/5 (NT-4/5) or ciliary neurotrophic factor (CNTF) (Logan et al., 2006; Mansour-Robaey et al., 1994; Mey and Thanos, 1993; Nakazawa et al., 2002; Oshitari and Adachi-Usami, 2003; Peinado-Ramon et al., 1996; Weise et al., 2000; Yan et al., 1999). CNTF in particular is also an effective axogenic factor for injured RGCs (Cui et al., 1999; Cui et al., 2003; Leaver et al., 2006). However, injury to RGCs also causes changes in receptor expression (Chen and Weber, 2004; Cui et al., 2002; Lindqvist et al., 2004) and may alter responsiveness to trophic signals (Goldberg et al., 2002; Shen et al., 1999). It is therefore important to ensure that responsiveness to such factors is maintained or even enhanced during the regenerative process. In this regard, raised intracellular cAMP can increase neurotrophin receptor levels in cell membranes (Meyer-Franke et al., 1998) and enhance neuronal responsiveness to diffusible growth factors (Cui et al., 2003; Li et al., 2003; Park et al., 2004).

After CNS injury, axonal regrowth is inhibited by the glial scar, which contains reactive astrocytes and secreted chondroitin sulfate proteoglycans (CSPGs). Regrowth is also inhibited by myelin-associated factors such as oligodendrocyte myelin glycoprotein (OMgp), myelin-associated glycoprotein (MAG) and Nogo (Grandpre and Strittmatter, 2001; Yiu and He, 2006). Many of these axonal growth inhibitory ligands act via a Rho GTPase signaling pathway (Borisoff et al., 2003; Fournier et al., 2003; Monnier et al., 2003; Sandvig et al., 2004) and inactivation of Rho or downstream effectors such as Rho-kinase (ROCK) enhance neural regeneration both *in vitro* and *in vivo*. The enzyme C3 transferase inactivates Rho by ADP ribosylation (Saito, 1997). Cell-permeable C3 fusion proteins stimulate neurite growth in tissue culture (Bertrand et al., 2005; Shearer et al., 2003) and *in vivo* promote some axonal growth when applied to optic nerve or spinal cord lesion sites (Bertrand et al., 2005; Dergham et al., 2002; Fournier et al., 2003; Lehmann et al., 1999; Monnier et al., 2003). Intracellular cAMP levels also influence neuronal responsiveness to these growth inhibitory molecules and can counteract their effects (Bandtlow, 2003; Gao et al., 2004; Gao et al., 2003; Lu et al., 2004; Qiu et al., 2002; Snider et al., 2002; Spencer and Filbin, 2004).

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There is increasing experimental evidence that more effective CNS repair can be elicited by combining pharmacotherapeutic and transplant strategies (Bregman et al., 2002; Fouad et al., 2005; Harvey et al., 2006). In the present study we examined such a combinatorial approach in the adult rat visual system. We tested whether intravitreal injections of a C3 fusion protein (C3-11) combined with CNTF and/or a non-degradable cell permeant cAMP analogue chlorphenylthio-cAMP (CPT-cAMP) (Cui et al., 2003) had synergistic effects on promoting adult RGC survival and axonal regeneration. We provided a relatively more permissive environment for regeneration of RGC axons by autologously grafting a segment of peripheral nerve (PN) onto the cut optic nerve (ON) (Cui et al., 2003; So and Aguayo, 1985). RGC viability and axonal regeneration were quantitatively assessed four weeks after PN transplantation using immunohistochemical and retrograde tracing techniques. We found that the effects of injecting C3-11, CNTF and CPT-cAMP were partially additive, increasing both the number of surviving RGCs and the proportion of viable RGCs that regenerated an axon.

Results

Treatment with C3-11 stimulates RGC survival and axonal regeneration into PN grafts

C3 transferase delivered to an ON lesion site (Lehmann et al., 1999) or to the cell bodies (Fischer et al., 2004) promotes adult RGC axonal regeneration within an inhibitory CNS environment. We first tested whether Rho antagonists promote RGC survival and axonal regeneration into a more permissive growth environment by injecting the cell-permeable Rho antagonist C3-11 (Bertrand et al., 2005) into the eye of young adult Fischer F344 rats at 4, 11, 18 days after ON transection and autologous PN transplantation. Four weeks after PN transplantation the number of surviving RGCs was assessed in retinal whole mounts using βIII-tubulin immunohistochemistry. This method provides an accurate assessment of RGC viability after ON injury (Ahmed et al., 2006; Cui et al., 2003; Koprivica et al., 2005; Leaver et al., 2006; Yin et al., 2003). RGCs with regenerating axons were retrogradely labeled with fluorogold (FG) after injection of the tracer into the distal end of each PN graft. Examples of βIII-tubulin and FG label in retinal whole mounts from saline and C3-11 injected eyes are shown in Figs. 1A, B and C, D, respectively. In the single (4 day) C3-11 injection group, the total number of surviving RGCs was significantly higher than saline control (Table 1, unpaired Student's t test with Welch's correction, $P=0.01$); however, the number of regenerating FG-labeled RGCs was not significantly different between the two groups ($P=0.24$) (Fig. 2).

Repeated doses of C3-11 enhanced both the survival and axonal regeneration of injured RGCs (Table 1, Fig. 2). In the double C3-11 injection group (day 4 and 11 injections), RGC survival and regeneration were significantly higher than in the comparable saline double injection group (unpaired Student's t test with Welch's correction, $P<0.0001$ for survival, $P=0.01$ for regeneration). In the triple C3-11 injection group (day 4, 11 and 18 injections), RGC survival and regeneration were not significantly greater than the double injected animals (Table 1, Fig. 2). Note that we previously found no significant difference between PN-ON graft only and saline injection groups (Cui et al., 2003; Park et al., 2004); thus, the intravitreal injection procedure does not by itself influence RGC viability in PN-grafted rats.

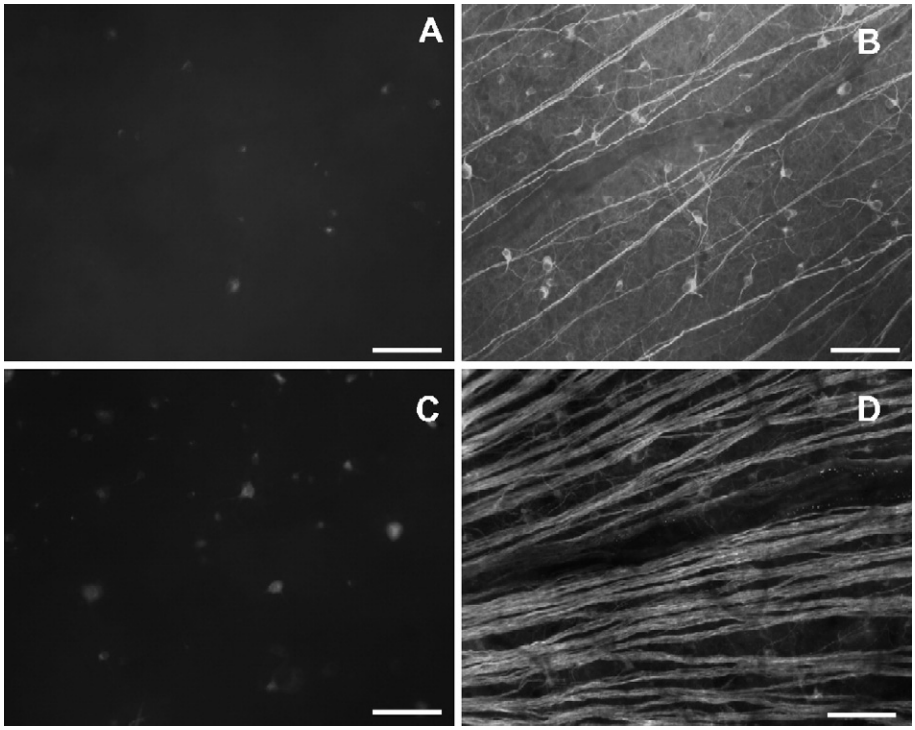

Fig. 1. Fluorogold (FG)-labeled regenerating (A, C) and viable βIII-tubulin-positive (B, D) RGCs in rats that received saline (A and B, same field) or C3-11 injections (C and D same field). Scale bars, 100 μm.