Development/Plasticity/Repair

Cellular Mechanisms Associated with Spontaneous and Ciliary Neurotrophic Factor–cAMP-Induced Survival and Axonal Regeneration of Adult Retinal Ganglion Cells

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We have shown previously that intraocular elevation of cAMP using the cAMP analog 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) failed to promote axonal regeneration of axotomized adult retinal ganglion cells (RGCs) into peripheral nerve (PN) grafts but significantly potentiated ciliary neurotrophic factor (CNTF)-induced axonal regeneration. Using the PN graft model, we now examine the mechanisms underlying spontaneous and CNTF/CPT-cAMP-induced neuronal survival and axonal regrowth. We found that blockade of the cAMP pathway executor protein kinase A (PKA) using the cell-permeable inhibitor KT5720 did not affect spontaneous survival and axonal regeneration but essentially abolished the CNTF/CPT-cAMP-induced RGC survival and axonal regeneration. Blockade of CNTF signaling pathways such as phosphotidylinositol 3-kinase (PI3K)/akt by 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) by 2-(2-diamino-3-methoxyphenyl-4H-1-benzopyran-4-one (PD98059), or Janus kinase (JAK)/signal transducer and activators of transcription (STAT3) by tyrphostin AG490 also blocked the CNTF/CPT-cAMP-dependent survival and regeneration effects. PKA activity assay and Western blots showed that KT5720, LY294002, and PD98059 almost completely inhibited PKA, PI3K/akt, and MAPK/ERK signal transduction, respectively, whereas AG490 substantially decreased JAK/STAT3 signal transduction. Intraocular injection of CPT-cAMP resulted in a small PKA-dependent increase in CNTF receptor α mRNA expression in the retinas, an effect that may facilitate CNTF action on survival and axonal regeneration. Surprisingly, in the absence of CNTF/CPT-cAMP, LY294002, PD98059, and AG490, but not KT5720, significantly enhanced spontaneous RGC survival, suggesting differential roles of these pathways in RGC survival under different conditions. Our data suggest that CNTF/CPT-cAMP-induced RGC survival and axonal regeneration are a result of multiple pathway actions, with PKA as an essential component, but that these pathways can function in an antagonistic manner under different conditions.

Key words: **ciliary neurotrophic factor; cAMP; protein kinase A; phosphotidylinositol 3-kinase; mitogen-activated protein kinase; Janus kinase**

Introduction

cAMP plays an important role in diverse neuronal functions including survival (Rydel and Greene, 1988; Hanson et al., 1998), modulation of axonal guidance (Ming et al., 1997; Nishiyama et al., 2003), and enhancement of neurite outgrowth (Jo et al., 1999; Kao et al., 2002). Recently, cAMP elevation was also shown to promote the regeneration of dorsal root ganglion axons into spinal cord (Neumann et al., 2002; Qiu et al., 2002). We reported recently that intraocular injection of ciliary neurotrophic factor (CNTF) increased the regeneration of adult retinal ganglion cell (RGC) axons into peripheral nerve (PN) grafts *in vivo* (Cui et al., 1999), and intraocular cAMP elevation using the cAMP analog 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) significantly potentiated the CNTF-induced axonal regeneration of RGCs (Cui et al., 2003). Similarly, cAMP elevation and neurotrophin-3 application have a synergistic effect on axonal regeneration in spinal cord (Lu et al., 2004).

cAMP acts on protein kinase A (PKA) to exert many of its biological functions (Li et al., 2000), effects that are blocked by the cell-permeable PKA inhibitor KT5720 (Qiu et al., 2002). *In vitro*, KT5720 has also been reported to influence other signaling pathways such as mitogen-activated protein kinase (MAPK)/extracellular signal-related kinase (ERK) and akt (Davies et al., 2000). CNTF elicits its primary biological actions via the Janus kinase (JAK)/signal transducer and activators of transcription (STAT3), phosphatidylinositol 3-kinase (PI3K)/akt, and MAPK/ERK pathways (Ip and Yancopoulos, 1996; Peterson et al., 2000; Alonzi et al., 2001; Dolcet et al., 2001; Goldberg et al., 2002; Guillet et al., 2002; Kaur et al., 2002). PI3K/akt plays an important role in mediating the survival of certain neurons (Vaillant et al., 1999; Namikawa et al., 2000; Campos et al., 2003), whereas the MAPK/ERK pathway has no influence (Vaillant et al., 1999;

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Namikawa et al., 2000; Alonzi et al., 2001) or a more minor influence (Mazzoni et al., 1999; Atwal et al., 2000) on cell viability. Nevertheless, both the PI3K/akt and MAPK/ERK pathways are involved in axonal regeneration *in vivo* and neurite outgrowth *in vitro* (Cowley et al., 1994; Miura et al., 1994; Namikawa et al., 2000; Desbarats et al., 2003).

The aim of the present study, using the PN-to-optic nerve (ON) approach in adult rats, was to elucidate the mechanisms underlying both spontaneous and CNTF/cAMP elevation-induced RGC survival and axonal regeneration. We injected intraocularly the PKA pathway inhibitor KT5720, the PI3K/akt pathway inhibitor 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), the MAPK/ERK pathway inhibitor 2-(2-diamino-3-methoxyphenyl-4H-1-benzopyran-4-one (PD98059), or the JAK/STAT3 pathway inhibitor AG490, a member of the tyrphostin family of protein kinase inhibitors (Sanchez-Margalet et al., 1994; Alessi et al., 1995; Dolcet et al., 2001; Goldberg et al., 2002). Using quantitative methods, we then determined whether inhibition of these pathways influenced the levels of spontaneous and CNTF/cAMP-induced RGC survival and axonal regeneration into PN grafts. Because cAMP elevation has been reported to upregulate the expression of CNTF receptor α (CNTFRα), the extracellular binding component of CNTF receptor complex (Ip and Yancopoulos, 1996), in certain neuroglial cells *in vitro* (Wewetzer et al., 2001), we also examined the effect of cAMP elevation on retinal CNTFRα mRNA expression in this *in vivo* regeneration model.

Materials and Methods

Young adult (8–10 weeks of age) Fischer 344 rats were used in this study. All surgical procedures were approved by the Animal Ethics Committees of The University of Western Australia and Shantou University Medical College.

Peripheral nerve grafting procedure. The PN–ON surgery procedure has been published previously (Cui et al., 1999, 2003; Yin et al., 2003). Briefly, rats were anesthetized with a 1:1 mixture (1.5 ml/kg) of ketamine (100 mg/ml) and xylazine (20 mg/ml). The left ON was exposed intraorbitally and transected within the sheath ~1.5 mm behind the optic disc. To preserve the retinal blood supply, care was taken not to damage the underlying ophthalmic artery. A 1.5 cm piece of peroneal nerve was dissected out from the left leg and sutured with 10/0 suture (Johnson and Johnson, North Ryde, Australia) onto the proximal stump of the transected ON. The distal part of the PN was placed over the skull and secured to connective tissue.

Experimental groups. PN-grafted animals were allocated to different experimental groups. The first group received no intravitreal injections, and the second group received intravitreal injections of saline; both groups served as controls. The rest of the animals received intravitreal injections of rat recombinant CNTF (1.5 μg per injection; PeproTech, Rehovot, Israel), the cell-permeable cAMP analog CPT-cAMP (0.1 mM; Sigma, St. Louis, MO), the PKA pathway inhibitor KT5720 (10 μM; Sigma), the PI3K/akt pathway inhibitor LY294002 (2 mM; Sigma), the MAPK/ERK pathway inhibitor PD98059 (5 mM; Sigma), or the JAK/STAT3 pathway inhibitor AG490 (2 mM; Calbiochem, La Jolla, CA) alone or in various combinations (see Fig. 1). For intravitreal injections, all rats received three posterior chamber eye injections of various molecules, 3 μl each, on days 3, 10, and 17 after PN–ON surgery (see Fig. 1). For each intravitreal injection, the micropipette was inserted in peripheral retina, just behind the ora serrata, and was deliberately angled to avoid damage to the lens (Leon et al., 2000).

Retrograde labeling of RGCs with regenerating axons. The fastest-regenerating RGC axons grow in PN grafts at a rate of ~2 mm/d, after an initial delay period of 4–5 d (Cho and So, 1987). To provide sufficient time for regrowing axons to reach the distal end of the PN grafts, the number of RGCs with regenerating axons was assessed 1 month after surgery. To retrogradely label these regenerating RGC axons and their parent cell bodies, 0.2 μl of 4% fluorogold (FG) (Fluorochrome, Denver, CO) was injected slowly into the distal end of the PN graft. Animals survived for another 3 d to maximize retrograde transport of the dye. After deeply anesthetizing the animals with sodium pentobarbitone (Nembutal, 60 mg/ml, i.p.; Merial Australia, Parramatta, Australia), the rats were perfused with 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Injection of a small volume of FG was essential to avoid diffusion of dye toward the optic disc and consequent staining of viable but non-axon-regenerating RGCs (Cui et al., 2003).

After removal from the eye cups, retinas were postfixed in 4% paraformaldehyde for 45 min, flat mounted, and temporarily coverslipped in Citifluor (Citifluor, London, UK). To determine the total number of FG-labeled RGCs, the outline of each retina was drawn on a computer screen using an MD2 microscope digitizer (Accustage, Shoreview, MN), and a grid was randomly placed over the drawing. A cursor was placed on each grid intersection, and the number of FG-labeled RGCs was counted at that point. Each sample field was 0.235 × 0.235 mm, and 60–80 fields were sampled per retina (Cui et al., 2003). The average number per field of RGCs with regenerating axons was determined, and the total FG-labeled RGC number was then obtained by multiplying this figure by the retinal area.

Immunohistochemical staining of viable RGCs. After FG counts were made, whole retinas were immunostained with an antibody to neuronal class βIII tubulin (TUJ1; Babco, Richmond, CA). TUJ1 staining in retinal whole mounts was shown to be adult RGC specific and was efficient in RGC identification (Cui et al., 2003; Yin et al., 2003). Coverslips were removed, and retinas were brushed off the slides in PBS. After PBS washes, retinas were blocked in 10% normal goat serum (Hunter Antisera, Jesmond, Australia) and 0.2% Triton X-100 (Sigma) for 1 hr and then incubated in the same medium with TUJ1 antibody (1:500) for 1 d at 4°C. After additional washes, retinas were incubated with cyanine 3-conjugated goat anti-mouse IgG (1:100; The Jackson Laboratory, Bar Harbor, ME) overnight at 4°C. Retinas were again flat mounted onto slides and coverslipped in Citifluor. The number of TUJ1-immunofluorescent cells in the ganglion cell layer was calculated using the sampling procedures described above for FG labeling.

cAMP-dependent protein kinase A activity assay. The effectiveness of intraocular KT5720 injection in blocking PKA activity was examined using the PepTag assay for nonradioactive detection of cAMP-dependent protein kinase (Promega, Madison, WI). The procedure was performed following the manufacturer's protocol.

Briefly, 3 d after PN–ON procedure, eyes were injected with saline ($n = 3$), a mixture of CNTF and CPT-cAMP ($n = 3$), or a mixture of CNTF, CPT-cAMP, and KT5720 ($n = 4$). Animals were allowed to survive for another 6 hr. Normal intact animals ($n = 3$) were used as controls. Total protein from retina was extracted with lysis buffer (1% Triton X-100, 0.5% NP-40, 1 mM EDTA, 150 mM NaCl, 1 mM PMSF, 5 mM sodium pyrophosphate, 0.2 mM sodium molybdate, 0.05 mM sodium fluoride, and 1 mM sodium orthovanadate), and cell supernatant was collected after centrifuging at 15,000 × g for 15 min, snap frozen, and kept at −80°C for later usage. For each sample, 5 μl of reaction buffer, A1 peptide (PKA substrate kemptide), and 1 μl of peptide protection solution were added, and the mixture was incubated at room temperature for 30 min. The reaction was stopped by placing the tube in a 95°C heating block for 10 min. The phosphorylated (phospho)-PKA downstream products (phospho-kemptide) were separated from unphosphorylated kemptide (unphospho-kemptide) in a 0.8% agarose gel, and bands were visualized under UV light. The gel was photographed and quantified using ScionImage (Scion, Frederick, MD) densitometric software.

Western blot analysis to confirm pathway inhibition. Western blot experiments were used to determine the efficacy of the intraocular LY294002, PD98059, and AG490 injections in inhibiting PI3K/akt, MAPK/ERK, and JAK/STAT3 pathways, respectively. We examined the levels of downstream products of these pathways: phospho-Akt in the PI3K/akt pathway, phospho-ERK1/2 in the MAPK/ERK pathway, and phospho-STAT3 in the JAK/STAT3 pathway. The levels of these products were determined at both 6 hr and 3 d after the intraocular injections of the inhibitors, which in turn had been injected 3 d after the PN graft procedure.

Retinas were homogenized using the same lysis buffer as above. The protein concentration of the supernatant was determined using the Bio-

Rad (Hercules, CA) protein assay reagent. Approximately 50 μg of protein was loaded and separated in a 10% acrylamide–Bis solution (Bio-Rad) gel. The protein was transferred onto Hybond-C Super membrane (Amersham Biosciences, Little Chalfont, UK) and blocked with 5% skim milk in 0.1% Tween 20 (ICN Biochemicals, Cleveland, OH) in TBS (TTBS). The membranes were incubated with phosphorylated or unphosphorylated primary anti-akt (1:1000), anti-ERK1/2 (1:2000 for phosphorylated and 1:1000 for unphosphorylated), or anti-STAT3 (1:1000) antibodies (all from Cell Signaling, Beverly, MA, except phospho-ERK1/2 antibody, which is from Promega) in 5% BSA in TTBS overnight at 4°C. After washing in TTBS, the membranes were incubated in biotinylated secondary antibody (Vector Laboratories , Burlingame, CA) at a 1:20,000 dilution for 1 hr at room temperature. The membranes were incubated with HRP-conjugated antibody (Babco) before the labeled proteins were detected using the ECL agent (Pierce, Rockford, IL), following the supplier's manual. Membranes were then exposed to Kodak (Rochester, NY) X-Omat AR film, and the images on the film were developed with Kodak film developer and fixed with Ilford fixer (Ilford, Mt. Waverley, Australia). Membranes were stripped by incubating them with stripping buffer (2% SDS, 7 μl of β-mercaptoethanol/ml in TTBS, pH 7.6) at room temperature for 30 min followed by two brief washes with TTBS. Membranes were then blocked again in 5% skim milk in TTBS before reprobing them with antibodies against unphospho-akt, against unphospho-ERK1/2, or against unphospho-STAT3 or monoclonal anti-β-actin antibody (Sigma), which was used as loading control. The antibodies were diluted 1:1000 in 5% BSA–TTBS. The intensity of each band was quantified using the ScionImage program. The relative levels of phospho-Akt, ERK1/2, and STAT3 were expressed as the ratio to β-actin.

Western blot analysis to examine whether cross-talk exits between PI3K/akt, MAPK/ERK, and JAK/STAT3 pathways. In PN–ON-only animals, RGC survival was increased after inhibition of the PI3K/akt, MAPK/ERK, or JAK/STAT3 pathways. It was possible that inhibition of one of these pathways led to increased activity in one of the other signaling cascades. Thus, we further investigated whether cross-talk exists between these affected signaling pathways. Using the same surgical and Western blot approaches described above, we measured PI3K/akt pathway activity after application of PD98059 and AG490, MAPK/ERK pathway activity after application of LY294002 and AG490, and JAK/STAT3 pathway activity after application of LY294002 and PD98059.

Reverse transcription and quantitative PCR study for CNTFRα expression. To investigate the effect of cAMP elevation on CNTFRα mRNA expression in retina, real-time PCR was used to examine the level of CNTFRα mRNA expression after certain treatments.

The surgical procedures were the same as above but with different intravitreal treatments. Briefly, 3 d after the PN–ON procedure, we performed intravitreal injections of saline, CNTF, CPT-cAMP at various concentrations, or combined application of CPT-cAMP and KT5720 in a 3 μl volume. There were four to five animals in each group, and all animals survived for another 3 d. After killing animals with a Nembutal overdose, the retinas were quickly dissected out and immediately put into RNAlater (Ambion, Austin, TX). They were stored at 4°C until processing. PCR procedures were then performed by an independent researcher (S.H.) who did not know the origin of the samples. RNA was isolated from each retina using 1 ml of RNAwiz (Ambion), and equal amounts of total RNA (1 μg) were reverse transcribed in 25 μl volumes with Moloney murine leukemia virus reverse transcriptase (RT) (Promega) and random hexamers (Promega), according to manufacturer's instructions. The reactions were then purified through columns (MoBio PCR Clean-up; MoBio Laboratories, West Carlsbad, CA) before quantitative PCR. Quantitative PCR was performed using the Roche (Basel, Switzerland) LightCycler with the following primers: CNTFRα (forward, 5′-AAGCCCGATCCTCCAGAAAATGTG-3′; reverse, 5′-CTCCATGTCCCAATCTCATTGTCC-3′) as described by Wewetzer et al. (2001) and L19 ribosomal protein RNA (forward, 5′-CTGAAGGTCAAAGGGAATGTG-3; reverse, 5′-GGACAGAGTCTTGATGATCTC-3′). Reactions were performed using 1 μl of FastStart DNA Master SYBR Green I (Roche) with 2 mM $MgCl_2$, 0.5 μM primers (CNTFRα) or with 3 mM $MgCl_2$, 0.5 μM primers (L19) in a total volume of 10 μl. Cycling conditions were: 95°C for 15 sec, 52°C for 5 sec, and 72°C for 10 sec for 45 cycles (CNTFRα) and 95°C for 15 sec, 52°C for 5 sec, and 72°C for 8 sec for 45 cycles (L19). Controls (with and without RT enzyme) were used to check for genomic DNA amplification.

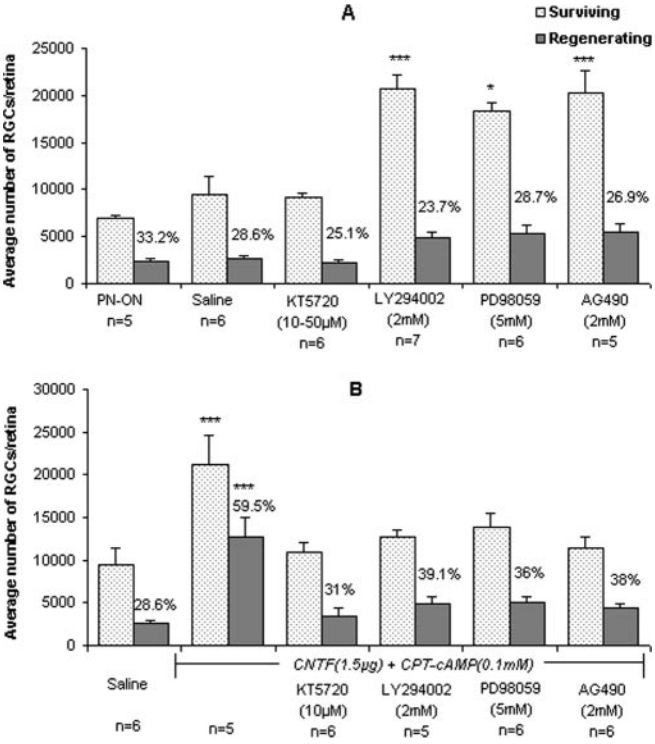

Figure 1. Average numbers of FG-labeled axon-regenerating and TUJ1-positive surviving RGCs under various experimental conditions in the absence (*A*) or presence (*B*) of CNTF/CPT-cAMP. $*p < 0.05$, $***p < 0.001$; Dunnett's test; comparisons were made against the saline group. Error bars represent SEM.

Statistical analysis. RGC data from the different groups were statistically analyzed using Dunnett's or Bonferroni tests after one-way ANOVA. Dunnett's test was used to compare mean values of experimental groups against the same control group (usually the saline treatment group), whereas the Bonferroni test was used to compare mean values among all intragroups. Both procedures have been used widely (Cui et al., 2003; Yin et al., 2003; Pearse et al., 2004).

Results

One month after the PN–ON procedure, the average numbers of surviving (TUJ1-positive) and axon-regenerating (FG-positive) RGCs were 6910 ± 277 (mean ± SEM) per retina and 2297 ± 323 (mean ± SEM) per retina in the PN–ON-only group and 9398 ± 2048 per retina and 2685 ± 328 per retina in the PN–ON plus intraocular injection of saline group, respectively (Fig. 1*A*). For comparison, note that there are ~110,000 RGCs per retina in normal adult Sprague Dawley rats (Cui et al., 2003). Intravitreal saline injections slightly increased both RGC survival and axonal regeneration; however, these increases were not significant. Examples of surviving and axon-regenerating RGCs are shown in Figure 2.

PKA pathway in spontaneous and CNTF/CPT-cAMP-induced RGC survival and axonal regeneration

PKA activity assays

PKA activity assays (Fig. 3*A*) and quantitative analysis of the results (Fig. 3*B*) showed that the PN–ON procedure and intravitreal injection of saline substantially reduced PKA activity in the affected eyes compared with the normalized level in intact animals. Intraocular injection of CNTF and CPT-cAMP signifi-

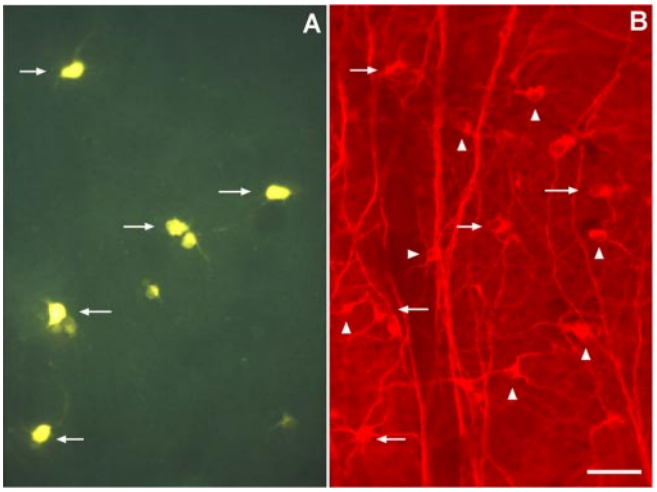

Figure 2. Fluorescent photomicrographs of a retinal whole mount showing characteristics of retrogradely FG-labeled RGCs that have regenerated an axon into a PN graft (*A*, arrows) and surviving TUJ1-immunoreactive RGCs (*B*). *A* and *B* are the same retinal field but using different fluorescent filters. FG-labeled RGCs are clearly also TUJ1 positive (*B*, arrows); however, many of the TUJ1-positive RGCs do not contain FG (arrowheads); they are therefore viable but do not have axons regenerating to the distal end of the PN graft. Scale bar, 50 μm.

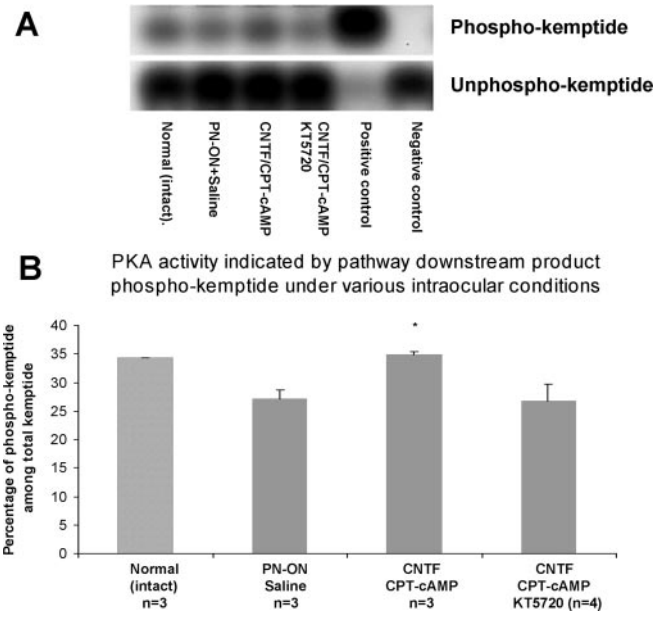

Figure 3. PKA activity assay (*A*) and quantitative analysis of the results (*B*). *A*, The top row shows the level of phosphorylated kemptide, a downstream PKA pathway product, whereas the bottom row depicts unphosphorylated kemptide. *B*, Proportions of phosphorylated kemptide among total kemptide. Intraocular injection of 3 μl of the PKA pathway inhibitor KT5720 at 10 μM effectively blocked PKA signaling activity in the retina. Error bars in *B* represent SEM; *$p < 0.05$.

cantly ($p < 0.05$; Dunnett's test) increased PKA activity in PN-grafted rats, returning PKA levels to those seen in unoperated animals. This CNTF- and CPT-cAMP-induced increase in PKA activity was completely blocked by the PKA pathway inhibitor KT5720 at 10 μM (Fig. 3), and the level of PKA activity appeared to be even lower than in the saline group, confirming that KT5720 was efficient in inhibiting the PKA pathway at this concentration in this *in vivo* adult CNS model.

RGC counts

Intraocular injections of KT5720 alone did not affect spontaneous survival and axonal regeneration of RGCs after the PN–ON procedure. Compared with the intraocular saline-injected group, the average number of both surviving (9107 ± 541 per retina) and axon-regenerating (2284 ± 304 per retina) RGCs in the KT5720 treatment group was not significantly different (Fig. 1*A*).

Compared with the PN–ON control and saline injection groups, combined intraocular injections of CNTF and the cAMP analog CPT-cAMP significantly increased the number of surviving (21,273 ± 3411 per retina; $p < 0.001$) and axon-regenerating (12,661 ± 2385 per retina; $p < 0.001$) RGCs (Fig. 1*B*). Relating these values to the total number of RGCs in normal unlesioned retinas, with CNTF/cAMP treatment, approximately one in five adult rat RGCs survived for at least 4 weeks after the PN–ON surgery, and of these, ~60% had regenerated axons into PN grafts. In contrast, the proportion of regenerating-to-surviving RGCs was 33.2% in the PN–ON group with no eye injections and 28.6% in the saline-injected group. These data thus confirm our previous finding that CNTF/cAMP promotes the survival of axotomized adult RGCs and significantly increases the proportion of viable RGCs that can regenerate their axons into a PN graft (Cui et al., 2003).

When KT5720 was applied together with CNTF/CPT-cAMP, the increase in CNTF/CPT-cAMP-induced RGC survival and axonal regeneration was almost completely blocked (Fig. 1*B*). Both the survival (10,912 ± 1179 per retina) and axonal regeneration (3386 ± 1070 per retina) of RGCs in the CNTF/CPT-cAMP/KT5720 treatment group were significantly lower ($p < 0.01$ for survival and $p < 0.001$ for regeneration; Bonferroni) than the CNTF/CPT-cAMP group. Furthermore, these values were not significantly different from the PN–ON control or saline injection groups (Fig. 1). Thus, in contrast to spontaneous survival and axonal regrowth, the PKA pathway plays a critical role in CNTF/CPT-cAMP-induced survival and axonal regeneration of adult rat RGCs.

PI3K/akt pathway in spontaneous and CNTF/CPT-cAMP-induced RGC survival and axonal regeneration

Western blots

Western blot analysis revealed slightly different results 6 hr or 3 d after intravitreal injection of the various molecules or combinations of molecules (Fig. 4). Compared with normal (intact) control retinas, the PN–ON procedure and intravitreal injection of saline did not render an immediate effect on PI3K activity in the retinas (Fig. 4*A*), as determined by the level of phospho-akt, but it did slightly increase PI3K activity at a later stage (3 d) (Fig. 4*B*). As expected, intraocular injection of CNTF significantly increased the level of phospho-akt, a downstream product of the PI3K/akt pathway at both the 6 hr ($p < 0.05$; Dunnett's test) (Fig. 4*A*) and 3 d ($p < 0.01$; Dunnett's test) (Fig. 4*B*) time points. The effect appeared to be gradual, because the levels of phospho-akt were higher at the 3 d than at the 6 hr time point. However, CPT-cAMP treatment also significantly increased the level of phospho-akt when measured at both 6 hr and 3 d after injection. In fact, the influence of CPT-cAMP was more obvious at 6 hr ($p < 0.01$; Dunnett's test) than at 3 d ($p < 0.05$; Dunnett's test). An additional increase, although not statistically significant, was seen at 6 hr when CNTF and CPT-cAMP were applied together (Fig. 4*A*). Importantly, the CNTF/CPT-cAMP-induced increase in phospho-akt was significantly ($p < 0.01$; Bonferroni) (Fig. 4*A*) blocked by the PI3K/akt pathway inhibitor LY294002 (2 mM) at 6 hr and completely blocked ($p < 0.001$; Bonferroni) (Fig. 4*B*) at the 3 d time point.

RGC counts

In the absence of exogenous CNTF and CPT-cAMP, intravitreal injections of LY294002 substantially increased rather than decreased RGC survival (20,640 ± 1548 per retina) (Fig. 1*A*). The number of TUJ1-positive RGCs was significantly higher than that seen in both the PN–ON control and saline-injected groups ($p < 0.001$; Bonferroni) (Fig. 1*A*). Treatment of LY294002 alone also increased, although not significantly, the average number of RGCs regenerating axons into PN grafts (4882 ± 645 per retina). The increased axonal regeneration is likely to be a consequence of the increased pool of surviving RGCs, because the proportion of axon-regenerating RGCs among surviving RGCs in the LY294002 treatment group (23.7%) was similar to the saline group (28.6%) (Fig. 1*A*).

In contrast to the above observations, when LY294002 was applied together with CNTF/CPT-cAMP, the CNTF/CPT-cAMP-induced RGC survival was effectively blocked. The average number of surviving RGCs in this group was 12,668 ± 892 per retina, not significantly higher than the saline-injected group (Fig. 1*B*). After treatment with LY294002, the level of CNTF/CPT-cAMP-induced axonal regeneration also substantially decreased (4959 ± 782 per retina). This value was significantly lower ($p < 0.001$; Bonferroni) than for the CNTF/CPT-cAMP treatment group (Fig. 1*B*). The average number of axon-regenerating RGCs in the CNTF/CPT-cAMP/LY294002 treatment group was higher than the saline group (2685 ± 328 per retina), but this difference did not reach significance. Similar amounts of RGC survival and axonal regeneration were seen after application of LY294002 at a lower concentration (0.5 mM) in the presence of CNTF/CPT-cAMP (data not shown).

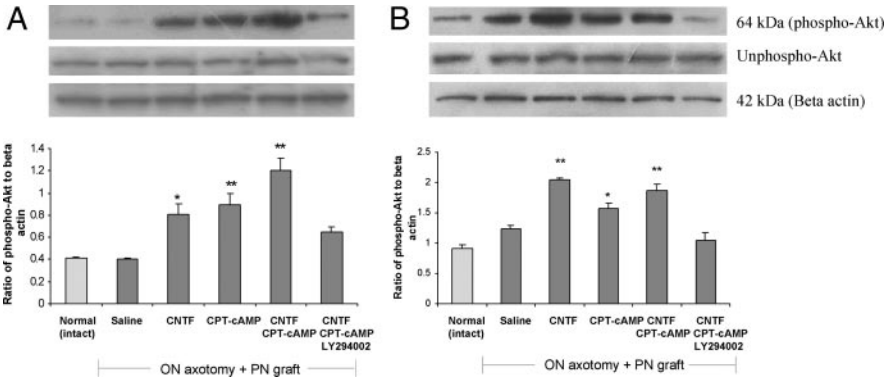

Figure 4. Western blots and the quantitative analysis on the PI3K pathway downstream product phosphorylated akt 6 hr (*A*) or 3 d (*B*) after various experimental interventions. The PI3K pathway inhibitor LY294002 blocked the CNTF/CPT-cAMP-induced increase in PI3K pathway activity substantially at 6 hr and completely at 3 d after intravitreal injection. *$p < 0.05$, **$p < 0.01$; Dunnett's test; comparisons were made against the saline group. Error bars represent SEM.

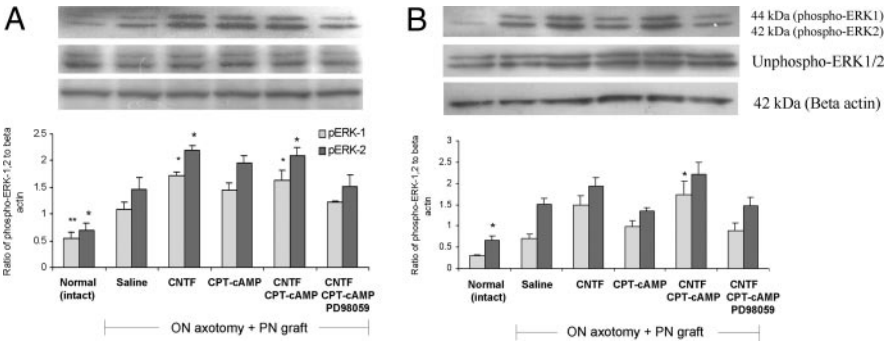

Figure 5. Western blotting study on the MAPK pathway downstream product phosphorylated ERK1/2 6 hr (*A*) or 3 d (*B*) after various experimental interventions. The MAPK pathway inhibitor PD98059 blocked the CNTF/CPT-cAMP-induced increase in MAPK/ERK pathway activity substantially at 6 hr and completely at 3 d after intravitreal injection. *$p < 0.05$, **$p < 0.01$; Dunnett's test; comparisons were made against the saline group. Error bars represent SEM.

MAPK/ERK pathway in spontaneous and CNTF/cAMP-induced RGC survival and axonal regeneration

Western blots

Western blot analysis of the MAPK/ERK pathway 6 hr and 3 d after various intravitreal treatments showed that the levels of phospho-ERK1/2, downstream products of the MAPK/ERK pathway, increased in the saline-injected group (Fig. 5*A*,*B*). At the 6 hr time point, this increase in phospho-ERK1 ($p < 0.01$; Dunnett's test) and ERK2 ($p < 0.05$; Dunnett's test) after saline injection was significant, whereas at the 3 d time point, only the increase in phospho-ERK2 was significant ($p < 0.05$; Dunnett's test). These data suggest an influential effect of the PN–ON procedure and eye injection on MAPK/ERK activity.

After CNTF injection, a significant increase in phospho-ERK1 and -ERK2 was seen 6 hr later (Fig. 5*A*), an increase that was still evident 3 d after treatment (Fig. 5*B*). CPT-cAMP by itself had a minor effect on ERK phosphorylation at the earlier time point (Fig. 5*A*). After combined application of CNTF and CPT-cAMP, no clear synergistic effect was seen at either the 6 hr or 3 d time points (Fig. 5*A*,*B*). The MAPK/ERK pathway inhibitor PD98059 almost entirely abolished the CNTF- and CPT-cAMP-induced increases in phospho-ERK1 ($p < 0.05$ and $p < 0.01$ at the 6 hr and 3 d time points, respectively; Bonferroni) and phospho-ERK2 ($p < 0.05$ at the 3 d time point; Bonferroni), reducing protein expression approximately to the levels seen in the saline-injected group (Fig. 5). These data confirmed the effectiveness of this molecule in inhibiting the MAPK/ERK pathway within the retina at this concentration.

RGC counts

Similar to our observations on RGC survival and axonal regeneration after LY294002 treatment, intravitreal injections of PD98059 significantly enhanced RGC survival (18,401 ± 903 per retina) in PN–ON-only animals. The twofold increase was significantly higher than saline-injected ($p < 0.05$; Bonferroni) and PN–ON control ($p < 0.001$; Bonferroni) groups (Fig. 1*A*). Again, because the number of surviving RGCs was greater, the number of axon-regenerating RGCs also increased after PD98059 treatment (5275 ± 909 per retina). This increase was not, however, significantly higher than in the saline group (Fig. 1*A*). Note that the proportion of axon-regenerating RGCs in this group was 28.7%, similar to the saline group, again suggesting that the increased number of regenerating axons was simply the result of increased overall viability of the injured RGC population.

In the presence of CNTF/CPT-cAMP, PD98059 substantially blocked the CNTF/CPT-cAMP-induced increase in RGC sur-

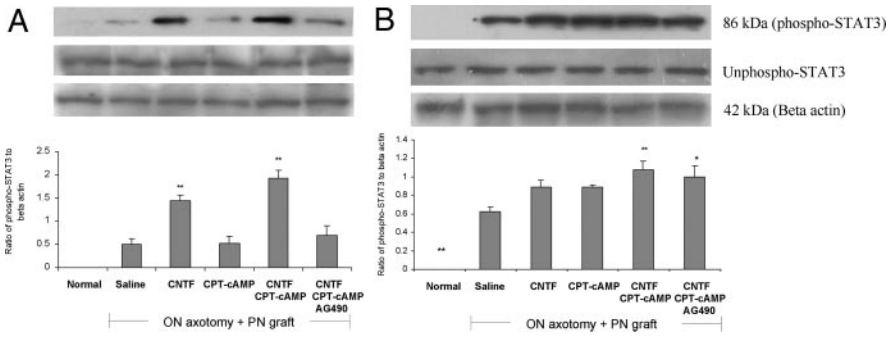

Figure 6. Western blotting study on the JAK pathway downstream product phosphorylated STAT3 6 hr (*A*) or 3 d (*B*) after various experimental interventions. The JAK pathway inhibitor AG490 substantially blocked the CNTF-induced increase in JAK pathway activity at 6 hr but failed to exert any effect on the CNTF/CPT-cAMP-induced increase in JAK/STAT3 pathway activity 3 d after intravitreal injection. $*p < 0.05$, $**p < 0.01$; Dunnett's test; comparisons were made against the saline group. Error bars represent SEM.

vival (13,848 ± 1679 per retina), a value not significantly higher than the saline group (Fig. 1*B*). The CNTF/CPT-cAMP-induced increase in axonal regeneration was also significantly ($p < 0.001$) blocked after PD98059 treatment, but the value (4983 ± 799 per retina), although not statistically significant, was still higher than the saline group (Fig. 1*B*). Similar effects on RGC survival and axonal regeneration were seen after application of PD98059 at a lower concentration (50 μM) in the presence of CNTF/CPT-cAMP (data not shown).

JAK/STAT3 pathway in spontaneous and CNTF/cAMP-induced RGC survival and axonal regeneration

Western blots

Western blot analysis revealed substantial differences in JAK/STAT3 pathway activity at 6 hr and 3 d, as determined by levels of the JAK/STAT3 pathway downstream product phospho-STAT3 (Fig. 6*A*,*B*). In normal intact retinas, no JAK/STAT3 activation was seen, but activity was slightly increased 6 hr after intravitreal injection of saline, suggesting a minor influence of the PN–ON procedure and intravitreal injection on this pathway at this early stage. However, intraocular CNTF injections significantly enhanced the level of phospho-STAT3 6 hr after injection ($p < 0.01$; Dunnett's test) (Fig. 6*A*). These *in vivo* results are thus consistent with previous data showing that biological activities of CNTF are mediated by the JAK/STAT3 pathway. Although an increase was also seen after intravitreal injection of CPT-cAMP, the difference was not statistically significant (Fig. 6*A*).

When CNTF and CPT-cAMP were applied together, a synergistic effect on the level of phospho-STAT3 was seen. Compared with the CNTF treatment group, the level of phospho-STAT3 in the combined treatment group was significantly higher ($p < 0.05$; Bonferroni) (Fig. 6*A*). These data thus suggest (1) that cAMP elevation might potentiate CNTF-induced JAK/STAT3 activity or (2) that CNTF and CPT-cAMP might act via different mechanisms to enhance JAK/STAT3 pathway activity at the early stage. The dramatic increase in phospho-STAT3 protein expression after CNTF and CPT-cAMP application was substantially blocked ($p < 0.001$; Bonferroni) by intravitreal injection of AG490 at 2 mM (Fig. 6*A*), confirming the effectiveness of this molecule in inhibiting the JAK/STAT3 pathway in our *in vivo* model.

Three days after the intravitreal injections, the level of phospho-STAT3 was substantially increased in saline-treated retinas ($p < 0.01$; Dunnett's test). Although greater levels of phospho-STAT3 were also seen in each of the CNTF and CPT-cAMP treatment groups at this time point, no statistical difference was seen when compared with the saline group (Fig. 6*B*), suggesting a slow upregulation process after the PN–ON procedure and the eye injection. However, compared with the saline treatment group, at least an additional significant increase in the level of phospho-STAT3 was seen after combined application of CNTF and CPT-cAMP ($p < 0.01$; Dunnett's test) (Fig. 6*B*). In addition, AG490 treatment failed to block the JAK/STAT3 pathway at this time, because its influence on a CNTF/CPT-cAMP-induced increase in phospho-STAT3 level was minimal (Fig. 6*B*). These data suggest (1) that the AG490 inhibitor had only temporary blocking effects and (2) that cAMP elevation had a slow but progressive influence on JAK/STAT3 pathway activity.

RGC counts

As with the other two pathway inhibitors, intravitreal injection of the JAK/STAT3 inhibitor AG490 (2 mM) enhanced RGC survival (20,640 ± 1548 per retina) (Fig. 1*A*) in control PN-grafted rats. This was significantly higher than the PN–ON control ($p < 0.001$; Bonferroni) and saline-injected ($p < 0.01$; Bonferroni) groups. The treatment also increased spontaneous axonal regeneration, but not significantly, resulting in a mean number of 5446 ± 949 axon-regenerating RGCs per retina, a twofold increase compared with the saline group (Fig. 1*A*). As before, this increased axonal regeneration was likely attributable to the increased pool of surviving RGCs; the proportion of axon-regenerating RGCs in the AG490 treatment group was 26.9%, very similar to the saline-injected group (28.6%).

In the presence of CNTF/CPT-cAMP, AG490 substantially blocked the CNTF/CPT-cAMP-induced increase in RGC survival (11,488 ± 1296 per retina), a value not significantly different from the saline group (Fig. 1*B*). The CNTF/CPT-cAMP-induced increase in axonal regeneration was also significantly ($p < 0.001$; Bonferroni) blocked after AG490 treatment (4360 ± 609 per retina), a value not significantly higher than the saline control (Fig. 1*B*). Note that the successful blockade of the CNTF/CPT-cAMP-induced survival and axonal regeneration by AG490 was achieved in the absence of complete inhibition of JAK/STAT3 signaling cascades.

Lack of cross-talk between PI3K/akt, MAPK/EERK, and JAK/STAT3 pathways after PN–ON procedure

Western blot results revealed that selective pharmacological inhibition of any one of these kinase pathways did not alter expression or activity in the remaining two pathways. Thus, the levels of phospho-akt and phospho-ERK1/2, phospho-akt and phospho-STAT3, and phospho-ERK1/2 and phospho-STAT3 remained unaffected in the retina after intravitreal injections of JAK/STAT3, MAPK/ERK, and PI3K/akt pathway blockers, respectively (Fig. 7*A*–*C*). These results indicate that the surprising increase in RGC survival after intravitreal injection of LY294002, PD98059, or AG490 was unlikely to be caused by a compensatory increase in activity in one of the other, noninhibited kinase signaling pathways.

Changes in CNTFRα expression in retina after intraocular intervention

CNTFRα mRNA expression was quantified 6 d after ON–PN transplantation and 3 d after intraocular injection of control or pharmacological agents. The ON–PN procedure itself appeared to have little impact on the level of CNTFRα mRNA expression, because there was only a slight decrease in the level of CNTFRα mRNA in the saline group compared with normal intact animals (Fig. 8). Intraocular CNTF injection also had little effect, but intraocular injection of CPT-cAMP (0.1 mM) did increase CNTFRα mRNA expression, an effect that was blocked by the PKA pathway inhibitor KT5720 (Fig. 8). Note that CPT-cAMP at higher dosages (0.5 and 1 mM) decreased the CNTFRα mRNA expression level, perhaps as a result of detrimental effects on the retinas. In fact, it was noticed that the retinas treated at these higher dosages appeared to be soft and disintegrated during the dissection procedure. Similar poor condition of the retinas after CPT-cAMP treatment at these high dosages was also observed in an ON crush model (data not shown). At the appropriate concentrations, the results do suggest, however, that cAMP analogs may increase CNTFRα mRNA expression in a PKA pathway-dependent manner.

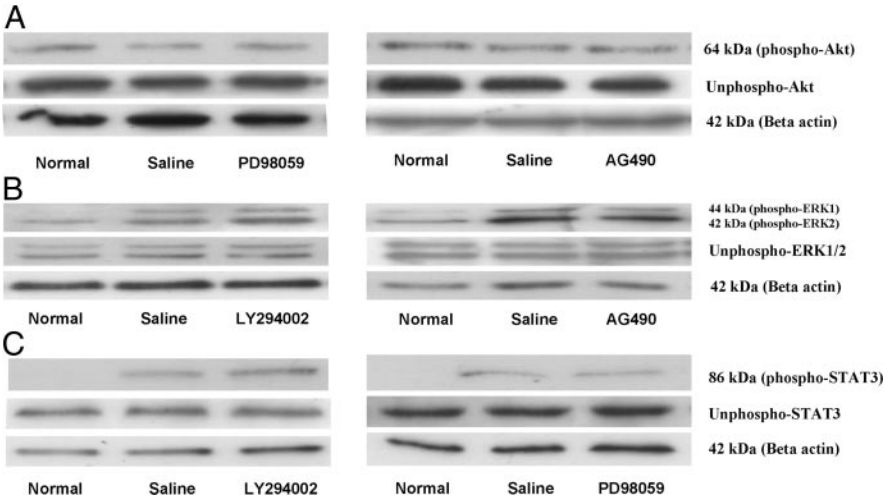

Figure 7. Western-blotting studies on cross-talk between PI3K/akt, MAPK/ERK, and JAK/STAT3 signaling pathways 6 hr after one or various experimental interventions. *A*, PI3K signaling activity after MAPK/ERK (left column) and JAK/STAT3 (right column) pathway inhibition. *B*, MAPK/ERK signaling activity after PI3K/akt (left column) and JAK/STAT3 (right column) pathway inhibition. *C*, JAK/STAT3 signaling activity after PI3K/akt (left column) and MAPK/ERK (right column) pathway inhibition. Inhibition of any of these pathways did not affect the signaling activity of the remaining two pathways.

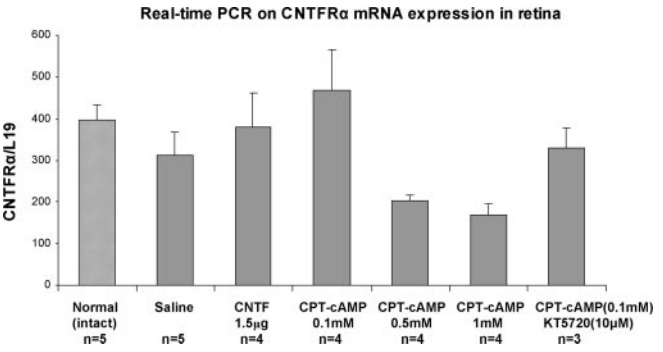

Figure 8. Real-time PCR studies showing the expression levels of CNTFRα against the housekeeping gene *L19* after various treatments. All groups received the PN–ON procedure and intraocular treatment (3 d later) except the normal intact control (striped bar). CNTFRα expression in the retina decreased after ON injury and saline treatment. Intraocular injection of the cAMP analog CPT-cAMP at the appropriate concentration (0.1 mM in 3 μl) substantially upregulated CNTFRα expression (nearly 50% increase compared with the saline group), an effect that was completely blocked by the specific PKA pathway inhibitor KT5720 (10 μM in 3 μl). CNTF treatment (1.5 μg) slightly increased the level of CNTFRα expression. Error bars represent SEM.

Discussion

In this study, we systematically analyzed the influence of major signaling pathways (PKA, PI3K/akt, MAPK/ERK, and JAK/STAT3) on CNTF and cAMP actions in promoting adult rat RGC viability and axonal regrowth. We showed that the CNTF/cAMP-induced enhancement of survival and axonal regeneration into PN grafts after ON injury is primarily PKA pathway dependent and involves PI3K/akt, MAPK/ERK, and JAK/STAT3 signaling pathways. In addition, upregulation of CNTFRα by cAMP elevation may also participate in the enhancement of RGC survival and axonal regeneration.

Importantly, PI3K/akt, MAPK/ERK, and JAK/STAT3 pathways appear to exert different biological functions under different conditions. After the PN–ON procedure, and in the absence of exogenous CNTF/cAMP elevation, these kinases appeared to participate in the process leading to RGC death, because inhibition of any of the pathways promoted RGC survival after axotomy and PN grafting. Interestingly, PKA inhibition by itself did not influence RGC viability in this group of animals. Although there were more adult RGCs that regenerated their axons into PN grafts in the LY294002-, PD98059-, and AG490-treated groups, this increase was most likely merely a consequence of the overall increase in RGC survival. This interpretation is based on the fact that, as a proportion of viable RGCs, the proportion of axon-regenerating RGCs among the LY294002, PD98059, and AG490 groups (23.7, 28.7, and 26.9%, respectively) was very similar to saline-injected controls (28.6%) and animals injected with KT5720 (25.1%). In comparison, combined injection of CNTF and CPT/cAMP clearly had a specific impact on RGC axonal regeneration, because in this group, the proportion of viable RGCs that regenerated an axon was 60% (cf. Cui et al., 2003).

The cell death data are in contrast to previous work that showed that PI3K/akt and MAPK/ERK are the major signaling executors for neuronal survival (Crowder and Freeman, 1999; Datta et al., 1999; Klocker et al.; 2000; Brunet et al., 2001; Rong et al., 2003). However, in renal epithelial cells, the PI3K/akt pathway inhibitor LY294002 increased bcl-2 protein and inhibited apoptosis (Carbott et al., 2002), and in cardiomyocytes, PI3K accelerated necrotic cell death during hypoxia (Aki et al., 2001). Furthermore, the MAPK/ERK pathway inhibitor PD98059 has been shown to be neuroprotective after focal cerebral ischemia (Alessandrini et al., 1999). In the absence of CNTF/cAMP after ON–PN transplantation, the PI3K/akt pathway inhibitor LY294002 may protect RGCs by enhancing the level of bcl-2, an important survival-promoting gene for neurons (Bähr, 2000; Nuydens et al., 2000; Yuan and Yankner, 2000), including RGCs (Huang et al., 2003; Isenmann et al., 2003). ERK has been shown to phosphorylate synapsin I, a major phosphoprotein found in nerve terminals (Jovanovic et al., 1996; Matsubara et al., 1996). Synapsin I maintains synaptic vesicles, and phosphorylation of

this protein may lead to the dissociation of the vesicles, resulting in the release of excitotoxic amino acids such as glutamate (Alessandrini et al., 1999). Thus, the MAPK/ERK pathway inhibitor PD98059 may protect RGCs by preventing excitotoxic amino acid release. It is also possible that the increased RGC survival is achieved by enhanced c-*fos* signal after PD98059 treatment, because increased c-*fos* signal, known to be associated with regulation of excitability and neuronal survival (Zhang et al., 2002) including RGCs (Oshitari et al., 2002), was seen in the cortex of PD98059-treated animals (Alessandrini et al., 1999).

Although it has been reported previously in other systems that MAPK/ERK and PI3K negatively regulate STAT-transcriptional activities (Krasilnikov et al., 2003) that and stimulation of the MAPK pathway inhibits STAT activation, perhaps via suppressor of cytokine signaling (Terstegen et al., 2000), the observed increases in RGC survival are unlikely to be attributable to cross-talk between PI3K/akt, MAPK/ERK, and JAK/STAT3 pathways, because we failed to detect any influence of inhibition of one particular pathway on the activity of the other two pathways. Interestingly, the MAPK/ERK signaling cascade has been shown recently to act in an antagonistic manner in sympathetic neurons, and the MAPK/ERK pathway inhibitor PD98059 enhances dendritic growth of sympathetic neurons *in vitro* (Kim et al., 2004).

It is also necessary to consider the possibility that indirect action by other cellular components in the retina after PI3K/akt, MAPK/ERK, or JAK/STAT3 pathway inhibition may have contributed to the observed increases in RGC survival. Intravitreal injection of kinase inhibitors will affect RGCs situated in the innermost ganglion cell layer, but if these molecules are sufficiently permeable, they may also influence signaling in glia and other retinal neurons located in deeper retinal layers. In the presence or absence of exogenous CNTF/CPT-cAMP, the other major cell populations in the retina, such as photoreceptors, Müller cells, amacrine cells, etc., may respond differentially to the different environments. These altered responses could result in changed neurotrophic factor production, metabolism, and/or energy consumption that could directly or indirectly affect RGC survival. Macrophages are of particular interest in this regard, because we have shown recently that factors from activated macrophages in the eye promoted RGC survival and axonal regeneration (Yin et al., 2003). It is possible that inhibition of PI3K/akt, MAPK/ERK, or JAK/STAT3 pathways might lead to signaling cascades that result in macrophage activation. Finally, production of CNTF in Müller cells (Chun et al., 2000) may vary under different experimental conditions and could also have contributed to different RGC survival and axonal regeneration.

Recently, Mao et al. (2004) reported a positive link between ionotropic glutamate receptors and ERK1/2 phosphorylation. Abnormal activation of glutamate receptors can compromise cell viability via cellular cascades resulting from alterations in Ca^{2+} and Na^{+} influx and K^{+} efflux (Yu et al., 1999; Zipfel et al., 2000; Arundine and Tymianski, 2003); thus, it is possible that changes in glutamate sensitivity–signaling were also involved in PI3K/akt, MAPK/ERK, or JAK/STAT3 pathway-dependent RGC death in PN-grafted rats (Skaper et al., 2001). In fact, PI3K has been shown recently to increase calcium channel trafficking to the plasma membrane and calcium influx in dorsal root ganglion neurons (Viard et al., 2004).

After intraocular CNTF/CPT-cAMP injections, inhibition of the PI3K, MAPK/ERK, or JAK/STAT3 pathways significantly blocked the pharmacologically induced axonal regeneration of axotomized adult RGCs. In this paradigm, the data are thus more consistent with the observations that PI3K, MAPK/ERK, and JAK/STAT3 signaling are associated with neuronal survival and axonal regeneration. The CNTF receptor is composed of an extracellular CNTF binding subunit, CNTFRα, and two transmembrane proteins, glycoprotein 130 and leukemia inhibitory factor receptor β (Ip et al., 1992; 1993; Davis et al., 1993). Via this receptor complex, CNTF activates multiple signaling pathways, including PI3K/akt, MAPK/ERK, and JAK/STAT3 signaling pathways (MacLennan et al., 2000; Alonzi et al., 2001; Dolcet et al., 2001). Activation of PI3K/akt and MAPK/ERK can result in phosphorylation (and thus inactivation) of proapoptotic Bad and activation of anti-apoptotic bcl-2 proteins (Alonzi et al., 2001; Brunet et al., 2001), both potentially working together to enhance RGC survival in this adult injury model. In addition, elevation of cAMP increases the responsiveness of purified embryonic or neonatal RGCs to a range of trophic factors (Meyer-Franke et al., 1995) via various mechanisms (Goldberg and Barres, 2000; Hansen et al., 2001; Reiriz et al., 2002) or membrane depolarization (Meyer-Franke et al., 1998). Greater RGC viability and improved long-term survival *in vitro* is obtained if cAMP is elevated in combination with a mixture of factors including BDNF, CNTF, and insulin growth factor-1. Elevation of cAMP in RGCs allows the translocation of MAPK to the nucleus in response to appropriate trophic stimulation (Meyer-Franke et al., 1998).

It is known that the response of a neurite to a given guidance or adhesion molecule can be modulated by changing cAMP levels and/or by blocking PKA (Ming et al., 1997; Song and Poo, 1999). Raised cAMP may also reduce the effect of growth-inhibitory factors on axon-regenerating adult RGCs (Cai et al., 1999; 2001). cAMP-induced activation of PKA phosphorylates and thus inhibits Rho signaling pathways (Lehmann et al., 1999). Recently, a number of studies have shown increased axonal regeneration after elevation of intracellular cAMP (Nikulina et al., 2004; Pearse et al., 2004). Inhibition of Rho pathway may be one of the mechanisms underlying cAMP elevation-induced axonal regeneration. Although we did not see increased axonal regeneration into a PN graft after elevation of cAMP level in the eye (Cui et al., 2003), enhanced axonal growth into the distal part of a crushed ON has been seen (our unpublished observation). This is consistent with a recent report in which intraocular injection of cAMP analog promotes axonal regeneration in the ON (Monsul et al., 2004).

Forskolin, which enhances intracellular cAMP levels, has been shown recently to increase CNTFRα mRNA expression in cultured neonatal olfactory ensheathing cells (Wewetzer et al., 2001). In our *in vivo* model, we found that elevation of cAMP also increased CNTFRα mRNA expression in the retina. In the presence of exogenous CNTF, the increased level of CNTFRα by cAMP elevation may facilitate the action of CNTF on neuronal survival and axonal regeneration.

In summary, PKA, PI3K/akt, MAPK/ERK, and JAK/STAT3 pathways are all involved in CNTF/CPT-cAMP-induced survival and axonal regeneration, with the PKA pathway appearing to be more influential. However, the exact roles of these signaling pathways vary under different conditions. In the visual system, the role of these pathways may be critically dependent on how the RGCs are injured and the nature of the conditions to which the injured RGCs are exposed (e.g., whether they are exposed to exogenous neurotrophic factors). Our data show (1) that CNTF/CPT-cAMP-induced RGC survival and axonal regeneration were PKA, PI3K/akt, MAPK/ERK, and JAK/STAT3 pathway dependent and (2) that in the absence of CNTF/CPT-cAMP, PI3K/akt, MAPK/ERK, or JAK/STAT3, pathway inhibition resulted in increased RGC survival. The possible cellular and/or molecular

mechanisms underlying this unexpected increase in RGC survival after intraocular kinase inhibition are currently under investigation.

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