Down-regulation of apoptosis mediators by RNAi inhibits axotomy-induced retinal ganglion cell death in vivo

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Summary

Transection of the optic nerve induces an apoptotic degeneration of retinal ganglion cells (RGC) in the rat retina. The immediate early gene c-Jun, the proapoptotic Bcl-2 family member Bax and the apopotosome constituent Apaf-1 have been shown previously to play major roles in the induction or execution of the apoptosis cascade. In this study we have designed and generated short interfering RNAs (siRNAs) against c-Jun, Bax and Apaf-1, which were injected into the optic nerve stump in order to inhibit axotomy-induced apoptosis. siRNAs were first tested in vitro to ensure silencing efficiency. In vivo, a clear neuronal localization of Cy3-labelled siRNA could be visualized in retinal flat mounts. Retinas that were injected with anti-Apaf-1- and anti-c-Jun-siRNA showed significantly more surviving RGC than non-injected or anti-EGFP-injected controls (2- to 3-fold, respectively). Anti-Bax-siRNA-injected retinas showed a trend towards an increased RGC number (not significant). Regulation of target proteins in situ could be visualized by immunohistochemical stainings. We conclude that (i) c-Jun and Apaf-1 play major roles in the apoptotic cascade of RGC and may represent useful targets for antiapoptotic strategies in RGC in vivo, and (ii) injection of siRNAs into the optic nerve stump is a new method to down-regulate target genes specifically in RGC.

Keywords: apoptosis; axotomy; gene silencing; retinal ganglion cell; RNA interference

Abbreviations: Apaf-1 = apoptotic protease-activating factor-1; Ced-4 = cell-death abnormality-4; DIV = in vitro; EGFP = enhanced green fluorescent protein; NeuN = neuronal nuclei; RGC = retinal ganglion cells; RGL = retinal ganglion cell layer; RNAi = RNA interference; siRNA = short interfering RNA


Introduction

Optic nerve transection is an established model for apoptotic cell death, resulting in degeneration of ~80–90% of retinal ganglion cells (RGC) by 14 days after nerve transection in adult rats (Berkelaar et al., 1994; Kermer et al., 2001). Axotomy-induced RGC death is associated with the activation of an intracellular signalling cascade, involving immediate early genes, pro- and anti-apoptotic members of the Bcl-2 family, and caspases (reviewed in Bähr, 2000).

Increased expression of immediate early genes, e.g. c-Fos and c-Jun, is one of the earliest events observed in axotomized RGC (Hüll and Bähr, 1994b; Martin-Villalba et al., 1998). Inhibition of c-Jun by a dominant-negative isoform attenuated apoptotic cell death of dopaminergic cells in an axotomy model of the nigrostriatal pathway (Crocker et al., 2001). However, c-Jun has also been shown to be up-regulated in regenerating neurons, enhancing the transcription of genes encoding growth promoting proteins. Thus, c-Jun may exert multiple functions depending on the stimulus and the examined paradigm (Robinson, 1995; Lu et al., 2003). It therefore remains to be elucidated whether c-Jun up-regulation in RGC represents a mere epiphenomenon or whether it plays a crucial role in apoptosis induction in this cell type.

Further downstream in the apoptotic cascade, Bax acts as a proapoptotic member of the multidomain subfamily of the Bcl-2 family proteins, leading to caspase activation via
cytochrome c release (Brady and Gil-Gomez, 1998). Bax expression is increased following optic nerve transection, and antisense oligonucleotides against Bax mRNA have been shown to decrease apoptosis in the optic nerve transection model (Isemann et al., 1997).

The mammalian cell-death abnormality-4 (Ced-4) homologue apoptotic protease-activating factor-1 (Apaf-1) is a key mediator of caspase-induced cell death, activating downstream caspases via apoptosome formation (Li et al., 1997; Zou et al., 1999). In a cell culture paradigm of human fibroblasts, silencing of Apaf-1 via RNA interference (RNAi) has been shown to inhibit apoptosis induced by ultraviolet (UV) irradiation, etoposide and cisplatin (Lassus et al., 2002).

For all three proteins, c-Jun, Bax and Apaf-1, there is evidence to suggest that their specific down-regulation may effectively interfere with the initiation or execution of the programmed cell death cascade in RGC.

The dissection of intracellular signalling cascades via lack-of-function analysis is a common experimental approach. Although regulation of protein function by dominant-negative protein homologues, antibodies or pharmacological inhibitors has been widely performed, all these methods share the inability to be completely specific towards their protein of interest. Gene silencing by RNAi offers the possibility to inhibit mRNA in a sequence-specific manner by degradation of the target gene. Gene silencing by RNAi offers the possibility to inhibit mRNA in a sequence-specific manner by degradation of the target gene. Although regulation of protein function by dominant-negative protein homologues, antibodies or pharmacological inhibitors has been widely performed, all these methods share the inability to be completely specific towards their protein of interest.

The sequence of the anti-enhanced green fluorescent protein (EGFP) siRNA used in our study has been published previously (Caplen et al., 2001). Other siRNA sequences were designed applying an algorithm proposed by Elbashir et al. (2001b), taking care to select siRNAs with a G/C-content <50%. Sequences were then checked for homologies using the NCBI BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), excluding all siRNAs that showed homologies to genes in the genome of Rattus norvegicus (reviewed in Hannon, 2002). A number of recent studies have shown that RNAi can successfully be utilized to silence target genes in mammalian neuronal cells in vitro and in vivo (reviewed in Lingor et al., 2004a).

In this study we selectively target proteins involved in apoptotic cell death in RGC. We demonstrate that a local application of short interfering RNA (siRNA) directed against genes involved in apoptosis regulation by injection into the optic nerve stump following axotomy is able to down-regulate the expression of the target genes in situ and thus increase the number of surviving RGC.

**Material and methods**

**siRNA sequences and generation**

The sequence of the anti-enhanced green fluorescent protein (EGFP) siRNA used in our study has been published previously (Caplen et al., 2001). Other siRNA sequences were designed applying an algorithm proposed by Elbashir et al. (2001b), taking care to select siRNAs with a G/C-content <50%. Sequences were then checked for homologies using the NCBI BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), excluding all siRNAs that showed homologies to genes in the genome of Rattus norvegicus other than the target gene.

Cy3-labelled RNA oligonucleotides were chemically synthesized (Biospring, Frankfurt/Main, Germany) and had the following sequences: anti-EGFP-sense Cy3 5'-GAC GUU AAC GCC CAC AAG UUC-3', anti-EGFP-antisense Cy3-5'-ACU GGU GCC GUC UUC CGC-3', anti-Bax #1-sense 5'-UUG GAG AUG AAC UGC ACA AUU-3', anti-Bax #1-antisense 5'-UUG UCC AGU UCU UCC AAU-3', anti-Bax #2-sense 5'-CUU GGU CCC GUA AUU AUU UCU-3', anti-Bax #2-antisense 5'-CUU GCC CAA UAU UAU UGU CCA GUU-3', anti-Apaf-1 #1-sense 5'-AGA ACU UGG UGC AAU AUU GUU-3', anti-Apaf-1 #1-antisense 5'-CAU UAA AGC ACA AAG UUC UUU-3', anti-Apaf-1 #2-sense 5'-UAU AGG CAU AUA CUG GAU GUU-3', anti-Apaf-1 #2-antisense 5'-CAU CCA GUU UAU AUU-3', anti-c-Jun #1-sense 5'-GUU GAA AAC CUU GGU GUU-3', anti-c-Jun #1-antisense 5'-CGG UUU CCC AAC GUU-3', anti-c-Jun #2-sense 5'-AGU CAU GAA CCA GUU UAU AUU-3', and anti-c-Jun #2-antisense 5'-GUU AAC GUG GUU CAU GAC UUU-3'. The siRNA yield was determined by spectrophotometry for each siRNA preparation and amounted to 400-650 ng/μl.

**RGC-5 cell culture, siRNA transfection and culture lysates**

The RGC-5 cell line has been derived by SV-40-induced immortalization of rat RGC (Krishnamoorthy et al., 2001). Cells were plated at a density of 40 000/cm² in 24-well culture plates and kept at 37°C, 5% CO₂ and saturated humidity. The culture medium was based on Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Gibco, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS Gold; PAA, Colbe, Germany), glutamine and antibiotics (penicillin/streptomycin; Gibco). On day in vitro (DIV), concanavalin A (Sigma–Aldrich, Munich, Germany) was added to the near-confluent culture for inhibition of further cell division. Apoptosis was induced by addition of 50 nM staurosporine (Sigma–Aldrich) at DIV 2.

siRNAs were transfected using LipofectAMINE® 2000 (Invitrogen, Karlsruhe, Germany) according to manufacturer’s instructions. Briefly, per well of a 24-well plate, 1 μl Lipofect AMINE® 2000 and 50 ng siRNA were each added to 50 μl DMEM/F12 without supplements. The two solutions were combined and the complexing reaction was allowed to continue for 20 min at room temperature. One hundred microlitres of the siRNA–liposome complex were added to each well. Cultures were kept for 12, 24 or 48 h after apoptosis induction, then washed twice with phosphate-buffered saline (PBS) and lysed (Fig. 1B).

**Fig. 1** Experimental setup for in vitro and in vivo studies. RGC-5 cultures were transfected with siRNAs on DIV 1 after plating and apoptosis was induced using staurosporine on DIV 2. (A) Cell lysates were prepared consecutively at 12, 24 and 48 h after staurosporine application. (B) For in vivo studies siRNA was injected into the optic nerve stump using a Hamilton syringe directly following axotomy.
Western blotting
The protein content of the samples was determined using the bicinchoninic acid assay (Pierce, Rockford, IL, USA) and equal amounts of protein (2 μg) were loaded in each lane for a sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were then transferred to a nitrocellulose membrane and blocked with 5% skimmed milk in Tris-buffered saline/Tween-20 (TBS-T) for 1 h. Membranes were then incubated with primary antibodies (anti-c-Jun, 1:1000, Cell Signaling Technologies; anti-Apaf-1, 1:1000, anti-Bax, 1:1000, both Santa Cruz Technology) followed by corresponding horseradish peroxidase-coupled secondary antibodies against rabbit IgG (1:2000 for 1 h at room temperature; Dianova, Hamburg, Germany). ECL-Plus reagent (Amersham, Arlington Heights, IL, USA) was applied on the membrane and the chemiluminescence was visualized and quantified via a Fluor-S-max imager (Bio-Rad, Munich, Germany).

Optic nerve transection, siRNA application and retrograde labelling
Optic nerve transection was performed as previously described in detail (Kermer et al., 2001). In brief, adult female Sprague–Dawley rats (200–250 g; Charles River, Sulzfeld, Germany) were anaesthetized by intraperitoneal injection of chloral hydrate (420 mg per kg body weight). The skin was incised close to the superior orbital rim and the orbita was opened leaving the supraorbital vein intact. The lacrimal gland was moved aside, the superior extraocular muscles detached from their tendinous insertion points and the eye rotated to expose the optic nerve. The optic nerve was exposed by longitudinal incision of the perineurium and then transected ~2 mm from the posterior eye pole without damaging the retinal blood supply.

Using a 26-gauge microlitre syringe (Hamilton, Bonaduz, Switzerland), 5 μl siRNA (2000 ng; 143 pmol) or 5 μl PBS, respectively, were injected slowly into the optic nerve stump directly following the axotomy procedure (Fig. 1A). The syringe was inserted such that its opening was completely covered by the optic nerve stump, and care was taken not to enter the optic disc or penetrate the retina. Some of the siRNA solution was found on the surface of the optic nerve stump after the injection procedure and served as an additional siRNA pool for retrograde axonal uptake. A 2 × 2 mm piece of gel foam was soaked in FluoroGold (5% dissolved in 0.9% NaCl; Fluorochrome Inc., Englewood, CO, USA) and placed on the optic nerve stump in order to retrogradely label RGC. Retinal blood supply was verified by fundoscopy and animals with persistent retinal ischaemia were excluded.

For RGC counts, four animals were used in each group, except for the c-Jun #2 group, where seven animals were used (see Table 1). For immunohistochemical visualization of protein regulation, two animals were injected for each condition and the contralateral eyes served as non-axotomized controls.

Retinal flat mounts and cell counting
On day 14 post-axotomy, animals received an overdose of chloral hydrate and the eyes were extracted. The cornea, the lens and the vitreous body were removed, and the remaining eye cup containing the retina was fixed in 4% paraformaldehyde (Sigma) for 1 h. Retinas were then extracted and flat-mounted in glycerol-PBS (1:1) on glass slides. The number of FluoroGold-positive RGC was determined by fluorescence microscopy (Zeiss-Axioplan, Oberkochen, Germany) using a UV filter (365/420 nm). Three fields of 62 500 μm² were counted in each retinal quadrant (at eccentricities of one-sixth, one-half and five-sixths of the retinal radius). RGC counts were performed by two different investigators according to a blinded protocol.

Immunohistochemistry
On day 4 post-axotomy, animals received an overdose of chloral hydrate and the eyes were extracted. The cornea, the lens and the vitreous body were removed and the remaining eye cup containing the retina was fixed in 4% paraformaldehyde for 1 h. The eye cups were then dehydrated in 30% sucrose at 4°C for 24 h and immersed in cryostat mounting liquid at −20°C. Retinal sections of 16 μm were prepared using a Leica cryostat, collected on gelatine-coated glass slides and kept at −20°C until further processing. Tissue sections were dehumidified at 37°C for 1 h and antigen retrieval was performed for 4 h in TBS-T (pH 9.0) at 60°C. Unspecific binding was blocked by application of 10% new-born goat serum, and primary antibodies (anti-c-Jun, Cell Signaling Technologies; anti-Apaf-1, anti-Bax, both Santa Cruz Technology) were applied in a 1:1000 dilution. After two 1:2000 antisera dilutions, sections were dehydrated at 37°C for 24 h and immersed in cryostat mounting liquid at −20°C. After 4 h, the sections were counter-stained with DAPI (4',6-diamidino-2-phenylindole) (Sigma–Aldrich) and mounted in Moviol (Hoechst, Frankfurt, Germany).

Table 1 Increased survival of RGC following optic nerve transection and siRNA injection directed against mediators of apoptosis in the adult rat

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>1/6 retinal radius</th>
<th>3/6 retinal radius</th>
<th>5/6 retinal radius</th>
<th>Average</th>
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<tr>
<td>Anti-Bax #1</td>
<td>4</td>
<td>260 ± 100</td>
<td>247 ± 89</td>
<td>221 ± 86</td>
<td>243 ± 46</td>
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<tr>
<td>Anti-Bax #2</td>
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<td>401 ± 222</td>
<td>364 ± 156</td>
<td>312 ± 157</td>
<td>359 ± 111</td>
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<tr>
<td>Anti-Apaf-1 #1</td>
<td>4</td>
<td>437 ± 129</td>
<td>407 ± 153</td>
<td>419 ± 173</td>
<td>421 ± 70</td>
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<tr>
<td>Anti-Apaf-1 #2</td>
<td>4</td>
<td>438 ± 237</td>
<td>472 ± 265</td>
<td>361 ± 151</td>
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<td>4</td>
<td>445 ± 167</td>
<td>401 ± 214</td>
<td>436 ± 176</td>
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<tr>
<td>Anti-c-Jun #2</td>
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<td>617 ± 318</td>
<td>609 ± 297</td>
<td>608 ± 294</td>
<td>612 ± 230</td>
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<tr>
<td>Anti-EGFP</td>
<td>4</td>
<td>290 ± 121</td>
<td>282 ± 115</td>
<td>279 ± 117</td>
<td>284 ± 105</td>
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<tr>
<td>Non-injected control</td>
<td>4</td>
<td>226 ± 67</td>
<td>204 ± 53</td>
<td>172 ± 48</td>
<td>200 ± 12</td>
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Data are given as mean ± standard error of the mean RGC density (cells/mm²) at one-sixth, one-half and five-sixths of the retinal radius and the average RGC density over the radius. Anti-Bax #1, anti-Bax #2, anti-Apaf-1 #1, anti-Apaf-1 #2, anti-c-Jun #1 and anti-c-Jun #2 correspond to siRNAs injected directly after axotomy. Anti-EGFP corresponds to the non-specific siRNA control used. Non-injected control animals were used to exclude injection-mediated effects on RGC survival. Statistics were performed using one-way ANOVA as given in Fig. 5.
All comparable images of a series were taken using identical exposure times. Adjustment for brightness and contrast was performed using Corel Draw™8 in an identical manner for all comparable images of one series.

Statistical analysis
Cell culture experiments were performed at least in duplicate. For in vivo studies the number of animals used is given in Table 1. All data are given as mean ± standard deviation. Values were compared using one-way analysis of variance (ANOVA) and considered as significantly different at $P < 0.05$, $P < 0.01$ and $P < 0.001$.

Results
Characterization of siRNAs in vitro
Although there is increasing knowledge about successful siRNA construction algorithms, no sufficient prediction of the silencing potential of single siRNAs has been possible up to now (Mittal, 2004). Therefore, two siRNAs for each target gene were constructed and tested for their silencing activity in an in vitro paradigm before in vivo evaluation. The rat RGC line RGC-5 was used as an in vitro model to most closely mimic the silencing behaviour in RGC in vivo. One day (DIV 1) after plating, near-confluent RGC-5 cultures were transfected with siRNA by lipofection and staurosporine was added on DIV 2 in order to induce apoptosis. Cultures were lysed 12, 24 and 48 h after apoptosis induction and protein regulation was determined by protein electrophoresis and blotting (Fig. 1A).

Baseline expression of c-Jun, Bax and Apaf-1 was observed in control cultures. A marked up-regulation and phosphorylation (48 kDa isofom) of c-Jun as well as an up-regulation of Apaf-1-levels was detectable already at 12 h after staurosporine application. Bax levels remained nearly unchanged after staurosporine treatment of the RGC-5 culture. Transfection of cultures with siRNAs directed against c-Jun, Bax or Apaf-1 resulted in differential decrease of target protein expression, which was most pronounced at the 48 h time point. Target protein expression was nearly completely abolished in anti-c-Jun #2 and anti-Bax #1 and anti-Bax #2 siRNA-treated cultures, whereas down-regulation of Apaf-1 by anti-Apaf-1 siRNAs was less pronounced compared with its baseline expression (Fig. 2). siRNA against EGFP used as control did not significantly reduce target protein expression. Down-regulation of target proteins was verified by band density measurements standardized against β-tubulin control bands (not shown). The in vitro data suggested anti-c-Jun #2, anti-Bax #2 and anti-Apaf-1 #2 siRNAs to have the most pronounced silencing effects.

siRNA localizes in retinal ganglion neurons after injection into the optic nerve stump
RGC are the only retinal cell type to send their axons via the optic nerve. Application of siRNA to the optic nerve stump thus selectively delivers siRNA to RGC, sparing other cell types. In order to demonstrate efficient siRNA delivery to RGC, Cy-3 labelled siRNA was injected to the optic nerve stump and native retinal flat mounts were evaluated at day 1 and day 3 post-injection (Fig. 1B). Cy-3 fluorescence was clearly visible in an RGC-like distribution pattern at both time points. However, we also noted that the intensity of Cy-3 fluorescence was variable in different retinal areas (Fig. 3A and B). We attributed this variable Cy-3 distribution to the injection technique, which did not allow targeting all axons in an equal manner. Immunohistochemistry for neuronal nuclei (NeuN) was performed in order to additionally demonstrate neuronal localization of injected Cy-3 siRNA. All Cy-3-positive cells expressed the neuronal marker NeuN (Fig. 3C–H).

Attenuation of target gene expression in situ
siRNAs with the most pronounced silencing activity (anti-Bax #2, anti-Apaf-1 #2 and anti-c-Jun #2) were used in order to demonstrate a silencing of the target genes in situ. siRNAs were injected directly after axotomy and animals were sacrificed at day 4 after injection.

A basal expression of Bax was observed in the retinal ganglion cell layer (RGL) in non-axotomized animals. Axotomy combined with injection of control siRNA (anti-EGFP) induced an increase in Bax immunoreactivity, mainly in the RGL. Anti-Bax siRNA maintained the Bax immunoreactivity at a level comparable to non-axotomized controls (Fig. 4A–C).
Apaf-1 showed a weak expression pattern in the control RGL, which was only slightly stronger in axotomized and control-siRNA injected animals. Injection of Anti-Apaf-1 siRNA resulted in down-regulation of Apaf-1 immuno-reactivity below non-axotomized controls (Fig. 4E–G).

Immunoreactivity for c-Jun was hardly detectable in non-axotomized retinas. Following axotomy, a marked increase of c-Jun immunostaining in the nuclei of cells located in the RGL could be observed, which was distinctly less pronounced in anti-c-Jun siRNA-injected animals (Fig. 4I–K).

Down-regulation of proapoptotic proteins attenuates axotomy-induced RGC death in vivo

Each of the two siRNAs designed to target either Bax, Apaf-1 or c-Jun, as well as one control siRNA targeting EGFP, were tested in the axotomy model in order to correlate silencing effects in vitro and in vivo.

At day 14 post-axotomy, control retinas from non-injected or anti-EGFP siRNA injected eyes contained 200 ± 12 or 284 ± 105 RGC per mm², respectively (not significantly different). Retinas injected with siRNA against Bax did not show any significant difference in RGC numbers compared with either control. Both siRNAs against Apaf-1 significantly increased RGC numbers in comparison with non-injected control retinas, to ~2-fold (421 ± 70 and 424 ± 100 RGC per mm², respectively). Retinas injected with siRNAs against c-Jun showed the most pronounced increase in RGC, containing ~2- to 3-fold more RGC than non-injected controls (428 ± 109 and 612 ± 230 RGC per mm², respectively). In anti-c-Jun #2-injected retinas the increase in RGC numbers was significant as compared with both non-injected and anti-EGFP-injected controls (Table 1 and Fig. 5A).

We noted that RGC numbers in different counting fields of siRNA-injected retinas showed a high variation, which was beyond well known physiological differences in RGC density between inner and outer retinal eccentricities, and thus resulted in unusually high standard deviations. Standard deviations of RGC numbers in non-injected control retinas were much less prominent. RGC counts from all counted fields were therefore plotted in a histogram. In control retinas (non-injected, anti-EGFP-injected) as well as the anti-Bax #1-injected retina RGC, numbers were mostly <500 per field. Anti-Bax #2- and both anti-c-Jun-injected retinas showed a markedly increased number of fields, with >500 RGC per field. Most remarkably, in anti-c-Jun #2-injected retinas only 37 out of 84 (44%) counted fields...
had <500 RGC per field, while 11 fields (13%) showed RGC numbers of >1000 (Fig. 5B). We attribute the increased standard deviation in RGC numbers of siRNA-injected retinas to the fact that, due to the siRNA-injection technique, not all regions of the retina were targeted in the same way (see also Fig. 3A and B). siRNA-mediated silencing and down-regulation of target genes thus occurs heterogeneously within each retina.

Fig. 4 Photomicrographs of retinal sections demonstrating regulation of target protein expression in vivo. Basal expression of c-Jun, Bax and Apaf-1 can be detected in the RGC layer of non-axotomized control animals (A, E, I). Axotomized and anti-EGFP siRNA-injected animals show an up-regulation of c-Jun, Bax and Apaf-1 in the RGC layer at day 4 post-axotomy (B, F, J). Retinas injected with siRNAs directed against c-Jun and Apaf-1 showed a marked down-regulation of protein expression at day 4 post-axotomy (C, G, K). Sections where the primary antibody has been omitted are shown as controls (D, H, L). Upper rows show target protein expression (Cy3), middle rows nuclear counter stain (DAPI) and lower rows merged images (Cy3/DAPI). Bar = 50 μm.
Discussion

Since the discovery of siRNAs as the key mediator of RNA-induced gene silencing, RNAi has rapidly evolved to become a tool for gene regulation in mammals (Elbashir et al., 2001a). Delivery of nucleic acids to target cells, however, has been one of the major problems encountered, especially in mammalian neurons. In vitro, many cell lines can be efficiently transfected using cationic liposomes, but primary neuron cultures suffer from pronounced cytotoxic effects and low transfection efficiency of liposome-based agents (Ohki et al., 2001). We have shown recently that application of non-conjugated siRNA to primary neuron
cultures results in its endosomal uptake, but only minimal specific regulatory effects (Lingor et al., 2004b). For in vivo studies, application of non-conjugated siRNAs by intravenous injection or electroporation has proven successful (Calegari et al., 2002; Song et al., 2003). Gene regulation via DNA-based RNAi vectors has been recently shown to successfully suppress expression of transcription factors when electroporated into the retina. Different retinal layers were transduced with varying efficiencies, but siRNA delivery by electroporation did not allow for specific targeting of RGC (Matsuda and Cepko, 2004).

We show here that injection of siRNA into the optic nerve stump is a specific means of siRNA delivery to the cytoplasm of RGC. Anatomically, RGC are the only retinal neurons extending their axons via the optic nerve, thus allowing them to be selectively retrogradely targeted (Kermer et al., 2001). By application of siRNAs to the axon stumps directly following axotomy we made use of the model-inherent disruption of cell-/axon-integrity, thus avoiding well known difficulties of siRNA delivery across cell membranes. Application of siRNA by intraocular injection to the vitreous was equally considered, since it represents a common method of drug delivery to RGC. Intraocular injection, however, would not exclude the targeting of cell types different from RGC, and necessitates a sufficient penetration of siRNA across the RGC membrane, which we found to be a limiting step in previous studies on primary neurons (Lingor et al., 2004b).

We noticed, however, that the distribution of the siRNA in the retina was not homogeneous, due to the injection technique itself (Fig. 3), and resulted in an increased statistical error in RGC counts (Fig. 5 and Table 1). The effects of siRNA on functional parameters are therefore likely to be underestimated, which has to be considered in the interpretation of the results.

In this study we applied the siRNA-mediated gene silencing technique in order to elucidate the role of proteins involved in different steps of the axotomy-induced apoptotic cell death cascade of retinal ganglion neurons.

An up-regulation of the immediate early gene c-Jun in RGC has been described previously in models of axotomy or ischaemia (Hüll and Bähr, 1994a; Lu et al., 2003; Yoshimura et al., 2003). However, it was not clear whether c-Jun up-regulation represents an important step in apoptotic RGC death or whether it is an epiphenomenon. In other models of neurodegeneration, inhibition of c-Jun successfully attenuated neuronal cell death thus favouring an active role of c-Jun in the induction of apoptosis: antibody-mediated inactivation of c-Jun reduced neuronal death induced by nerve growth factor deprivation in vitro and adenoviral expressed dominant-negative c-Jun delayed the degeneration of dopaminergic nigral axons in the striatum after medial forebrain bundle axotomy in vivo (Estus et al., 1994; Crocker et al., 2001). We show here that inhibition of c-Jun by a single siRNA injection increases the number of RGC at 14 days post-axotomy by 2- to 3-fold. As we have shown, Cy3-siRNA is strongly visible in RGC at 24 and 72 h post-transsection (Fig. 3), which is indicative of fast retrograde transport of siRNA to the cell soma. The presence of siRNA in RGC coincides with known expression kinetics of c-Jun following optic nerve transection with a maximal c-Jun expression at day 3 post-axotomy (Hüll and Bähr, 1994). Our data thus support the hypothesis that c-Jun activation after axotomy represents an active step in the induction of apoptotic cell death in RGC of adult rats rather than being a secondary phenomenon.

Together with cytochrome c (Apaf-2) and pro-caspase-9 (Apaf-3), Apaf-1 is an essential mediator of caspase-induced cell death allowing for the formation of the apoptosome. RNAi techniques have been successfully employed in order to down-regulate Apaf-1 and thus inhibit apoptosis in vitro (Lassus et al., 2002). Down-regulation of Apaf-1 in RGC has not yet been shown. We show that down-regulation of Apaf-1 in RGC partially inhibits axotomy-induced cell death, thus attributing a role for Apaf-1 in the apoptotic cascade in RGC.

Bax plays an important role in ontogenetic and lesion-induced apoptotic neuron death (White et al., 1998). In the retina, an up-regulation of Bax has been demonstrated in RGC following optic nerve crush (Isenmann et al., 1997) and ischaemic retinal damage (Kaneda et al., 1999). Furthermore, down-regulation of Bax using antisense oligonucleotides decreased apoptotic RGC death after axotomy (Isenmann et al., 1999). In our paradigm, both Bax siRNAs showed a pronounced down-regulation of Bax protein in the in vitro paradigm, while regulation of Bax expression in situ was less markedly observed. In vivo anti-Bax #2 siRNA-injected retinas showed a trend towards increased RGC numbers without reaching significance levels.

Failure to significantly regulate RGC numbers in vivo while showing effective regulation in vitro are likely to be attributed to limited availability of siRNA due to the singular injection. Although siRNA-mediated gene silencing has been shown to last for up to 3 weeks in mammalian neurons in vitro (Omi et al., 2004), the kinetics of siRNA degradation may prevent sufficient regulation of genes with a long half-life or protracted expression.

We thus propose that RNAi-mediated gene silencing in vivo markedly depends on (i) the time course of expression of target proteins, and (ii) the kinetics of siRNA application. While c-Jun is known to be rapidly up-regulated following an apoptosis inducing lesion a single siRNA injection has been shown to be sufficient to attenuate c-Jun expression and RGC death. Acting further downstream, Bax and Apaf-1 are activated at later time points in the apoptotic cascade, when the extent of down-regulation by a single siRNA injection may not be completely sufficient (Bähr, 2000). Application of siRNA at multiple time points or more prolonged siRNA expression using hairpin vectors (Miyagishi and Taira, 2004) could additionally result in more powerful silencing effects, and such approaches are currently being tested by our group.
Overall, our results indicate that siRNAs can be used successfully to elucidate functional roles of proteins within the apoptotic cascade in vivo. In the future, RNAi may thus not only have roles in the dissection of intracellular pathways, but likely may be used for therapeutic targeting of proteins involved in neurodegeneration.

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