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Preconditioning-Induced Protection from Oxidative Injury is Mediated by Leukemia Inhibitory Factor Receptor (LIFR) and its Ligands in the Retina

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Abstract

Preconditioning with moderate oxidative stress (e.g., moderate bright light or mild hypoxia) can induce changes in retinal tissue that protect photoreceptors from a subsequent dose of lethal oxidative stress. The mechanism underlying this induced protection is likely a general mechanism of endogenous protection which has been demonstrated in heart and brain using ischemia and reperfusion. While multiple factors like bFGF, CNTF, LIF and BDNF have been hypothesized to play a role in preconditioning induced endogenous neuroprotection, it has not yet been demonstrated which factors or receptors are playing an essential role. Using quantitative PCR techniques we provide evidence that in the retina, LIFR activating cytokines leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1) and cardiotrophin like cytokine (CLC) are strongly up regulated in response to preconditioning with bright cyclic light leading to robust activation of signal transducer and activator of transcription-3 (STAT3) in a time-dependent manner. Further, we found that blocking LIFR activation during preconditioning using a LIFR antagonist (LIF05) attenuated the induced STAT3 activation and also resulted in reduced preconditioning-induced protection of the retinal photoreceptors. These data demonstrate that LIFR and its ligands play an essential role in endogenous neuroprotective mechanisms triggered by preconditioning-induced stress.

Keywords

bright light preconditioning; leukemia inhibitory factor receptor (LIFR); glycoprotein 130 (gp130); endogenous neuroprotection; photoreceptor; antagonist; kinetics

Introduction

Photoreceptor cells are the sensory neurons in eye that absorb light to initiate vision. Since these cells are post mitotic, loss of photoreceptors results in permanent blindness. Insults that can kill photoreceptors include genetic mutations, mechanical injury and oxidative damage

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(Bird 1995; Faktorovich *et al.* 1992; Lavail *et al.* 1992; Penn *et al.* 1987). Prolonged bright light exposure can also induce oxidative damage which, when severe, kills photoreceptors (Noell *et al.* 1966; Penn *et al.* 1987). However, under such unfavorable conditions, retinal cells initiate a response to rescue photoreceptors by recruiting or secreting a variety of antioxidants, cytokines and/or neurotrophic factors (Chaum 2003; Lavail *et al.* 1992; Liu *et al.* 1998; Penn *et al.* 1987; Steinberg *et al.* 1995; Wen *et al.* 1995; Wen *et al.* 1998). This has been clearly demonstrated in models where exposure to subtoxic levels of stress (e.g., bright cyclic light) induced changes in retinal tissue that protect photoreceptors from a subsequent dose of lethal stress (Li *et al.* 2001; Li *et al.* 2003; Liu *et al.* 1998).

Factors that were shown to be up regulated under oxidative stress include basic fibroblast growth factor (bFGF), ciliary neurotrophic factor (CNTF), brain derived neurotrophic factor (BDNF), LIF, and CLC (Chaum 2003; Faktorovich *et al.* 1992; Lavail *et al.* 1992; Rattner *et al.* 2008; Samardzija *et al.* 2006; Zachary 2005). While these are hypothesized to play a role in preconditioning-induced endogenous neuroprotection, it has not yet been demonstrated which factors or receptors are essential for the protection. Intriguingly, among the up regulated molecules LIF, CNTF, and CLC belong to the same family and signal through heterodimerization of leukemia inhibitory factor receptor (LIFR) and glycoprotein 130 (gp130). Since these ligands and receptors are functional in the retina (Sherry *et al.* 2005; Ueki *et al.* 2008; Wen *et al.* 1998), our hypothesis is that activation of LIFR: gp130 complex plays an essential role in preconditioning-induced endogenous protection of retinal photoreceptors. This hypothesis predicts that inhibiting the activation of these receptors during stress would make the photoreceptor cells more susceptible to oxidative damage. LIF05, a mutant LIF molecule, antagonizes LIF, CNTF, CT-1 and CLC activities by competitively binding and blocking the LIFR dimerization with gp130 (Hudson *et al.* 1996; Vernallis *et al.* 1997). In this study, we tested our hypothesis by delivering LIF05 during preconditioning. The data show that inhibiting LIFR activation blocks the protective effects of preconditioning resulting in increased photoreceptor sensitivity to oxidative stress.

Materials and Methods

Recombinant protein expression and purification

Expression and purification of human LIF and LIF05 was performed as described previously (Robinson *et al.* 1994b). Briefly, LIF and LIF05 were expressed as glutathione-S-transferase (GST) fusion proteins in *E. coli* strain JM109. Cultures were grown in LB plus ampicillin (100µg/ml) at 37°C and 300 rpm until they reached midlog phase ($A_{600} = 0.6$). Isopropyl β -D-1-thyogalactopyranoside (IPTG) was then added to the culture to a final concentration of 0.1 mM and induction was carried out for additional 3 h at room temperature. Intracellular fusion protein was recovered from cell extracts by affinity binding to a slurry of glutathione-Sepharose 4B beads (GE Healthcare, Uppsala, Sweden). Washes were carried out as described by the manufacturer's protocol. Isolation of LIF or LIF05 was achieved by cleavage of the fusion protein with human thrombin (Amersham Biosciences, Piscataway, NJ) in 1X PBS (pH 7.3) overnight at room temperature. Following cleavage, the elution containing hLIF or LIF05 was pooled with additional 4 batch washes (1X PBS, pH 7.3). Cleaved hLIF or LIF05 was further purified by fast protein liquid chromatography (FPLC) using a Mono-S cationic exchange column (Amersham Biosciences, Piscataway, NJ). Elution was carried out with a linear gradient of 0 – 1 M NaCl in 20 mM MES buffer (pH 6.0). Eluted fractions were analyzed using SDS-PAGE and the fractions containing enriched LIF or LIF05 were pooled and concentrated by ultrafiltration (Millipore Corporation, Billerica, MA). Purities of LIF and LIF05 were >90% as evaluated by SDS-PAGE. Purified LIF and LIF05 were tested on human retinal Müller cell cultures to determine biological activity and rule out contamination with

endotoxins. Concentrations were determined using BCA assay as described by the manufacturer's protocol (Thermo Scientific, Rockford IL)

Kinetic analysis of LIFR and gp130 interaction with LIF and LIF05

Interactions of the cytokine receptor domains with immobilized LIF or LIF05 were analyzed by surface plasmon resonance (SPR) using the SensiQ system (ICX Technologies, Oklahoma City, OK) as described by the manufacturer. A carboxyl sensor, with two channels, was installed in SensiQ and allowed to thermally equilibrate for about 15 min. The sensor was cleaned with a 3 min injection of 0.1 M HCl. An activation solution of 2 mM 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and 0.5 mM N-hydroxysulfosuccinimide (NHS) was prepared in deionized water immediately before injection. This activation solution was injected over both channels for approximately 3 min. Activated carboxyl surface was incubated with a 11 min injection of 50 µg/mL anti-GST in 10 mM acetate buffer pH 5.0 over both channels. Any remaining NHS esters were capped with a 3 min injection of 1 M ethanolamine, pH 8.0. A solution of GST-LIF or GST-LIF05 was prepared for each experiment at 50 nM in running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween-20). The solution was injected over channel 1 until an appropriate amount of LIF or LIF05 was captured. Channel 2 was left unmodified to serve as a reference for non-specific binding to the anti-GST surface. A series of concentrations of soluble LIFR (# 249-LR-040/CF, R&D Systems, Minneapolis, MN) and/or soluble gp130 (# 228-GP-050/CF, R&D Systems, Minneapolis, MN) in running buffer were injected over the sensor chip to measure binding parameters with LIF and LIF05 surfaces. Following dissociation, the surfaces were regenerated by injecting 20 mM NaOH for 2 min.

Animals and bright cyclic light preconditioning

All procedures were performed according to the ARVO statement for the use of animals in ophthalmic and vision research. BALB/cJ mice (5-6 weeks of age) were obtained from Jackson laboratories and maintained in 60 lux cyclic light at cage level (12h ON: 12h OFF) for 7 days. Animals were then maintained in 600 lux cyclic bright light for preconditioning (12h ON: 12h OFF) for 6 days followed by a 4 day recovery period under normal cage room conditions (i.e., 60 lux: 12h ON: 12h OFF) (Figure 1).

Bright-light induced damage

For bright light damage, unanesthetized mice were exposed to diffuse white fluorescent light maintained at 4000 lux at cage level for 4 h immediately after the bright light exposure (600 lux) on the 6th day of preconditioning (See Figure 1). Control mice were maintained in normal cage room light condition (60 lux) prior to exposure to 4000 lux damaging light. After light exposure, all mice were allowed to recover for 8 hours in the dark and then kept in normal cage room lighting for 4 days before analysis by ERG and histology.

Electroretinograms (ERGs)

ERGs were recorded using a Colordome ERG instrument (Diagnosys, Littleton, MA) to measure the function of surviving photoreceptors. Analysis was carried out as described previously by Ueki et al. (Ueki *et al.* 2008). Briefly, after an 8-hour dark adaptation, mice were deeply anesthetized with a single intraperitoneal injection of xylazine (7 mg/kg) and ketamine (40 mg/kg). Mice were then placed on a 37°C heating pad throughout the experiment. Pupils were dilated with tropicamide and phenylephrine. Full-field ERGs were recorded from both eyes using gold wire electrodes placed centrally on the cornea. A platinum reference electrode was attached in the mouth, and a platinum needle electrode in the tail served as a ground. Electrode positioning was monitored throughout the measurements using an infrared camera. A series of increasing flash intensities over a range of 6 logarithmic units of intensity (0.001,

0.01, 1, 100, 200 and 400 cd.s/m²) were used. The amplitude of the a-wave was measured from the baseline to the trough of a-wave. After the ERG recording, retinas were harvested for histological analysis.

Histological analysis

Mice were euthanized with CO₂ and the eyes were enucleated and marked with a green dye on dorsal surface to mark the superior hemisphere. Eyes were then fixed in PERFIX (20% isopropanol, 2% trichloroacetic acid, 4% paraformaldehyde, and 2% zinc chloride) overnight and placed in 70% ethanol at room temperature for 4 days followed by embedding in paraffin. Sagittal sections through the center of the eye, including the optic disc, were cut at 5µm thickness. After hematoxylin and eosin (H&E) staining, rows of photoreceptor nuclei were counted using light microscopy at nine equidistant points beginning at the optic nerve head toward both the inferior and superior retinal hemispheres.

Intravitreal Injections

Normal BALB/cJ mice were deeply anesthetized with a single intraperitoneal injection of xylazine (7 mg/ kg) and ketamine (40 mg/kg). One microliter of LIF, LIF05 or PBS (vehicle control) were injected into the vitreous chamber of the eye using a 36 gauge needle (World Precision Instruments, Sarasota, FL) through the temporal lymbus of the eye. For inhibition studies during preconditioning, 8 µg of LIF05 were injected into the right eye of BALB/cJ mice in a volume of 1 µl PBS after 2 days of bright cyclic light preconditioning. One microliter of PBS was injected into the right eye which served as control. Following injection, the mice were returned to their cages for 4 more days of bright cyclic light preconditioning. After a total of 6 days of preconditioning, mice were euthanized and the eyes were enucleated and subjected to western blot analysis.

Western blots

Retinas were harvested immediately after animals were euthanized, and homogenized in a lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% (v/v) NP-40, 5% (v/v) glycerol, and protease inhibitor cocktail (Calbiochem, San Diego, CA). Protein content was measured using BCA protein assay (Pierce, Rutherford, IL). Total protein from each sample (15 µg) was electrophoresed on 4-20% gradient SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA), and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were incubated in blocking buffer [5% BSA in TBST (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween-20)] for 1 h at room temperature, and then incubated overnight at 4°C with rabbit polyclonal antiphospho-STAT3 antibody (#9131, Cell Signaling Technology, Beverly, MA), in blocking buffer, followed by 1-h incubation at room temperature with HRP-conjugated goat anti-rabbit secondary antibody (Amersham Biosciences, Piscataway, NJ). Signals were visualized using SuperSignal West Dura extended duration substrate (Pierce, Rutherford, IL) and quantified by conventional digital image analysis using an ImageStation 4000R (Eastman Kodak, Rochester, NY). Blots were stripped and reprobed with rabbit polyclonal anti-STAT3 (#9132, Cell Signaling Technology, Beverly, MA) and anti-β actin (ab6276, Abcam) followed by appropriate secondary antibodies for quantification of bands.

Real-time PCR

mRNA levels of selected genes were measured using real-time PCR with cDNA extracted from retinas as templates. Primers (Table I) were designed using PrimerQuest software (Integrated DNA Technologies, Coralville, IA, USA) spanning the intron–exon boundary to amplify the corresponding mRNAs without amplifying potentially contaminating genomic DNA. Real-time PCR was carried out with the SYBR green PCR master mix (Bio-Rad Lab., Hercules,

CA, USA) using the MyiQ Single-Color Real-Time PCR detection system (Bio-Rad Lab., Hercules, CA, USA) following the manufacturer's instructions. Electrophoresis of PCR products was performed to identify that a single band of the correct size had been amplified.

Statistical Analysis

All statistical analyses are done using SigmaStat 3.10 (Systat Software, Inc. Richmond, CA). Results are expressed as mean $\pm$ standard deviation (SD). Differences between two groups were assessed using either paired or unpaired t-tests while differences between more than two groups were assessed using Analysis of Variance (ANOVA) followed by Holm-Sidak *post hoc* test. A *p* value less than 0.05 was considered significant.

Results

Preconditioning-induced protection

Functional and morphological evaluation of photoreceptor cell protection by bright cyclic light preconditioning was carried out using ERG and quantitative histological analysis. BALB/cJ mice, with or without prior bright cyclic light preconditioning were subjected to severe light stress before returning them to room light conditions for 4 days of recovery followed by ERG and histological analysis. Figures 2A and 2B show amplitudes of ERG a-waves in response to different intensities of light flashes. a-waves represent the electric response generated in photoreceptors and thus serve as a measure of photoreceptor function in response to light. As expected, mice that were exposed to severe light stress without preconditioning exhibited almost complete loss of photoreceptor function. However, mice that were preconditioned with bright cyclic light (600 lux; 6AM to 6PM for 6 days) prior to the severe light stress were able to retain almost 83% of their retinal function. Figures 2C and 2D show representative sections of the superior retina and quantitative analysis of photoreceptor numbers in the outer nuclear layer (ONL), respectively. As reflected in the ERG analysis, animals that were subjected to light stress without preconditioning exhibited severe photoreceptor degeneration. Compared to normal, undamaged retina that contain 10-11 rows of photoreceptor nuclei in the ONL, these animals retained only 1-2 rows of photoreceptor nuclei following bright light exposure. However, in animals that were subjected to bright cyclic light preconditioning, the degeneration was modest, as mice retained 9-10 rows of photoreceptor nuclei in spite of the severe light stress. These data demonstrate that preconditioning with subtoxic light stress protects the retina from a subsequent dose of lethal light damage.

Expression of neuroprotective factors during preconditioning

Previous reports have shown that injury or stress in the retina can induce expression of neuroprotective factors (Cao *et al.* 2001; Cao *et al.* 1997; Faktorovich *et al.* 1990; Faktorovich *et al.* 1992; Lavail *et al.* 1991; Wen *et al.* 1995). To determine which factors could be responsible for preconditioning-induced protection in our model, we measured gene expression changes of neuroprotective factors that include LIF, CNTF, Oncostatin M (OSM), CT-1, CLC and BDNF. Ribosomal protein RPL19 was used as control. Results show that LIF, OSM, CT-1 and CLC exhibit a strong upregulation (166, 74, 21 and 47 fold respectively) with bright cyclic light preconditioning in a time-dependent manner (Figure 3). Surprisingly, unlike in rats (Liu *et al.* 1998) we did not observe any upregulation of CNTF. BDNF, another neuroprotective factor, exhibited an 11 fold increase in its levels by the end of the 6th day of preconditioning. We then tested whether this strong upregulation of LIF, OSM, CT-1, CLC and BDNF is reflected at a functional level by measuring activation of a common downstream signaling molecule, STAT3. Results show a robust time-dependent activation of STAT3 with bright cyclic light preconditioning (Figures 3B and 3C). STAT3 reached peak activation by the end of 4 days of preconditioning and remained elevated with 6 days of preconditioning. Two other pathways that are known to signal downstream of gp130 include Akt and ERK. However, we

did not observe significant changes in activation of these pathways with preconditioning (data not shown) suggesting that preconditioning is more strongly associated with STAT3 activation. Activation of STAT3 could be mediated by LIFR:gp130 heterodimer (LIF, CT-1, CLC), OSMR:gp130 heterodimer (OSM) or the TrkB receptor (BDNF). Therefore, we tested whether LIFR:gp130 activation is essential for preconditioning-induced STAT3 activation and protection.

Antagonism of LIFR attenuates preconditioning-induced STAT3 activation

hLIF has two active sites, site II and site III that bind to gp130 and LIFR respectively (Gearing *et al.* 1991; Gearing *et al.* 1992b; Gearing *et al.* 1992a; Hudson *et al.* 1996; Plun-Favreau *et al.* 2003; Robinson *et al.* 1994a). LIF first binds LIFR and then recruits gp130 forming a high affinity heterodimer complex (Gearing *et al.* 1992b; Gearing *et al.* 1992a). This heterodimerization leads to the activation of Jak mediated STAT3 pathway (Ernst *et al.* 1994; Stahl *et al.* 1994; Taniguchi 1995). Hudson *et al.*, (Hudson *et al.* 1996; Vernallis *et al.* 1997) have mutated the gp130 binding sites on hLIF generating the antagonist LIF05, which binds LIFR but can no longer bind gp130 to activate the STAT3 pathway. It thus acts as a competitive inhibitor for all cytokines that recruit LIFR (Vernallis *et al.* 1997). If injected *in-vivo*, LIF05 is predicted to block the activities of LIFR activating cytokines LIF, CNTF, CT-1 and CLC. Unlike in humans, mOSM can signal only through OSMR:gp130 (type II receptor complex) but not LIFR:gp130 (type I receptor complex) (Ichihara *et al.* 1997). Thus, LIF05 cannot antagonize OSM activities in mice. To determine whether LIFR activation was responsible for preconditioning-induced STAT3 activation we injected 2, 5 or 8 µg of LIF05 into the right eyes of mice after 2 days of preconditioning while left eyes were injected with PBS to serve as controls. Following injection, mice were preconditioned 4 additional days in bright cyclic light. After a total of 6 days of preconditioning, STAT3 activation was measured using Western blot analysis. Figure 4 shows that 2 µg of LIF05 was able to inhibit 45% of the preconditioning-induced STAT3 activation while 5 µg and 8 µg of LIF05 were able to block the activation by about 60% and 70%, respectively. This clearly demonstrates that LIF05 is able to inhibit the preconditioning-induced STAT3 activation in a dose-dependent manner by antagonizing LIFR activation. Injection of 12 µg LIF05 did not further decrease the STAT3 activation compared to 8 µg LIF05 (data not shown). This residual activation could be due to other factors including OSM and BDNF which LIF05 cannot antagonize. Clearly LIFR activation is playing an essential role in preconditioning induced STAT3 phosphorylation. Surprisingly, preconditioning induced a 2-fold increase in total STAT3 levels (Figure 3B). This increase was not reduced by LIF05 (Figure 4) suggesting that the increase in total STAT3 is a result of activation of pathways independent of LIFR such as OSMR or TrkB.

Antagonism of LIFR blocks preconditioning induced protection

In order to assess the role of LIFR in preconditioning-induced protection, we injected LIF05 during bright cyclic light preconditioning and determined whether blocking LIFR abolishes the preconditioning-induced protection. After 2 days of preconditioning, PBS or LIF05 (8µg) were injected intravitreally and the mice were returned to their cages for additional bright cyclic light preconditioning. After the 6th day of preconditioning (i.e., 4 days after injection), animals were subjected to severe light stress (4000 lux for 4h; 6PM to 10PM) immediately after the bright light adaptation and allowed to recover from the stress for 4 days under normal cage room light conditions (60 lux; 6AM to 6PM). Following recovery, animals were subjected to functional analysis using ERG and the eyes were harvested immediately for quantitative histological analysis. Figures 5A and 5B show that LIF05 injection reduced preconditioning induced preservation of the retinal function by 44% (compared at the brightest flash intensity, LIF05+PC+LD vs. PC+LD). This decrease in retinal function is caused by reduced protection of photoreceptors in the presence of LIF05 since we observe a significant increase in photoreceptor cell loss following light stress in LIF05 injected eyes (Figures 5C and 5D, 10

layers in PC+LD vs. 6 layers in LIF05+PC+LD). While PBS injection caused a slight decrease in the retinal function (Figures 5A and 5B) it did not lead to a significant decrease in photoreceptor survival (Figures 5C and 5D). The mechanism for the loss of function in PBS injected eyes without a loss in photoreceptor cells is currently unknown, but similar results have been observed in the absence of light stress where PBS injection causes a transient reduction in retinal function without causing any cell death. As expected, PBS injected control eyes that were subjected to light damage without preconditioning (PBS+LD), exhibited dramatic loss in retinal function and the photoreceptors, but was slightly protective compared to non-injected light damaged eyes (LD). Together, these results clearly suggest that blocking LIFR activation during preconditioning attenuates the endogenous protective mechanism triggered by bright cyclic light preconditioning.

Effect of LIF05 on normal retinal function

In order to rule out the possibility that injection of LIF05 itself kills photoreceptors, we injected 8 µg of LIF05 intravitreally into a normal BALB/cJ mice and measured the retinal function and morphology using ERG and histological analysis 8 days after injection. LIF05 did not induce any change in retinal function or morphology compared to PBS control (Figures 6A and 6B). This demonstrates that LIF05 had no toxic effects on retinal function and morphology up to dosages of 8 µg.

LIF05 inhibits LIF induced STAT3 activation

To demonstrate that LIF05 functions as an antagonist specifically against LIFR ligands, we intravitreally injected 0.1 µg of LIF, a quantity that was previously shown to activate STAT3 in the retina (Ueki *et al.* 2008), with or without the antagonist LIF05. PBS injected eyes served as negative controls. 2 days after injection, we measured the levels of STAT3 activation using Western blot analysis. While 2 µg of LIF05 was able to inhibit 50% of the LIF induced STAT3 activation 5µg and 8µg of LIF05 were able to inhibit STAT3 activation by 60% and 90% respectively (Figure 7). This clearly demonstrated that LIF05 inhibits LIFR activation by agonists *in-vivo* in a dose dependent manner. However, significantly larger amounts of LIF05 were needed (8µg) to effectively antagonize smaller doses of LIF (0.1 µg). To determine why such large quantities of antagonist are needed, we performed receptor binding assays using surface plasmon resonance (SPR).

LIF forms a high affinity stable complex with LIFR and gp130

Figures 8A and 8B show the SPR analysis of LIFR interaction towards LIF and LIF05. Recombinant soluble LIFR and gp130 were injected over immobilized GST-LIF or GST-LIF05 to determine the kinetic rate constants for association and dissociation. Results show that both LIF and LIF05 display high affinity towards LIFR, exhibiting fast association and dissociation rates yielding an equilibrium dissociation constant (K_D) of 2.93nM and 4.72nM respectively (See Table II). Also, LIF displays a weaker affinity towards gp130 exhibiting a slow association and fast dissociation from gp130 compared to LIFR yielding an equilibrium dissociation constant of 9.02nM (See Table II). Because of its mutations, as expected, LIF05 did not exhibit any affinity towards gp130 (Figure 8D; Table II). When LIFR and gp130 are injected together, LIF binds the receptors with fast association rate and exhibits a very slow dissociation rate suggesting the formation of a high affinity stable complex facilitated by the co-operative binding between LIF, LIFR and gp130 (Figure 8C). On the other hand, LIF05 because of its inability to bind gp130 cannot form a similar high affinity complex and thus dissociates from LIFR at a fast rate (Figure 8D). LIF bound to the heterodimer LIFR:gp130 is thus a more stable complex than LIF05 bound to LIFR. This justifies the need for high doses of LIF05 to antagonize a small quantity of LIF (Vernallis *et al.* 1997).

Discussion

Previous work and our results show that preconditioning with moderate light stress protects retinal photoreceptors from a subsequent exposure to damaging light (Li *et al.* 2001; Li *et al.* 2003; Liu *et al.* 1998; Penn *et al.* 1987) (Figure 2). However, the mechanism for this inducible protection is far from fully understood. Previous studies have shown that exposure to light stress results in a strong upregulation of CNTF, bFGF (in rats), LIF and CLC (in mice) (Liu *et al.* 1998; Samardzija *et al.* 2006). Separate studies have also shown that injection of these factors (CNTF, LIF or bFGF) in the absence of preconditioning can also protect photoreceptors from light damage (Faktorovich *et al.* 1992; Lavail *et al.* 1991; Lavail *et al.* 1992; Ueki *et al.* 2008) suggesting that their upregulation during stress is functionally connected to protection. In addition, other reports in rats have shown that photoreceptors near sites of mechanical injury are protected from a subsequent light stress. This protection was again accompanied by increases in the levels of bFGF and CNTF at the site of injury (Cao *et al.* 2001; Cao *et al.* 1997; Faktorovich *et al.* 1992; Wen *et al.* 1995). These findings clearly indicate that the retina has a self defense mechanism that helps it cope with unfavorable changes in the retinal tissue environment. However, the multiple factors that are up regulated can activate a variety of receptors and signaling pathways. It is thus far not clear yet, which of these pathways are primarily involved in the endogenous protective mechanism.

In our experiments with the bright cyclic light preconditioning model, we observed a strong upregulation of neuroprotective factors LIF, OSM, CT-1 and CLC which recruit either LIFR:gp130 or OSMR:gp130 complex for signaling (Figure 3A). However, unlike in rats, we did not notice any similar upregulation of CNTF in mice in response to bright cyclic light preconditioning (Figure 3A). A similar observation was made by Samardzija *et al.*, (Samardzija *et al.* 2006; Wenzel *et al.* 2005). This difference in the type of neuroprotective factors that regulate endogenous protection is probably attributed to species differences. Intriguingly LIF, CNTF, OSM, CT-1, and CLC, all belong to the same IL-6 family of cytokines that utilize gp130 receptor complexes to elicit similar and overlapping physiological responses mediated by STAT3 activation (Heinrich *et al.* 1998; Heinrich *et al.* 2003). So, the final neuroprotective responses in both mice and rats could be very similar. Also, we observed a modest increase in another neuroprotective factor, BDNF (Figure 3A) which signals via TrkB receptors. The robust activation of STAT3 in a preconditioning-dependent manner suggests that these expressed cytokines (LIF, OSM, CT-1 and CLC) are functional and activating the signal transducing receptor gp130 leading to STAT3 phosphorylation (Figure 3B and 3C). We thus hypothesized that activation of LIFR: gp130 complex plays an essential role in preconditioning-induced protection of retinal photoreceptors.

In mice LIF, CNTF, CT-1 and CLC bind to LIFR and gp130 to induce signaling while OSM utilizes OSMR and gp130. Blocking LIFR activation during preconditioning significantly attenuated induced STAT3 activation and protection of photoreceptor cells in the retina (Figures 4 and 5). These results confirm that LIFR is a vital part and plays an essential role in the endogenous protective mechanism inside the retina leading to photoreceptor survival and that the protection is likely mediated by its ligands LIF, CT-1 or CLC. In agreement with our observations, Joly *et al.* (Joly *et al.* 2008) have recently shown that LIF knockout mice have accelerated photoreceptor degeneration in a mouse model of inherited autosomal dominant retinitis pigmentosa. Together, these results demonstrate an important role for LIFR in an endogenous protective mechanism.

Our previous observations that intravitreal LIF injections can lead to signal transduction in photoreceptors demonstrates that LIF can penetrate retinal cell layers to directly activate LIFR/gp130 complexes on photoreceptors (Ueki *et al.* 2008). Our binding data demonstrates that LIF:LIFR:gp130 form a high affinity stable complex. In support of this, we have observed that

a single intravitreal injection of LIF resulted in STAT3 activation that persisted more than 6 days (data not shown). This would also suggest that LIF:LIFR:gp130 complexes are either not internalized at a fast rate or that they are recycled to the cell surface quickly. The formation of a stable complex between LIF, LIFR and gp130 (Figure 8), and persistent signal transduction suggests that this would make an effective ligand-receptor system for induction of long-term protection from a chronic stress.

Finally, the observation that LIF05 did not induce photoreceptor cell death under normal conditions (Figure 6) together with the evidence that there is no STAT3 activation at such conditions (Figures 3B and 3C, 0 days) suggests that LIFR is not active and its activation is not required for the survival of photoreceptors at physiological conditions. It is only under stressed conditions that this receptor is activated and leads to photoreceptor survival via STAT3 mediated survival pathways (Amaravadi and Thompson 2005). Endogenous inducibility, cooperative binding and prolonged signaling of LIF receptor system thus makes it ideally suited for long-term protection.

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References

- Amaravadi R, Thompson CB. The survival kinases Akt and Pim as potential pharmacological targets. *J. Clin. Invest* 2005;115:2618–2624. [PubMed: 16200194]
- Bird AC. Retinal photoreceptor dystrophies LI. Edward Jackson Memorial Lecture. *Am. J. Ophthalmol* 1995;119:543–562. [PubMed: 7733180]
- Cao W, Li F, Steinberg RH, Lavail MM. Development of normal and injury-induced gene expression of aFGF, bFGF, CNTF, BDNF, GFAP and IGF-I in the rat retina. *Exp. Eye Res* 2001;72:591–604. [PubMed: 11311051]
- Cao W, Wen R, Li F, Lavail MM, Steinberg RH. Mechanical injury increases bFGF and CNTF mRNA expression in the mouse retina. *Exp. Eye Res* 1997;65:241–248. [PubMed: 9268592]
- Chaum E. Retinal neuroprotection by growth factors: a mechanistic perspective. *J. Cell Biochem* 2003;88:57–75. [PubMed: 12461775]
- Ernst M, Gearing DP, Dunn AR. Functional and biochemical association of Hck with the LIF/IL-6 receptor signal transducing subunit gp130 in embryonic stem cells. *EMBO J* 1994;13:1574–1584. [PubMed: 8156996]
- Faktorovich EG, Steinberg RH, Yasumura D, Matthes MT, Lavail MM. Photoreceptor degeneration in inherited retinal dystrophy delayed by basic fibroblast growth factor. *Nature* 1990;347:83–86. [PubMed: 2168521]
- Faktorovich EG, Steinberg RH, Yasumura D, Matthes MT, Lavail MM. Basic fibroblast growth factor and local injury protect photoreceptors from light damage in the rat. *J. Neurosci* 1992;12:3554–3567. [PubMed: 1527595]
- Gearing DP, Comeau MR, Friend DJ, Gimpel SD, Thut CJ, McGourty J, Brasher KK, King JA, Gillis S, Mosley B. The IL-6 signal transducer, gp130: an oncostatin M receptor and affinity converter for the LIF receptor. *Science* 1992a;255:1434–1437. [PubMed: 1542794]
- Gearing DP, Thut CJ, VandeBos T, Gimpel SD, Delaney PB, King J, Price V, Cosman D, Beckmann MP. Leukemia inhibitory factor receptor is structurally related to the IL-6 signal transducer, gp130. *EMBO J* 1991;10:2839–2848. [PubMed: 1915266]
- Gearing DP, VandenBos T, Beckmann MP, Thut CJ, Comeau MR, Mosley B, Ziegler SF. Reconstitution of high affinity leukaemia inhibitory factor (LIF) receptors in haemopoietic cells transfected with the cloned human LIF receptor. *Ciba Found. Symp* 1992b;167:245–255. [PubMed: 1425017]

- Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem. J* 2003;374:1–20. [PubMed: 12773095]
- Heinrich PC, Behrmann I, Muller-Newen G, Schaper F, Graeve L. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem. J* 1998;334(Pt 2):297–314. [PubMed: 9716487]
- Hudson KR, Vernallis AB, Heath JK. Characterization of the receptor binding sites of human leukemia inhibitory factor and creation of antagonists. *J. Biol. Chem* 1996;271:11971–11978. [PubMed: 8662595]
- Ichihara M, Hara T, Kim H, Murate T, Miyajima A. Oncostatin M and leukemia inhibitory factor do not use the same functional receptor in mice. *Blood* 1997;90:165–173. [PubMed: 9207450]
- Joly S, Lange C, Thiersch M, Samardzija M, Grimm C. Leukemia inhibitory factor extends the lifespan of injured photoreceptors in vivo. *J. Neurosci* 2008;28:13765–13774. [PubMed: 19091967]
- Lavail MM, Faktorovich EG, Hepler JM, Pearson KL, Yasumura D, Matthes MT, Steinberg RH. Basic fibroblast growth factor protects photoreceptors from light-induced degeneration in albino rats. *Ann. N. Y. Acad. Sci* 1991;638:341–347. [PubMed: 1785811]
- Lavail MM, Unoki K, Yasumura D, Matthes MT, Yancopoulos GD, Steinberg RH. Multiple growth factors, cytokines, and neurotrophins rescue photoreceptors from the damaging effects of constant light. *Proc. Natl. Acad. Sci. U. S. A* 1992;89:11249–11253. [PubMed: 1454803]
- Li F, Cao W, Anderson RE. Protection of photoreceptor cells in adult rats from light-induced degeneration by adaptation to bright cyclic light. *Exp. Eye Res* 2001;73:569–577. [PubMed: 11825027]
- Li F, Cao W, Anderson RE. Alleviation of constant-light-induced photoreceptor degeneration by adaptation of adult albino rat to bright cyclic light. *Invest Ophthalmol. Vis. Sci* 2003;44:4968–4975. [PubMed: 14578424]
- Liu C, Peng M, Laties AM, Wen R. Preconditioning with bright light evokes a protective response against light damage in the rat retina. *J. Neurosci* 1998;18:1337–1344. [PubMed: 9454843]
- Noell WK, Walker VS, Kang BS, Berman S. Retinal damage by light in rats. *Invest Ophthalmol* 1966;5:450–473. [PubMed: 5929286]
- Penn JS, Naash MI, Anderson RE. Effect of light history on retinal antioxidants and light damage susceptibility in the rat. *Exp. Eye Res* 1987;44:779–788. [PubMed: 3653273]
- Plun-Favreau H, Perret D, Diveu C, Froger J, Chevalier S, Lelievre E, Gascan H, Chabbert M. Leukemia inhibitory factor (LIF), cardiotrophin-1, and oncostatin M share structural binding determinants in the immunoglobulin-like domain of LIF receptor. *J. Biol. Chem* 2003;278:27169–27179. [PubMed: 12707269]
- Rattner A, Toulabi L, Williams J, Yu H, Nathans J. The genomic response of the retinal pigment epithelium to light damage and retinal detachment. *J. Neurosci* 2008;28:9880–9889. [PubMed: 18815272]
- Robinson RC, Grey LM, Staunton D, Vankelecom H, Vernallis AB, Moreau JF, Stuart DI, Heath JK, Jones EY. The crystal structure and biological function of leukemia inhibitory factor: implications for receptor binding. *Cell* 1994b;77:1101–1116. [PubMed: 8020098]
- Robinson RC, Grey LM, Staunton D, Vankelecom H, Vernallis AB, Moreau JF, Stuart DI, Heath JK, Jones EY. The crystal structure and biological function of leukemia inhibitory factor: implications for receptor binding. *Cell* 1994a;77:1101–1116. [PubMed: 8020098]
- Samardzija M, Wenzel A, Auenberg S, Thiersch M, Reme C, Grimm C. Differential role of Jak-STAT signaling in retinal degenerations. *FASEB J* 2006;20:2411–2413. [PubMed: 16966486]
- Sherry DM, Mitchell R, Li H, Graham DR, Ash JD. Leukemia inhibitory factor inhibits neuronal development and disrupts synaptic organization in the mouse retina. *J. Neurosci. Res* 2005;82:316–332. [PubMed: 16206277]
- Stahl N, Boulton TG, Farruggella T, Ip NY, Davis S, Witthuhn BA, Quelle FW, Silvennoinen O, Barbieri G, Pellegrini S. Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. *Science* 1994;263:92–95. [PubMed: 8272873]
- Steinberg RH, Song Y, Cheng T, Matthes MT, Yasumura D, Lavail MM, Wen R. Exposure to constant light upregulates the expression of bFGF, CNTF, FGFR-1 and GFAP mRNAs in the rat retina. *Invest Ophthalmol. Vis. Sci* 1995;36:S637.
- Taniguchi T. Cytokine signaling through nonreceptor protein tyrosine kinases. *Science* 1995;268:251–255. [PubMed: 7716517]

- Ueki Y, Wang J, Chollangi S, Ash JD. STAT3 activation in photoreceptors by leukemia inhibitory factor is associated with protection from light damage. *J. Neurochem* 2008;105:784–796. [PubMed: 18088375]
- Vernallis AB, Hudson KR, Heath JK. An antagonist for the leukemia inhibitory factor receptor inhibits leukemia inhibitory factor, cardiotrophin-1, ciliary neurotrophic factor, and oncostatin M. *J. Biol. Chem* 1997;272:26947–26952. [PubMed: 9341130]
- Wen R, Cheng T, Song Y, Matthes MT, Yasumura D, Lavail MM, Steinberg RH. Continuous exposure to bright light upregulates bFGF and CNTF expression in the rat retina. *Curr. Eye Res* 1998;17:494–500. [PubMed: 9617544]
- Wen R, Song Y, Cheng T, Matthes MT, Yasumura D, Lavail MM, Steinberg RH. Injury-induced upregulation of bFGF and CNTF mRNAs in the rat retina. *J. Neurosci* 1995;15:7377–7385. [PubMed: 7472491]
- Wenzel A, Grimm C, Samardzija M, Reme CE. Molecular mechanisms of light-induced photoreceptor apoptosis and neuroprotection for retinal degeneration. *Prog. Retin. Eye Res* 2005;24:275–306. [PubMed: 15610977]
- Zachary I. Neuroprotective role of vascular endothelial growth factor: signalling mechanisms, biological function, and therapeutic potential. *Neurosignals* 2005;14:207–221. [PubMed: 16301836]

Figure 1.
Schematic diagram of bright cyclic light preconditioning

Figure 2.

Functional and morphological evaluation of preconditioning-induced protection of retinal photoreceptors. A) Representative ERG traces and B) a-wave amplitudes of BALB/cJ mice that have been light damaged (LD) (4000 lux for 4 h) with ($\circ$) or without ($\square$) prior bright cyclic light preconditioning (600 lux; 6 AM to 6 PM) for 6 days. Control eyes are represented in solid squares ($\blacksquare$). $n=6$; value = mean $\pm$ SD. (* $p < 0.001$, vs. LD eyes, paired t-test). C) Representative sections and D) quantification of number of rows of photoreceptor nuclei in the outer nuclear layer (ONL) along the vertical meridian of the retina. $n=6$; value = mean $\pm$ SD (* $p < 0.001$, vs. LD eyes, paired t-test). ONL – Outer nuclear layer; INL – Inner nuclear layer; GCL – Ganglion cell layer; ON – Optic nerve.

Figure 3.

Preconditioning with bright cyclic light leads to strong upregulation of neuroprotective factors. A) Time-dependent quantitative evaluation of cytokine mRNA upregulation using real time RT-PCR in response to bright cyclic light preconditioning (600lux; 6AM to 6PM). Members of IL-6 family cytokines (LIF, OSM, CT-1 and CLC) are upregulated 166, 74, 21 and 47 fold in response to bright cyclic light preconditioning. While CNTF did not exhibit a similar upregulation, BDNF exhibited an 11-fold increase in its mRNA levels by the end of the 6th day of preconditioning. Ribosomal protein RPL19 served as a control. $n=4$; value = mean $\pm$ SD. (* $p<0.01$, paired t-test). B) Representative Western blots and C) quantification of STAT3 activation. $n=3$; value = mean $\pm$ SD. We observed a robust preconditioning-dependent STAT3

activation which reached peak activation by the end of 4 days of preconditioning and remained elevated throughout the preconditioning exposure.

Figure 4.

LIF05 inhibited preconditioning-induced STAT3 activation. A) After 2 days of bright cyclic light preconditioning (600lux; 6Am to 6PM), mice were injected with PBS in the left eye (control) and LIF05 (2 μ g, 5 μ g or 8 μ g) in the right eye and returned to their cages for additional bright cyclic light preconditioning. After the 6th day of preconditioning, retinas were harvested and STAT3 activation was analyzed using Western blotting. PBS injected eyes without preconditioning served as controls for basal levels of STAT3 activation. PC – Preconditioning. B) Bands were quantified by conventional digital image analysis using a KODAK Image Station 4000R. n=3; value = mean $\pm$ SD. (*p<0.05, vs. PBS injected eyes with preconditioning, one way ANOVA and *post hoc* Holm-Sidak test for multiple comparisons).

Figure 5.

LIF05 inhibited preconditioning-induced protection of retinal photoreceptors. A) Representative ERG traces and B) quantification of a-wave amplitudes of BALB/cJ mice that are light damaged (LD) (4000lux for 4h) with (○, ▲, ◆) or without (△, □) prior bright cyclic light preconditioning (600lux; 6AM to 6PM for 6 days). Compared to uninjected eyes with preconditioning (○), LIF05 injected eyes lost 44% at the brightest flash intensity. $n=6$; value = mean $\pm$ SD (* $p<0.05$, vs. PC+LD eyes, paired t-test). C) Representative sections from superior retina and D) quantification of the number of rows of photoreceptor nuclei in the outer nuclear layer (ONL) along the vertical meridian of the retina. $n=6$; value = mean $\pm$ SD (* $p<0.05$, vs. PC+LD eyes, paired t-test). ONL – Outer nuclear layer; INL – Inner nuclear layer; GCL – Ganglion cell layer; ON – Optic nerve.

Figure 6.

Evaluation of LIF05 toxicity towards retinal function and morphology. A) Representative ERG traces and B) quantification of a-wave amplitudes of BALB/cJ mice 8 days after injection with PBS (■) or LIF05 (◇). C) Representative sections from superior retina and D) quantification of the number of rows of photoreceptor nuclei in the outer nuclear layer (ONL). n=3, value = mean ± SD. ONL – Outer nuclear layer; INL – Inner nuclear layer; GCL – Ganglion cell layer; ON – Optic nerve.

Figure 7.

LIF05 inhibits LIF stimulated STAT3 activation in vivo. A) 0.1 μ g of LIF was injected intravitreally with 0, 2, 5, or 8 μ g of the antagonist LIF05. After 2 days of injection, retinas were harvested and STAT3 activation levels were analyzed using Western blot analysis. Staining for total STAT3 and β -actin served as loading controls. A) Bands were quantified by conventional digital image analysis using a KODAK Image Station 4000R. $n=3$; value = mean $\pm$ SD (* $p<0.05$, vs. LIF only injected eyes (LIF 0.1 μ g), one way ANOVA and *post hoc* Holm-Sidak test for multiple comparisons).

Figure 8.

Kinetic analysis of LIFR and gp130 interaction towards LIF and LIF05. Soluble LIFR at concentrations of 1.5 nM, 3 nM, 6.25 nM, 12.5 nM, 25 nM or 50 nM were injected over an SPR sensor chip with anti-GST immobilized A) GST-LIF or B) GST-LIF05 at flow rates of 25 μ l/min. Responses obtained were corrected for background signal using a control flow cell. Association and dissociation rates are derived by global analysis of the response curves fit to a 1:1 kinetic model using QDat software (BioLogic Software, Ltd. Knoxville TN and Nomadics, Inc. Stillwater, OK) using 1:1 stoichiometry. Models are indicated in smooth gray line overlaid over response curve traces. Soluble gp130 (100 nM) was injected over C) GST-LIF or D) GST-LIF05 either as a mixture with soluble LIFR (10 nM) or separately at flow rates of 25 μ l/min.

Table I

Forward and reverse primers used in RT-PCR analysis. (F – Forward, R – Reverse, bp – base pairs)

Name		Sequences (5' to 3')	Product size (bp)
RPL19	F	TCACAGCCTGTACCTGAAGG	151
	R	TCGTGCTTCCTTGGTCTTAG	
LIF	F	TGAGATGCAGGGATTGTGCCCTTA	187
	R	AAATGAAGAGAGCATTGGCGCTGC	
OSM	F	TTTGACCCTCAGTCTCCTCATCCT	134
	R	AGGGCTCCAAGAGTGATTCTGTGT	
CT1	F	AAGACCACCAGACTGACTCCTCAA	126
	R	GCTGCACGTATTCCTCCAGAAGTT	
CLC	F	ACGAGCCTGACTTCAATCCTCCT	147
	R	ACGCAAGTAACACAGGAGGTGACT	
CNTF	F	AGAGCAATCACCTCTGACCCTTCA	189
	R	ATCTCACTCCAGCGATCAGTGCTT	
BDNF	F	AGAAGGTTTCGGCCCAACGAAGAAA	145
	R	GACATGTTTGCGGCATCCAGGTAA	

Table II
Comparison of association (k_a), dissociation (k_d) and estimated equilibrium dissociation (K_D) constants for the interaction of LIFR and gp130 with LIF and LIF05.

	LIFR (analyte)			gp130 (analyte)		
	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
LIF	3.66×10^5	1.07×10^{-3}	2.93	1.88×10^5	1.7×10^{-3}	9.02
LIF05	5.62×10^5	2.65×10^{-3}	4.72	ND	ND	ND