Cellular/Molecular

The Hormone Prolactin Is a Novel, Endogenous Trophic Factor Able to Regulate Reactive Glia and to Limit Retinal Degeneration

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Retinal degeneration is characterized by the progressive destruction of retinal cells, causing the deterioration and eventual loss of vision. We explored whether the hormone prolactin provides trophic support to retinal cells, thus protecting the retina from degenerative pressure. Inducing hyperprolactinemia limited photoreceptor apoptosis, gliosis, and changes in neurotrophin expression, and it preserved the photoresponse in the phototoxicity model of retinal degeneration, in which continuous exposure of rats to bright light leads to retinal cell death and retinal dysfunction. In this model, the expression levels of prolactin receptors in the retina were upregulated. Moreover, retinas from prolactin receptor-deficient mice exhibited photoresponsive dysfunction and gliosis that correlated with decreased levels of retinal bFGF, GDNF, and BDNF. Collectively, these data unveiled prolactin as a retinal trophic factor that may regulate glial–neuronal cell interactions and is a potential therapeutic molecule against retinal degeneration.

Key words: **gliosis; prolactin; retinal degeneration; trophic factor**

Introduction

Retinal degeneration results from the gradual destruction of retinal cells. Such deterioration occurs in aging retinas and accompanies a range of prominent human retinal disorders including age-related macular degeneration, glaucoma, and diabetic retinopathy (Zhang et al., 2012a). These disorders lead to some degree of visual disability, and their treatment remains an unmet need (Zhang et al., 2012a). Photoreceptors act as a fuse of sorts, since their irreversible loss has been identified as a cause of blindness in most degenerative retinopathies (Zack, 2000). Nevertheless, all retinal cells can contribute to disease activity as they eventually become dysfunctional or die.

Retinal homeostasis strongly relies on glial activity (Bringmann et al., 2006; Langmann, 2007). Glia help determine cell fate in the retina by contributing to the net secretion of proapoptotic and antiapoptotic signals, including growth factor and neurotrophins (Caffé et al., 1993; LaVail et al., 1998; Bringmann et al., 2006; Unterlauft et al., 2012). In addition to being neuroprotective, reactive glial cells can also damage neurons (Bringmann et al., 2006; Barres, 2008). Nevertheless, there is still no agreement on whether lessening gliosis, which is detected in every neurological disease and also in genetically determined and experimental models of inner and outer retinal degeneration (Bringmann et al., 2006; Grieshaber et al., 2012), is helpful or harmful (Barres, 2008). Though clear evidence emerged about a decade ago that the glia–neuron network is heavily involved in the survival of surrounding neurons *in vivo* (Bringmann et al., 2006; Barres, 2008), most aspects of its regulation remain to be elucidated. The identification of endogenous molecules able to modulate the function of this network will help promulgate retinal health. One such molecule may be prolactin, the hormone fundamental for lactation.

High levels of circulating prolactin in pregnant women are associated with increased electroretinogram (ERG) B-wave amplitude (De Luca Brunori et al., 1985). The primary role of prolactin in the retina, however, appears to be the regulation of its development, as prolactin gene deletion in zebrafish results in a thinner retinal inner nuclear layer (Huang et al., 2009). Also, prolactin promotes visual pigment synthesis in teleosts (Crim, 1975a,b; Allen and Cristy, 1978) and in amphibians (Crim, 1975b). In rodents and nonhuman primates, prolactin receptors

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 0270-6474/14/341868-11$15.00/0

Table 1. Primers used for real-time RT-PCR

mRNA	NCBI accession number	Direction	Sequence	Amplicon size (bp)	Primer efficiency (%)
m BNDF	EF125669.1	F	ATTACCTTCCTGCATCTGTTG	179	103.85
		R	TGTCCGTGGACGTTTACTTC		
r BDNF	NM_001270636.1	F	TCCACCAGGTGAGAAGAGTGATG	159	79.17
		R	TCACGCTCTCCAGAGTCCCATG		
m bFGF	NM_008006.2	F	ACTTAGAAGCCAGCAGCCG	154	99.84
		R	CCCGACGGCCGCGTGGAT		
r bFGF	NM_019305.2	F	GTCTCCCGCACCCTATCC	121	93.07
		R	ACAACGACCAGCCTTCCA		
m CNTF	NM_170786.2	F	CTCTATCTGGCTAGCAAGGA	146	72.81
		R	TCATCTCACTCCAGCGATCA		
r CNTF	NM_013166.1	F	GGACCTCTGTAGCCGTTCTA	161	101.35
		R	TCATCTCACTCCAACGATCA		
m GDNF	NM_010275.2	F	AATGTCCAACTGGGGGTCTA	180	133.16
		R	GCCGAGGGAGTGGTCTTC		
r GDNF	NM_019139.1	F	AGAGGGAAAGGTCGCAGAG	91	128.39
		R	AGCCCAAACCCAAGTCAGT		
m/r GFAP	NM_001131020.1/NM_017009.2	F	AGGCAGAAGCTCCAAGATGA	178/178	99.95
		R	GTTCTCGAACTTCCTCCTCA		
m HPRT	NM_013556.2	F	GTAATGATCAGTCAACGGGGGAC	177	98.39
		R	CCAGCAAGCTTGCAACCTTAACCA		
r HPRT	NM_012583.2	F	GACCGGTTCTGTCATGTCG	61	99.60
		R	ACCTGGTTCATCATCACTAATCAC		
m NGF	S62089.1	F	GGACGCAGCTTTCTATACTG	145	102.34
		R	TTCAGGGACAGAGTCTCCTT		
r NGF	NM_001277055.1	F	TGCATAGCGTAATGTCCATGTTG	149	159.97
		R	CTGTGTCAAGGGAATGCTGAA		
m PEDF	AF036164.1	F	TCCAACTTCGGCTACGATCT	143	103.8
		R	CGGTGAATGACAGACTCTGT		
r PEDF	NM_177927.2	F	CCAACTCTTTGCAGGACATG	78	97.67
		R	TCACAGGTTTGCCGGTAATC		
m rhodopsin	NM_145383.1	F	ATTCACCACCACCCTCTACA	155	94.80
		R	GCTTGCAGACCACCACGTA		
r prolactin R	NM_012630.2	F	CCAGGAGAGTTCCGTTGAAA	153	93.61
		R	GGTGGAAAGATGCAGGTCAT		

m, Mouse; r, rat; F, forward; R, reverse.

are present in both outer and inner nuclear layers of the retina and also in the ganglion cell layer (Rivera et al., 2008). Pioneering studies in rats proposed that prolactin modulates photoreceptor susceptibility to light-induced degeneration (O'Steen and Kraeer, 1977). Additionally, antibodies or antisense oligonucleotides targeting prolactin provoke retinal neovascularization and vasodilation (Aranda et al., 2005). Together, these data identify various types of retinal cells as prolactin targets, but studies remain sparse.

Considering that prolactin has been recently reported to be neuroprotective in the hippocampus (Morales, 2011) and that it signals through pathways similar to those triggered by CNTF, which is one of the neurotrophic factors most studied for neuroprotection of the retina (Wen et al., 2012), we evaluated the hypothesis that prolactin mitigates retinal degeneration through trophic actions.

Materials and Methods

Ethics statement. All experiments were approved by the Bioethics Committee of the Institute of Neurobiology from the National University of Mexico (clave NOM-062-ZOO-1999) in accordance with the rules and regulation of the Society for Neuroscience: Policies on the Use of Animals and Humans in Neuroscience Research. All efforts were made to minimize the number of animals used and their suffering.

Animals. Male albino rats (Wistar, 250–300 g), and 28 male and 28 female albino mice (3 months old) were fed *ad libitum* and reared in normal cyclic light conditions (12 h light/dark cycle) with an ambient light level of ~400 lux. Only rats were subjected to bright constant light exposure (BCL).

Rats. Hyperprolactinemia was induced by implanting two anterior pituitary (AP) glands under the kidney capsule, as previously described (Adler, 1986), and sham rats were subjected to similar surgery without implantation. Rats were then transferred to cages that allow light to enter unimpeded. BCL was achieved using two cold white fluorescent light sources positioned 50 cm above the cages (40 W), at an intensity of ~1200 lux at the cage floor. BCL exposure was maintained over a period of 48 h, after which time the animals were immediately returned to normal cyclic conditions. Ambient light-reared animals were used as negative controls. After a 24 h dark adaptation period, rats were evaluated by ERG or killed by CO_2 inhalation and decapitation. Eyes were enucleated and processed for apoptosis or RNA extraction.

Mice. Mice heterozygous for prolactin receptor ($prlr^{+/-}$; Ormandy et al., 1997; 129Sv background) were crossbred, and prolactin receptor-null ($prlr^{-/-}$) mice were selected by tail biopsy genotyping (Binart et al., 2000). Wild-type (7 females and 15 males), $prlr^{+/-}$ (10 females and 2 males), and $prlr^{-/-}$ (11 females and 11 males) mice were subjected to normal cyclic light. After a 24 h dark adaptation period, mice were evaluated by ERG or killed by CO_2 inhalation and decapitation. Eyes were enucleated and processed for histology, apoptosis, immunostaining, or RNA extraction.

Serum prolactin. Serum prolactin was measured in rats by the Nb2 cell bioassay, a standard procedure based on the proliferative response of the Nb2 lymphoma cells to prolactin as previously described (Tanaka et al., 1980).

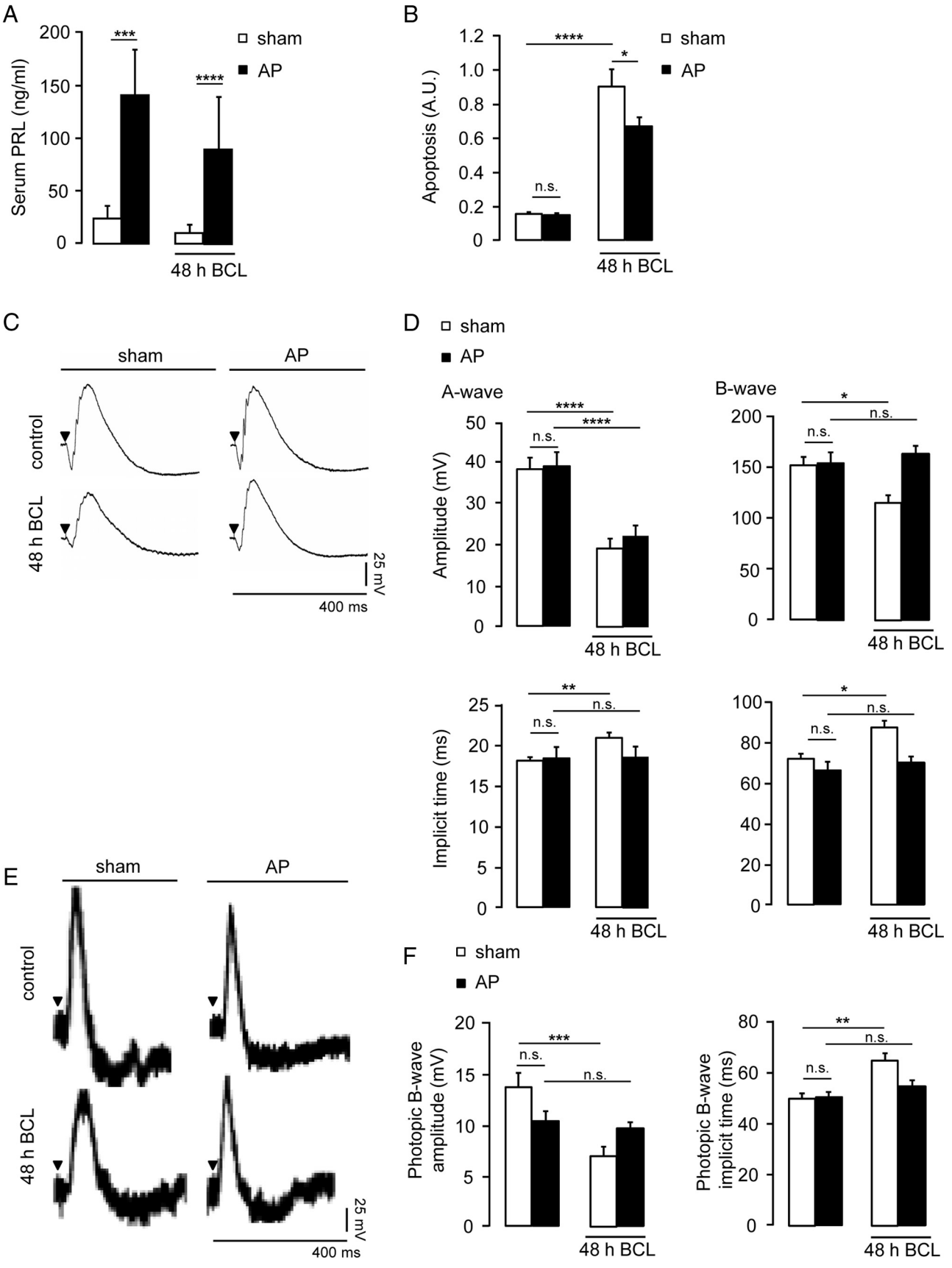

Figure 1. Hyperprolactinemia prevents retinal cell apoptosis and photoresponsive dysfunction associated with light damage. ***A–F***, Serum prolactin levels (***A***), retinal apoptosis (***B***), representative ERG responses under scotopic conditions (***C***), averaged amplitudes and implicit times of A- and B-waves under scotopic conditions (***D***), representative ERG responses under photopic conditions (***E***), and averaged amplitudes and implicit times of the photopic B-wave in sham and hyperprolactinemic (AP) rats exposed or not to BCL for 48 h (***F***). ERG analysis was performed on responses registered at maximal intensity stimulation (1.2 log cd.s/m ^{2}). Serum prolactin levels correspond to the mean ± SD of 4–14 animals per group. Other data correspond to (*Figure legend continues*.)

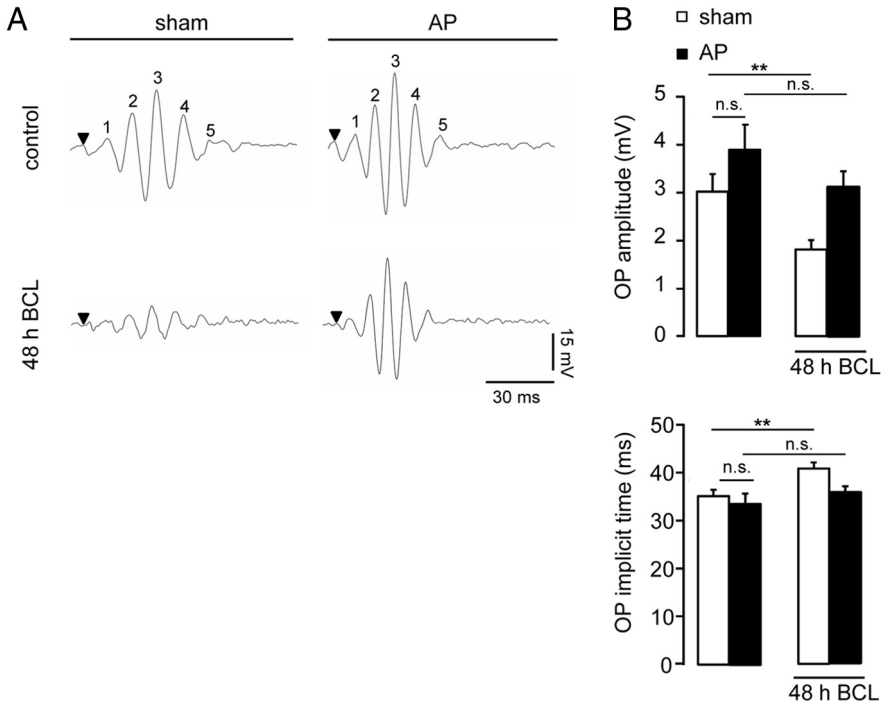

Figure 2. Hyperprolactinemia protects against alterations of OPs caused by light damage. ***A***, ***B***, Representative OP recordings (***A***) and averaged OP amplitude and implicit time in response to the maximal light stimulus (1.2 log cd.s/m^2) under scotopic conditions (***B***), in sham and hyperprolactinemic (AP) rats exposed or not exposed to BCL for 48 h. Data correspond to the mean ± SEM ($n = 9–18$ per group, three independent experiments; $^{**}p < 0.01$ as indicated; two-way ANOVA with Bonferroni's *post hoc* test). OP1 to OP5 have been labeled as 1–5. n.s., Not significant.

ERG recordings. Animals were anesthetized with 70% ketamine and 30% xylazine (1 μl/g body weight, intraperitoneally), and pupils were dilated with tropicamide-phenylephrin. ERG responses were recorded with a silver chloride ring electrode placed on the cornea. Two reference electrodes were positioned subcutaneously near the eye. The light stimulation included 0.7 ms flashes of increasing intensities (0.3, 0.6, 0.9, and 1.2 log cd.s/m^2; PS33 Plus PhotoStimulator, GRASS Technologies). The bandpass was set at 10 Hz to 1 kHz (amplifier, A-M Systems). Sixteen responses were averaged, with flashes at 10 s intervals. Before ERG assessment under photopic conditions, animals were adapted to normal light (400 lux) for 20 min. The light stimulation under photopic conditions included 0.7 ms flashes of 1.2 log cd.s/m^2 intensity. For quantitative analysis, the B-wave amplitude was measured between A- and B-wave peaks. For each animal, the ERG was recorded simultaneously in both eyes, and the recordings from the two eyes were averaged. Oscillatory potentials (OPs) were digitally isolated from the scotopic B-wave using a 100–500 Hz digital filter.

The amplitude of OPs was determined at maximal illumination intensity (1.2 log cd.s/m^2), as the sum of the amplitudes of OP1–OP5 in rats and of OP1–OP4 in mice. Of note, separate analyses for male and female mice revealed no significant differences in ERG response (data not shown).

Apoptosis determination. The apoptotic cell death detection ELISA (Roche Diagnostics) was used according to the manufacturer's instructions to quantify fragmented nucleosomal DNA associated with apoptotic cell death in harvested rat and mouse retinas.

Histology. Mouse eyes were fixed in Davidson solution (40% formalin, 95% ethanol, glacial acetic acid, and distilled water), dehydrated, and embedded in paraffin for sectioning (8 μm thick) and H&E staining. To quantify the thickness of the retina, images were taken at equivalent retinal eccentricities from the optic nerve head. Layer thickness was measured in at least four areas from each retina in three animals per group.

RNA isolation and cDNA synthesis. Retinas were dissected for mRNA analysis. All RNA was isolated using TRIzol (Life Technologies) according to the manufacturer's instructions. Total RNA was quantified. Contaminating genomic DNA was removed using 1 μl of RNase-free DNase I (Boehringer Mannheim)/10 μg of RNA at 37°C for 30 min. A 1 μg sample was reverse transcribed in a 20 μl reaction using the high-capacity cDNA reverse transcription kit (Applied Biosystems) in a Mastercycler (Eppendorf).

Quantitative PCR. Primer sequences are documented in Table 1, and the conditions used for the PCRs were as follows: PCR products were detected and quantified with Máxima Green/ROX qPCR Master (Thermo Scientific) in a 10 μl final reaction volume containing template and 0.5 μm of each primer. Amplification, performed in a PCR CFX96 thermocycler (Bio-Rad), included a denaturation step of 10 min at 95°C, followed by 40 cycles of amplification (10 s at 95°C, 30 s at primer pair-specific annealing temperature, and 30 s at 72°C). The PCR data were analyzed by the $2^{-\Delta\Delta CT}$ method, and cycle thresholds normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase were used to calculate the mRNA levels of interest.

Statistical analysis. All results were replicated in three or more independent experiments. Serum prolactin levels were presented as the mean ± SD, and all others as the mean ± SEM; all data showed normal distribution or equal variance according to D'Agostino–Pearson omnibus and Levene's tests, respectively. Statistical differences between two groups were determined by a two-tailed Student's *t* test, among three groups with one variable by one-way ANOVA followed by Bonferroni's *post hoc* comparison test, and among four groups with two variables by two-way ANOVA followed by Bonferroni's *post hoc* comparison test (SigmaStat version 7.0, SYSTAT). Differences in means with $p < 0.05$ were considered statistically significant.

Results

Hyperprolactinemia protects against retinal degeneration and photoresponsive dysfunction associated with light damage

Light damage has been used for >4 decades as an experimental model for human retinal degenerative diseases (Organisciak and Vaughan, 2010). We used a 48 h moderate BCL exposure in rats to evaluate the role of prolactin. Because systemic prolactin is taken up into the eye (O'Steen and Sundberg, 1982), we increased systemic levels of prolactin by placing AP implants under the kidney capsule of rats (Fig. 1*A*) and quantified apoptosis in BCL-exposed retinas by ELISA. BCL exposure induced a sixfold increase in retinal apoptosis ($p < 0.0001$; Fig. 1*B*). Hyperprolactinemia reduced by 25 ± 6% ($p < 0.05$) the levels of apoptosis in damaged retinas (Fig. 1*B*). Alone, hyperprolactinemia did not modify the basal levels of retinal apoptosis (Fig. 1*B*). Next, we addressed whether prolactin modifies the impact of light damage on retinal function. Figure 1*C* shows representative ERG recordings under scotopic conditions, corresponding to the massed light responses of rod and cone photoreceptors and of the ON bipolar cells. Quantitative analysis was performed at maximal illumination intensity (1.2 log cd.s/m^2), given that no significant

←

(*Figure legend continues.*) the mean ± SEM ($n = 16–20$ per group, three independent experiments; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$, as indicated; two-way ANOVA with Bonferroni's *post hoc* test). n.s., Not significant; PRL, prolactin.

difference was observed at lower intensities (data not shown). Hyperprolactinemia did not prevent the reduction in amplitude of the A-wave registered in BCL-exposed retinas but did protect against the delay in A-wave implicit time caused by BCL (14 ± 3% of controls, $p < 0.01$; Fig. 1*D*). Also, hyperprolactinemia prevented the reduction in amplitude and the delay in implicit time of the B-wave registered in BCL-exposed retinas (23 ± 5% and 12 ± 3% of controls, respectively; $p < 0.05$; Fig. 1*D*). ERGs were then recorded under photopic conditions to test the responses of cones (Fig. 1*E*). BCL reduced and delayed the B-wave amplitude and implicit time by 48 ± 6% (of controls, $p < 0.001$) and 16 ± 5% (of controls, $p < 0.01$), respectively (Fig. 1*F*). Hyperprolactinemia did limit the reduction of B-wave amplitude and prevented the delay in B-wave implicit time induced by BCL (Fig. 1*F*). Alone, hyperprolactinemia did not modify any ERG parameters.

Next, we further assessed the neuroprotective role of prolactin in the retina. Because the A- and B-waves cannot be used to evaluate inner retinal function, we analyzed OPs, the high-frequency components of the ERG that are considered to partly reflect inner retinal activity and are known to be altered in retinal degenerative disorders (Wachtmeister, 1998; Walter et al., 1999; Rangaswamy et al., 2006). Representative traces of the five OP components are shown in Figure 2*A*. The reduction in the amplitudes of OPs caused by BCL (45 ± 8% of control, $p < 0.01$; Fig. 2*B*) was prevented in hyperprolactinemic animals. In addition, the prolonged latencies of OPs (15 ± 3% of control, $p < 0.05$; Fig. 2*B*) associated with BCL were eliminated by hyperprolactinemia. Alone, hyperprolactinemia did not modify OP amplitude or implicit time.

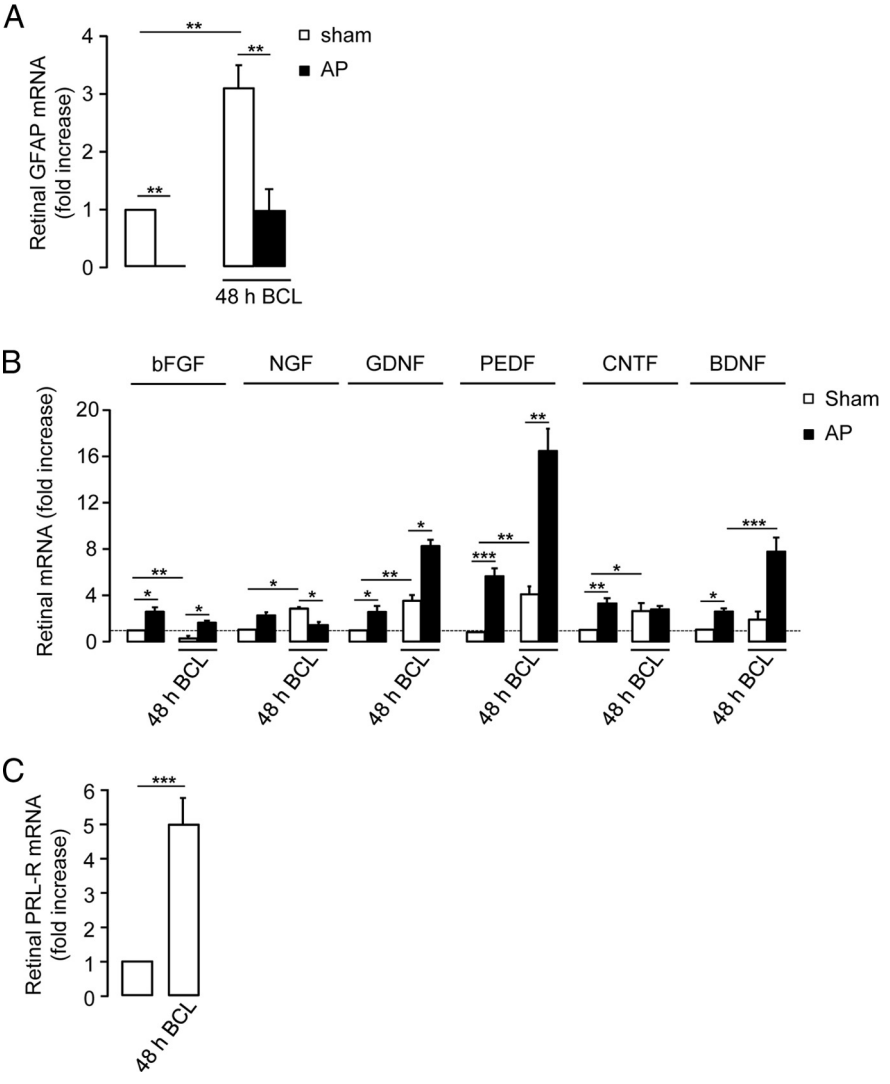

Figure 3. High levels of circulating prolactin limit retinal gliosis and neurotrophin expression changes associated with light damage, which upregulates the prolactin receptor in the retina. ***A***, Quantitative PCR (qPCR)-based quantification of GFAP mRNA levels in retinas from sham and hyperprolactinemic (AP) rats exposed or not exposed to BCL for 48 h. The data are presented as the mean ± SEM ($n = 4$–9 per group, three independent experiments; $^{**}p < 0.01$ as indicated; two-way ANOVA with Bonferroni's *post hoc* test). ***B***, qPCR-based quantification of bFGF, NGF, GDNF, PEDF, CNTF, and BDNF mRNA levels in retinas from sham and hyperprolactinemic (AP) rats exposed or not exposed to BCL for 48 h. The data are presented as the mean ± SEM ($n = 4$–9 per group, three independent experiments; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ as indicated; two-way ANOVA with Bonferroni's *post hoc* test). ***C***, qPCR-based quantification of prolactin receptor (PRL-R) mRNA levels in retinas from rats exposed or not exposed to BCL for 48 h. The data are presented as the mean ± SEM ($n = 4$–9 per group, three independent experiments; $^{***}p < 0.001$ as indicated, Student's *t* test).

High levels of circulating prolactin limit retinal gliosis and neurotrophin expression changes associated with light damage, which, in turn, upregulates the prolactin receptor in the retina

Because retinal neuron survival and function strongly depend on glial activity, we next evaluated whether prolactin modifies the gliosis that accompanies retinal injury. Gliosis, classically defined as the aggregation of glial cells at the sight of injury, is usually associated with increased synthesis of GFAP monomers that assemble into intermediate filaments in both astrocytes and Müller cells (Takamiya et al., 1988). Real-time RT-PCR analysis of the GFAP gene showed that hyperprolactinemia prevented the threefold increase in GFAP mRNA levels induced by BCL compared with levels in sham rats ($p < 0.01$; Fig. 3*A*). Alone, hyperprolactinemia drastically reduced (by 94 ± 0.1%; $p < 0.01$) the retinal content of GFAP transcript. Also, relative to unexposed animals, the BCL-induced increase in retinal GFAP mRNA levels is greater in hyperprolactinemic than in normoprolactinemic rats (20-fold vs 3-fold, respectively).

The response of glia to acute retinal damage is known to be mediated by several neurotrophic factors, including bFGF, GDNF, BDNF, CNTF, NGF, and pigment epithelium-derived factor (PEDF; Harada et al., 2000; Bringmann et al., 2006; Wen et al., 2012). Because prolactin promotes and reduces retinal cell survival and gliosis, respectively, we therefore studied whether prolactin upregulates the synthesis of retinal neuroprotective factors. Figure 3*B* shows that light damage decreased the bFGF mRNA levels by 74 ± 2% of controls ($p < 0.01$); increased the NGF, GDNF, PEDF, and CNTF mRNA levels by 180 ± 3% ($p < 0.01$), 240 ± 7% ($p < 0.01$), 320 ± 13% ($p < 0.01$), and 171 ± 8% ($p < 0.05$) of controls, respectively; but did not alter BDNF levels. Hyperprolactinemia fully blocked the BCL-mediated re-

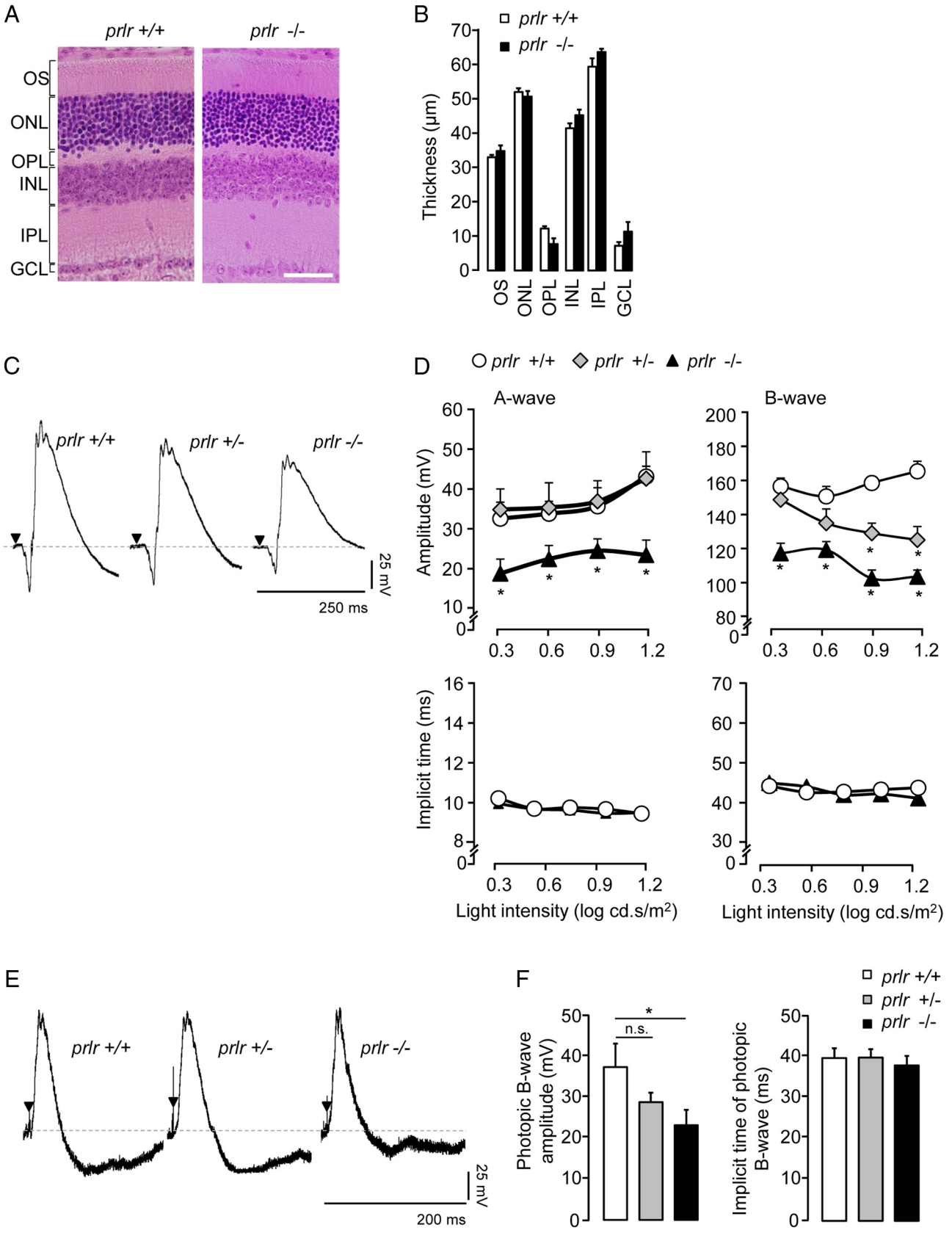

Figure 4. *prlr* $^{-/-}$ mice show photoresponsive dysfunction. ***A***, ***B***, Representative H&E-stained retinas (***A***) and averaged thickness of each layer of the retina (***B***) from *prlr* $^{+/+}$ *and prlr* $^{-/-}$ mice. Four sections per retina from each of six animals per group were analyzed in three separate experiments. Scale bar, 50 μm. ***C***, Representative scotopic ERG response in *prlr* $^{+/+}$, *prlr* $^{+/-}$, and *prlr* $^{-/-}$ mice at 1.2 log cd.s/m ^{2}. ***D***, Averaged amplitudes and implicit times of the scotopic A- and B-waves in *prlr* $^{+/+}$, *prlr* $^{+/-}$, and *prlr* $^{-/-}$ mice at increasing stimulus intensities. Data are presented as the mean $\pm$ SEM ($n = 3$–13 per group, seven independent experiments; *$p < 0.05$ vs values in *prlr* $^{+/+}$ mice; one-way ANOVA with Bonferroni's *post hoc* test). ***E***, Representative photopic ERG response in *prlr* $^{+/+}$, *prlr* $^{+/-}$, and *prlr* $^{-/-}$ mice. ***F***, Averaged amplitude and implicit time of the photopic B-wave in *prlr* $^{+/+}$, *prlr* $^{+/-}$, and (*Figure legend continues*.)

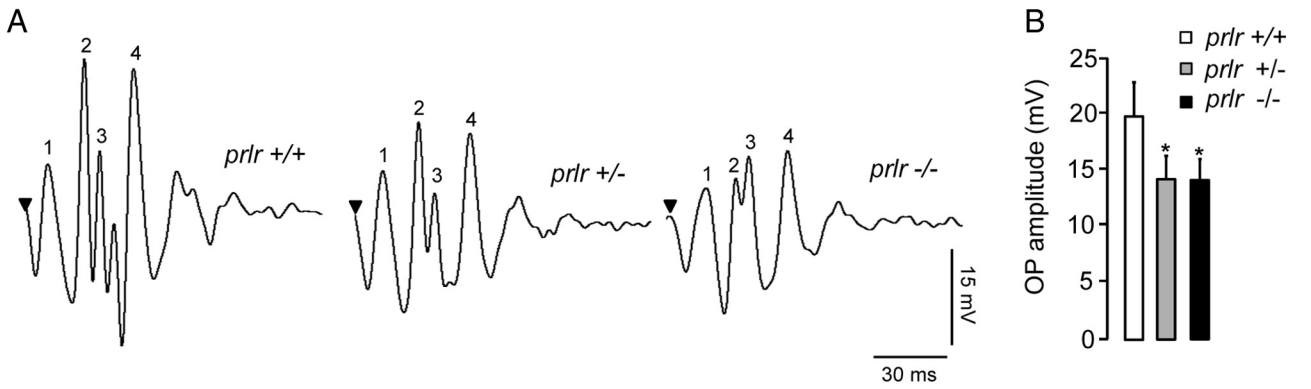

Figure 5. *prlr* $^{-/-}$ mice show altered OPs. ***A***, Representative OPs in *prlr* $^{+/+}$, *prlr* $^{+/-}$, and *prlr* $^{-/-}$ mice in response to the maximal light stimulus (1.2 log cd.s/m^2). OP1–OP4 have been labeled from 1 to 4. ***B***, Averaged OP amplitude in *prlr* $^{+/+}$, *prlr* $^{+/-}$, and *prlr* $^{-/-}$ mice at 1.2 log cd.s/m^2. Data correspond to the mean ± SEM ($n = 3$ per group, three independent experiments; *$p < 0.05$ vs values for *prlr* $^{-/-}$ mice; one-way ANOVA with Bonferroni's *post hoc* test).

duction and increase of bFGF and NGF retinal mRNA levels, respectively, observed in BCL-exposed retinas of normoprolactinemic animals. Additionally, the GDNF, PEDF, and BDNF mRNA levels in BCL-exposed retinas from hyperprolactinemic rats were upregulated by twofold ($p < 0.05$), fourfold ($p < 0.01$), and approximately threefold ($p < 0.01$), respectively, compared with values observed in the damaged retinas of normoprolactinemic animals. The CNTF mRNA levels in BCL-exposed retinas from hyperprolactinemic rats were not different from those in damaged retinas from normoprolactinemic animals. In the absence of BCL, hyperprolactinemia increased the bFGF, GDNF, PEDF, CNTF, and BDNF mRNA levels by approximately threefold ($p < 0.05$), approximately threefold ($p < 0.05$), approximately sevenfold ($p < 0.001$), threefold ($p < 0.01$), and approximately threefold ($p < 0.05$; relative to the control values), respectively, but did not alter NGF levels.

The long and short isoforms of the rat prolactin receptor differ in the length of their cytoplasmic tail. Both are determinant of the responsiveness of cells to prolactin and are present in the retina (Rivera et al., 2008). Moreover, the expression of these receptors is regulated by stress-related stimuli in certain regions of the CNS (Babenko et al., 2012; Benatti et al., 2012). Therefore, we next evaluated whether light damage regulates the expression of prolactin receptor in retinas using primers that recognize both isoforms. The prolactin receptor mRNA levels in retinas from rats exposed to BCL were fivefold greater than the levels in controls ($p < 0.001$; Fig. 3*C*).

prlr $^{-/-}$ mice show photoresponsive dysfunction

Next, we studied the retinas of mice with a genetic deletion of *prlr* (Ormandy et al., 1997). The histology of *prlr* $^{-/-}$ retinas is similar to that in wild-type mice (*prlr* $^{+/+}$; Fig. 4*A*) and *prlr* $^{+/-}$ mice (data not shown). The retina is composed of cone and rod photoreceptor outer segments and of three cellular layers: the outer nuclear layer, comprising cone and rod somata; the inner nuclear layer, comprising horizontal, bipolar, and amacrine interneurons, and Müller glial cells; and the ganglion cell layer, containing retinal ganglion cells and displaced amacrine cells. These cell layers are separated from each other by synaptic layers: the outer plexiform layer comprises processes of and synapses among photoreceptors, horizontal cells, and bipolar cells; while the inner plexiform layer is constituted by processes of and synapses among bipolar, amacrine, and retinal ganglion cells (Wässle, 2004; Sanes and Zipursky, 2010). *prlr* $^{-/-}$ and *prlr* $^{+/+}$ mice showed retinal layers of comparable width (Fig. 4*B*). Under scotopic conditions (Fig. 4*C*), the amplitudes of the A- and B-wave in *prlr* $^{-/-}$ retinas was reduced at all light intensities by an average of 37 ± 3% and 35 ± 4% ($p < 0.05$), respectively, compared with values in *prlr* $^{+/+}$ mice (Fig. 4*D*). The B-wave in *prlr* $^{+/-}$ retinas was decreased (by 21 ± 4% of wild-type, respectively, $p < 0.05$) but only at the most intense illuminations. Under photopic conditions (Fig. 4*E*), *prlr* $^{-/-}$ retinas showed decreased B-wave amplitude (35 ± 7% lower than *prlr* $^{+/+}$, $p < 0.05$) at maximal illumination intensity (1.2 log cd.s/m^2; Fig. 4*F*). No difference in implicit times was found.

We next analyzed OPs as sensitive indicators of inner retinal function (Fig. 5*A*). The amplitudes of OPs in *prlr* $^{-/-}$ and *prlr* $^{+/-}$ retinas were reduced by an average of 28 ± 4% and 28 ± 3%, respectively, compared with wild-type values ($p < 0.05$; Fig. 5*B*). The amplitudes of OPs did not differ between *prlr* $^{+/-}$ and *prlr* $^{-/-}$ retinas. The implicit times of OPs were not modified (data not shown).

Retinas from *prlr* $^{-/-}$ mice display increased GFAP levels and decreased expression of bFGF, GDNF, and BDNF

Alterations of ERG parameters can reflect retinal degeneration. Analysis of whole retinas showed that *prlr* deletion did not affect the levels of retinal apoptosis (Fig. 6*A*). In addition, we quantified the mRNA levels of rhodopsin, which are considered to correlate, though indirectly, with photoreceptor health. The rhodopsin mRNA levels in *prlr* $^{-/-}$ retinas were reduced by ~2.5-fold ($p < 0.05$) compared with rhodopsin mRNA from *prlr* $^{+/+}$ retinas (Fig. 6*B*).

Having demonstrated that increasing prolactinemia limits gliosis and neurotrophin expression changes upon retinal injury, we then investigated whether increased gliosis and reduced overall synthesis of neurotrophins correlate with ERG alterations in *prlr* $^{-/-}$ mice. The GFAP mRNA levels in *prlr* $^{-/-}$ retinas were approximately twofold ($p < 0.05$) greater than those in *prlr* $^{+/+}$ and *prlr* $^{+/-}$ retinas (Fig. 6*C*). Moreover, the bFGF, GDNF, and BDNF mRNA levels were reduced in *prlr* $^{-/-}$ retinas by 67 ± 4%, 75 ± 1%, and 63 ± 5% ($p < 0.01$), respectively, compared with values from *prlr* $^{+/+}$ retinas (Fig. 6*D*). The expression levels of

←

(*Figure legend continues.*) *prlr* $^{-/-}$ mice at 1.2 log cd.s/m^2. Data are presented as the mean ± SEM ($n = 3$ per group, three independent experiments; *$p < 0.05$ as indicated; one-way ANOVA with Bonferroni's *post hoc* test). n.s., Not significant; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; OS, outer segment; GCL, ganglion cell layer.

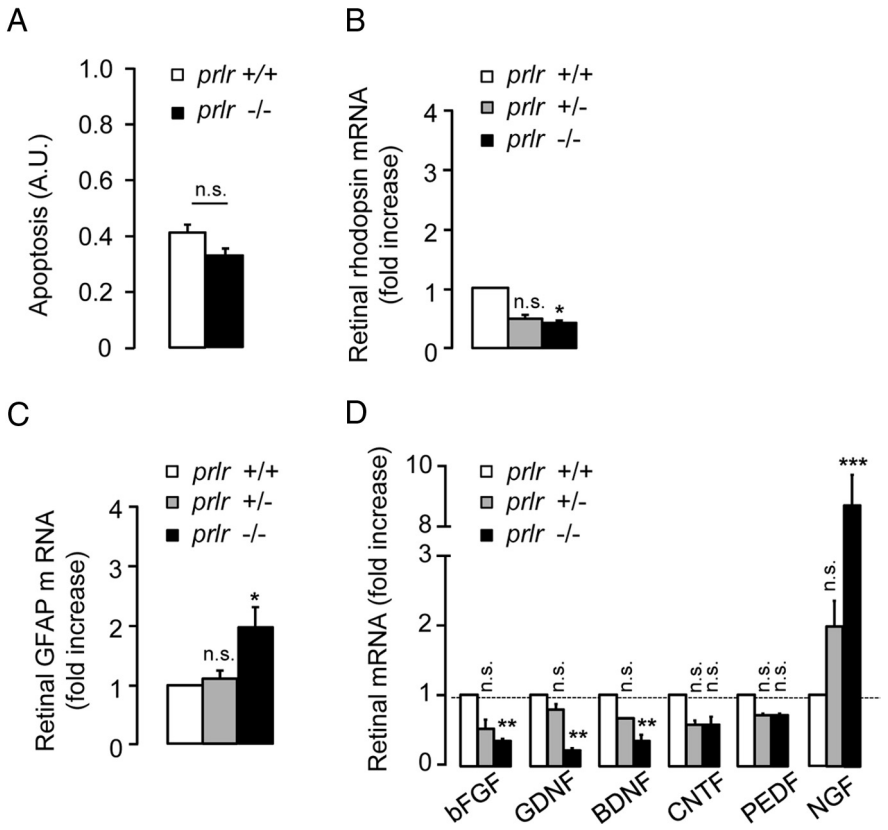

Figure 6. Retinas from *prlr* $^{-/-}$ mice show decreased expression levels of bFGF, GDNF, and BDNF, and a tendency to increased gliosis. ***A***, Retinal apoptosis from *prlr* $^{+/+}$ *and prlr* $^{-/-}$ mice. ***B***, Quantitative PCR (qPCR)-based quantification of rhodopsin mRNA levels in retinas from *prlr* $^{+/+}$, *prlr* $^{+/-}$, and *prlr* $^{-/-}$ mice. Apoptosis data correspond to the mean ± SEM ($n = 4$–9 per group, two independent experiments. qPCR data correspond to the mean ± SEM ($n = 4$–9 per group; $^{*}p < 0.05$ vs values for *prlr* $^{+/+}$ mice; one-way ANOVA with Bonferroni's *post hoc* test). ***C***, qPCR-based quantification of GFAP mRNA levels in retinas from *prlr* $^{+/+}$, *prlr* $^{+/-}$, and *prlr* $^{-/-}$ mice. The data are presented as the mean ± SEM ($n = 4$–9 per group, three independent experiments; $^{*}p < 0.05$ vs values for *prlr* $^{+/+}$ mice; one-way ANOVA with Bonferroni's *post hoc* test). ***D***, qPCR-based quantification of bFGF, GDNF, BDNF, CNTF, PEDF, and NGF mRNA levels in retinas from *prlr* $^{+/+}$, *prlr* $^{+/-}$, and *prlr* $^{-/-}$ mice. The data are presented as the mean ± SEM ($n = 4$–9 per group, three independent experiments; $^{**}p < 0.01$ and $^{***}p < 0.001$ vs values in *prlr* $^{+/+}$ mice; one-way ANOVA with Bonferroni's *post hoc* test). n.s., Not significant.

CNTF and PEDF were the same in the retinas of *prlr* $^{-/-}$ and *prlr* $^{+/+}$ mice. However, NGF mRNA levels in *prlr* $^{-/-}$ retinas were approximately eightfold greater ($p < 0.001$) than those in *prlr* $^{+/+}$ mice.

Discussion

Retinal homeostasis strongly relies on glial activity, and Müller cells are a major source of neurotrophin release in response to neural death (Bringmann et al., 2006). Postmitotic photoreceptor cells cannot compensate for losses during aging and retinal degenerative disorders including age-related macular degeneration, retinitis pigmentosa, glaucoma, and diabetic retinopathy (Nag and Wadhwa, 2012). In these diseases, loss of neurotrophic support leads to neural death, and excessive light exposure can also enhance the progression and severity of neural damage (Cruickshanks et al., 1993). Therefore, natural promoters of the neurotrophic activity in the retina are potential therapeutic agents against degeneration. Here, we have identified prolactin signaling as necessary for proper photoresponse and as a molecular mechanism underlying neurotrophic support in the retina.

This is the first report showing that the inactivation of *prlr* has a phenotype in adult retinal function. Mice lacking the *prlr* gene have an overall reduced ERG response, independent of the lighting conditions, implicating prolactin signaling as necessary for retinal function and consequently, visual perception. Targeted disruption of the prolactin gene in zebrafish did not compromise early eye formation, but the inner retinal neurons were insufficiently differentiated (Huang et al., 2009).

Consistent with previous studies (for review, see Organisciak and Vaughan, 2010), we show that BCL reduced the amplitudes and prolonged the latencies of ERG waves and OPs, in association with retinal cell apoptosis and glia activation. This BCL model recapitulates essential attributes of alterations observed in experimental retinal degeneration but also in retinal diseases (Jones et al., 2003; Marc et al., 2008; Bringmann et al., 2009). Most significantly, both BCL exposure and *prlr* deletion resulted in reduced ERG and OP magnitudes, effects that were mostly prevented by increasing systemic prolactin. Prolactin treatment also opposed the BCL-induced delays in ERG and OPs, and it reduced retinal cell apoptosis. Though *prlr* $^{-/-}$ retinas do not exhibit increased levels of apoptosis, previous findings showed that prolactin promotes neuronal cell survival (Morales, 2011) and decreases apoptosis in non-neuronal cells (Tessier et al., 2001; Bailey et al., 2004). In particular, prolactin upregulates the anti-apoptotic protein Bcl-2 (Leff et al., 1996), whose expression is reduced in retina and brain by stresses like exposure to damaging light (Grosche et al., 1995). Because Bcl-2 overexpression inhibits photoreceptor degeneration (Quiambao et al., 2001), increasing prolactinemia may limit retinal cell death by activating the Bcl-2 pathway. Further, our data may be explained by the incorporation of systemic prolactin into the retina (O'Steen and Sundberg, 1982), and it shows that prolactin limits the BCL proapoptotic effect at concentrations (0.06–0.16 μg/ml) close to those (0.2–0.3 μg/ml) circulating during pregnancy and lactation (Ben-Jonathan et al., 2008).

Abnormal ERG parameters provide information about the origin of retinal dysfunction. Photopic ERG reflects cone response while scotopic ERG includes both cone and rod responses if light intensity is sufficient. The 1.2 log cd.s/m^2 stimulus evoked large ERG responses in the photopic recordings; therefore, it was intense enough to stimulate cones under scotopic conditions. In the *prlr* $^{-/-}$ eyes, cone function is compromised, because we found reduced scotopic A-wave amplitude at all illumination intensities in association with reduced photopic B-wave amplitude. Consistent with a defect in cone photoreceptor activity, the scotopic B-wave is reduced. Additionally, *prlr* $^{-/-}$ mice have decreased B-wave amplitudes under both scotopic and photopic conditions, which are known to involve Müller glia activity (Bringmann et al., 2006). We also found that the magnitude of summed OPs is reduced in the *prlr* $^{-/-}$ eyes. This indicates that the inner retinal activity is impaired, but there is no significant

change in the number of retinal cells and processes since *prlr*$^{-/-}$ and *prlr*$^{+/+}$ mice showed retinal layers of comparable width. Although the specific cellular origins of the OPs must still be established, the circuitry associated with the OPs in the mature retina includes "ON," "OFF," and feedback pathways (Dong et al., 2004), and many retinal cells influence OP characteristics (Wachtmeister, 1998). Collectively, these findings indicate that prolactin signaling helps maintain the activity of most retinal cells and implicate prolactin as a natural retinal trophic factor.

Photoreceptor degeneration alters the neuron–glial network to modify the production of neurotrophic factors required for an environment that minimizes damage (Harada et al., 2002). Our data support this notion by showing that BCL exposure increases the retinal expression of neuroprotective factors GDNF, PEDF, CNTF, and NGF, while decreasing that of bFGF. These neurotrophin changes help explain the presence of apoptosis in the BCL-exposed retinas. Neurotrophic rescue of photoreceptors is mediated by the action of neurotrophic factors like NGF, BDNF, CNTF, PEDF, and GDNF on Müller cells and microgliocytes that, in response, produce bFGF and GDNF, which act directly on the photoreceptor to promote survival (Fontaine et al., 1998; Wexler et al., 1998; Harada et al., 2000; Wahlin et al., 2000; Bringmann and Reichenbach, 2001). For example, blocking NGF signaling increases the levels of Müller cell-derived bFGF, thereby preventing light-induced photoreceptor death (Harada et al., 2000, 2002; Zack, 2000). We found that increasing prolactinemia prevented the BCL-induced increase and decrease of NGF and bFGF, respectively. Hyperprolactinemia also enhanced the BCL-induced expression of the prosurvival factors GDNF, PEDF, and BDNF, consistent with its anti-apoptotic effect. Moreover, the differences in growth factor mRNA production between *prlr*$^{-/-}$ and BCL-exposed animals may explain the lack of retinal cell apoptosis and layer disorganization in *prlr*$^{-/-}$ retinas. We assume that the reduced rhodopsin mRNA levels in the *prlr*$^{-/-}$ retinas do not reflect retinal cell degeneration, although this issue requires further studies. Despite decreases in bFGF, GDNF, and BDNF mRNA levels in *prlr*$^{-/-}$ retinas, CNTF and PEDF levels were maintained, and NGF level was increased compared with the wild-type, indicating that *prlr*$^{-/-}$ mice may have compensatory mechanisms responsible for retinal cell survival. The discrepancies between growth factor mRNA levels in hyperprolactinemic and *prlr*$^{-/-}$ animals may be attributed to the timing and duration of prolactin excess (the entire lifespan including early development in *prlr*$^{-/-}$ mice vs transient adult onset in AP-implanted rats), as previously reported (Ding et al., 2013). These data further indicate that in our two models, while the mRNA levels of certain growth factors (e.g., bFGF, GDNF, and BDNF) are primarily regulated by prolactin, others (NGF, PEDF, and CNTF) may be regulated by prolactin as well as other factors. The fact that bFGF, GDNF, BDNF, CNTF, PEDF, and NGF protect against retinal degeneration (Wahlin et al., 2000; Wen et al., 2012; Kolomeyer et al., 2013) supports the neuroprotective effects of prolactin. Previous investigations did not observe the protective effect of prolactin against photic damage (Olafson and O'Steen, 1976; O'Steen and Kraeer, 1977), but they did not assess retinal function.

Our working model proposes that prolactin contributes to retinal homeostasis and neuroprotection by strengthening the neurotrophic activity of retinal glial cells and limiting their gliosis. Indeed, GFAP mRNA levels are threefold lower in hyperprolactinemic animals than in normoprolactinemic animals exposed to BCL. Relative to unexposed animals, the BCL-induced increase in retinal GFAP mRNA levels is higher in hyperprolactinemic than in normoprolactinemic rats (20- vs 3-fold, respectively). However, this difference is due to the much lower GFAP expression found in nonexposed hyperprolactinemic rats. BCL exposure upregulates prolactin receptor in the retina. The signaling events that govern the retinal synthesis of prolactin receptor are unknown, although upregulation of the prolactin receptor may result directly from local photoreceptor death, as already proposed for the receptor of the neuroprotective leukemia inhibitory factor (Rattner and Nathans, 2005; Joly et al., 2008). Alternatively, or perhaps concurrently, prolactin receptor synthesis may be stimulated by the upregulation of TNF-α and IL-1β occurring in retinal glia under degenerative pressure (Busik et al., 2008; Zhang et al., 2012b). These proinflammatory cytokines increase prolactin receptor expression (Corbacho et al., 2003, 2004), and also prolactin expression in brain tissue (De Bellis et al., 2005) and damaged brain (Chioléro et al., 1988; DeVito et al., 1995; Agha et al., 2004; Möderscheim et al., 2007). Notably, prolactin promotes reactive gliosis (Möderscheim et al., 2007; Simard and Rivest, 2007; Azcoitia et al., 2010), stimulates astrocyte proliferation (DeVito et al., 1995), and may target microgliocytes (Möderscheim et al., 2007); prolactin induces inducible nitric oxide (NO) synthase expression and increases NO production in brain (Popeski et al., 1999; Raso et al., 1999; Dogusan et al., 2001; Vega et al., 2010), and NO produced at a lesion has been proposed as a stop signal for microglia to accumulate (Chen et al., 2000). While the role of increasing prolactinemia in retinal gliosis requires further research, our results show that hyperprolactinemia reduces GFAP expression per se and can limit the gliosis that accompanies retinal injury.

Together with the presence of prolactin receptor in nonhuman primate retina (Rivera et al., 2008), these findings may suggest a role for prolactin in the human retina. High levels of circulating prolactin are associated with increased ERG B-wave amplitude in pregnant women (De Luca Brunori et al., 1985) and protect against diabetic retinopathy (Larinkari et al., 1982; Moloney and Drury, 1982; Bhatnagar et al., 2009). The present study provides a rationale to further investigate hormonal regulation of neuron–glia interactions under physiopathological conditions.

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