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Endogenous regeneration of damaged retinal pigment epithelium following low dose sodium iodate administration: An insight into the role of glial cells in retinal repair

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ABSTRACT

The retinal pigment epithelium (RPE) has been reported to demonstrate feasible self-regenerative potential under specific conditions. However, the precise underlying mechanisms involved in this process are still elusive. Here, we performed a sequential morphological, molecular, and functional analysis of retinal injury and subsequent tissue regeneration after intravenous administration of a low dose of sodium iodate (15 mg/kg) in mice over long-term observation, up to 3 months post-injury. To assess the kinetics of the injury/recovery process, the electroretinography (ERG) responses were correlated with ongoing alterations in retinal structure and the global gene expression profile of injured retinas using genome-wide RNA microarray technology, western blotting and immunohistochemical analyses.

We observed considerable improvement in the rod cell-mediated ERG response, which was accompanied by the regeneration of RPE within the injury site by the 3rd month post-injury. Our results confirm that the repairing mechanisms within injured retinas involve a significant glial cell reaction marked by glial cell proliferation, migration from their original location toward the injury site, followed by a significant overproduction of NTs such as BDNF, GDNF and NT-3. The global gene expression analysis revealed that initially up-regulated genes associated with cell death, apoptosis, acute response to stress pathways underwent considerable down-regulation in the late post-injury period. Accordingly, the genes implicated in nervous tissue remodeling and neuron development, the regulation of synaptic transmission and the establishment of localization were substantially induced by the 3rd month. Collectively, our observations support the view that Müller glial cells might well play an active role not only in retinal cell reorganization following injury but potentially also in RPE regeneration, which appears to be the key event in retinal reparative process. Furthermore, we provided novel compelling evidence of the crucial role of neurotrophins in the pathophysiology of retinal repair and identified the signaling pathways that are activated during this process.

1. Introduction

It has long been believed that the adult mammalian retina is incapable of regeneration. However, in recent experimental studies, it has been demonstrated that endogenous regenerative mechanisms are activated in pathologic conditions (Sherpa et al., 2011; Thomas et al., 2012; Xia et al., 2011). Likewise, mature retinal pigmented epithelium (RPE) cells retain an ability to divide throughout life. In particular, proliferating peripheral RPE cells migrate into central senescent regions of the retina to complete age-related central RPE loss (Kokkinopoulos et al., 2011). Interestingly, the mature RPE is held in a state of quiescence by the adjacent neural retina, and RPE cells begin to proliferate in response to retinal degeneration (Al-Hussaini et al., 2008). Although considerable progress has been achieved in this field, the mechanisms that are responsible for retinal regeneration are not fully understood. Importantly, the possible mechanisms that govern the differentiation and maturation of newly generated retinal cells remain to be elucidated.

The application of sodium iodate, which selectively injures the RPE, represents a useful model for the evaluation of endogenous

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mechanisms of RPE degeneration and its potential subsequent recovery. We recently provided evidence that $NaIO_3$-induced retinal damage triggers a sequence of pathophysiological events that support the self-regeneration of injured retinal tissue (Machalińska et al., 2011). However, when using a high dose of sodium iodate, a toxic effect has been associated with potentially irreversible damage to the tissue microenvironment, leading to permanent defects in RPE and photoreceptor cells that result in the loss of the repair signals and environmental guidance cues that are required for regeneration. Therefore, we previously speculated that when administrating the higher dose of sodium iodate, the locally based protective processes as well as the systemic organism responses were not sufficient to restore normal retinal function after the injury period. Interestingly, both our group and other authors have recently found that when $NaIO_3$ is used in lower doses, retinal cells may retain an intrinsic capacity to divide and regenerate, thus producing a noticeable regenerative effect that can be globally observed in the short-term post-injury period (Mizota and Adachi-Usami, 1997; Redfern et al., 2011; Xia et al., 2011). However, long-term observation has not yet determined which cell populations are directly or indirectly responsible for this regenerative phenomenon, nor have the mechanisms involved been elucidated.

The regenerative process in the retina involves the differential expression of many genes, including those involved in retinal neurogenesis, cell differentiation, and re-establishment of the morphologic structure. Of the many different putative pathophysiological processes that have been hypothesized to play a crucial role in retinal tissue repair, the production and secretion of soluble neuroprotective factors deserve special attention. The elucidation of their physiological roles in the maintenance of central nervous system (CNS) homeostasis as well as the regeneration of damaged tissue have ignited human expectations of using such factors to treat different neurodegenerative diseases, including retinal degeneration (Bazan, 2008; Kassen et al., 2009; MacDonald et al., 2007; Thanos and Emerich, 2005). The majority of neurotrophic factors act via different classes of receptors, leading to the subsequent activation of various signaling pathways in the target cells. Those signaling pathways may affect the survival, differentiation, and metabolic function of cells within the injured retina. Five major neurotrophins (NTs) have been identified in mammals: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and glial-derived neurotrophic factor (GDNF), and importantly, it was found that these factors might be locally produced in the neural retina (Cui et al., 2002; Seki et al., 2005; Ugolini et al., 1995; Vecino et al., 2002). However, the activity and physiological roles of NTs are still unknown. Moreover, the molecular mechanisms underlying the protective effects of NTs and the signaling pathways that support the survival of photoreceptors and RPE in the $NaIO_3$-related chemical injury models have not been yet subjected to in-depth investigation.

Consequently, in this study, we attempted to characterize the role of NTs in endogenous photoreceptor/RPE regeneration over the course of an experimental retinal injury. Hence, here we provide a systematic histological and molecular analysis as well as a functional study of the effects of $NaIO_3$ administrated intravenously at a low dose (15 mg/kg) on the mouse retina through long-term observation (a 3-month period). The principal aim of this study was to identify and characterize the pathophysiologic mechanisms that might participate in neuroprotection and retinal regeneration by re-entering the mitotic cell cycle or reprogramming the specific gene expression profile to recapitulate the sequence of normal retinal histogenesis in the post-injury period.

2. Methods

2.1. Ablation of RPE with sodium iodate

Mice at 8–10 weeks of age were anesthetized and injected intravenously into the retro-orbital sinus with sodium iodate (Sigma–Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS) at a dose of 15 mg/kg body weight. Untreated animals were injected with a comparable volume of PBS. Mice ($n = 5$ for each group) were monitored for RPE and general retinal degeneration and regeneration by histological and molecular analysis and by ERG at various time points following injection. All animal procedures were performed according to the regulations in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and have been approved by the local ethics committee.

2.2. Histological and immunofluorescence analysis

For cross-sections, the eyes were embedded in paraffin, cut into 5 μm-thick sections, and stained with hematoxylin and eosin (Sigma–Aldrich, USA). For immunofluorescence analysis performed by employing sequential stainings, the 5 μm-thick sections were deparaffinized in xylene (2 × 15 min) followed by hydration in decreasing ethanol concentration solutions (100, 95, 85, 70, and 50%) and antigen retrieval (20 min of boiling in citrate buffer, pH 6.0). After blocking in 10% normal serum (goat or donkey, depending on the host species of the secondary antibody) for 30 min in room temperature, the sections were incubated with the primary antibodies (abs) in PBS complemented with 1% BSA: goat anti-PCNA (1:100) (SC Biotechnology, Santa Cruz, CA., USA), rabbit anti-GFAP (1:50) (Novus Biologicals, Littleton, CO, USA), respectively, at 4 °C overnight. Next, incubation with secondary abs for 1 h at room temperature in the dark was performed: donkey anti-goat-Alexa Fluor-647 (1:50) (Life Technologies, Paisley, UK) or goat anti-rabbit-TR (1:50) (Vector Laboratories, Burlingame, CA, USA). For co-expression with PCNA, slides were additionally incubated with the primary rabbit anti-GFAP ab followed by the incubation with the secondary ab: goat anti-rabbit-TR (1:50). For coexpression with GFAP, slides were additionally incubated with Isolectin GS-IB_4 conjugated to Alexa Fluor-488 (Life Technologies, Paisley, UK). Moreover, for double staining procedures, the tyramide signal amplification (TSA) assay (Life Technologies, Paisley, UK) was also conducted following the manufacturer's protocol with minor modifications. Briefly, after standard deparaffinization and antigen retrieval, slides were incubated with 1% H_2O_2 for 1 h and subsequently blocked with 1% blocking reagent. Next, incubation with primary abs: rabbit anti-GFAP (1:1000) (Novus Biologicals) or goat anti-PCNA (1:1000) (SC Biotechnology) was performed at 4 °C overnight. Next, incubation with secondary abs was performed: goat anti-rabbit HRP (1:100) (Life Technologies, Paisley, UK) or donkey anti-goat HRP (1:100) (Merck Millipore, Billerica, MA, USA), respectively, for 1 h at room temperature. Lastly, the tertiary Alexa Fluor-594-tyramide ab was used for final visualization (from TSA kit #5). For co-expression with GFAP, slides were incubated, for 1 h at room temperature in dark, with following primary abs: rabbit anti-BDNF (1:1000; Acris Antibodies, San Diego, CA, USA), rabbit anti-Otx2 (1:500; Aviva Systems Biology, San Diego, CA, USA), rabbit anti-recoverin (1:500; Novus Biologicals) or mouse anti-rhodopsin (1:500; Abcam, Cambridge, UK). For co-expression with PCNA, slides were additionally incubated with primary ab: mouse anti-RPE65 (1:1000; LifeSpan Biosciences, Seattle, WA, USA). Next, incubation with the following secondary abs was performed: goat anti-rabbit HRP (1:100) and goat anti-mouse HRP (1:100) (both from Life Technologies). Lastly, the tertiary Alexa Fluor-488-tyramide ab was used for final visualization (from TSA

kit #12). Upon termination, all sections were then counterstained with DAPI solution (Thermo Scientific, Pittsburgh, PA, USA), mounted, and subjected to microscopy analysis using a LSM700 confocal system (Carl Zeiss, Jena, Germany).

2.3. RNA isolation, Affymetrix GeneChip microarray and data analysis

Total RNA was isolated from mouse retinas using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA isolates from four retinas per each time point (control, 7, 28 days and 3 months post-sodium iodate injection) were pooled to generate four samples for subsequent experimental procedures. A sense-strand cDNA generated from the total RNA using an Ambion WT Expression Kit (Life Technologies, Paisley, UK) was subjected to fragmentation and labeling using GeneChip® WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA), then hybridized onto an Affymetrix WT Array Strip. Hybridization as well as subsequent fluidics and scanning steps were performed with the use of an Affymetrix GeneAtlas™ system (Affymetrix, Santa Clara, CA, USA). The differences in expression of the chosen genes and Gene Ontology terms were analyzed with the R programming environment and Bioconductor packages.

2.4. Western blot analysis

For western blot analysis, retinas were homogenized in M-Per lysing buffer (Pierce, Rockford, IL) containing protease and phosphatase inhibitors (Sigma–Aldrich, St. Louis, MO: 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM sodium fluoride, and 2 mM Na_3VO_4). The mixture was centrifuged, supernatants were isolated, and protein concentrations were determined using the Bradford protein assay (Sigma–Aldrich). Equal amounts of protein (20 μg/well) were loaded and separated by 4–20% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE, mini-PROTEAN II electrophoresis system, Bio-Rad) and then transferred to a 0.2 um polyvinylidene fluoride (PVDF) membrane (Bio-Rad) and probed with the following antibodies (Santa Cruz Biotechnology): 1:1000 NGF (polyclonal), 1:1000 BDNF (polyclonal) and 1:1000 NT3 (polyclonal), 1:1000 GDNF (polyclonal), 1:500 TrkA (polyclonal), 1:600 TrkB (polyclonal), 1:1000 TrkC (polyclonal), and 1:10,000 beta-actin (monoclonal). Immunoreactive bands were detected using horseradish peroxidase-conjugated secondary Ab (Santa Cruz Biotech) specific for the primary Ab that were used in the previous step of analysis. Chemiluminescence detection was performed using the ECL Advance Detection Kit (Amersham Life Sciences, Buckinghamshire, UK), and bands were subsequently visualized with a UVP camera (Gel DOC-It Imaging system; Bio-Rad, Hercules, CA). Equal loading in the lanes was evaluated by stripping the blots for 2 h at 37 °C and then overnight at RT (IgG Elution Buffer; Thermo Scientific, Rockford, IL) and reprobing them in analogous way with GAPDH/ACTB/B2M monoclonal IgG Ab and then with a secondary HRP-conjugated antibody.

2.5. ERG recording

Scotopic and photopic ERGs were recorded 7, 18, 28 days and 3 months after sodium iodate administration using the same individual mice during the time of the experiment. After 4-h-long dark adaptation, mice were anesthetized with an intraperitoneal injection of ketamine (40 mg/kg) and xylazine (4 mg/kg). Then, the cornea was anesthetized (Alcaine; Alcon), and the pupils were dilated with 1% atropine. Retinal responses were recorded with the use of gold ring contact electrodes (LKC Technologies, Gaithersburg, USA). Needle electrodes placed under the scalp between the eyes and in the tail served as the reference and ground leads, respectively. ERGs were differentially amplified (0.05–1500 Hz), averaged, and stored using an LKC UTAS BigShot system. ERGs were recorded in response to strobe flash stimuli presented in the LKC Ganzfeld bowl, similar to the protocols used for human testing. For the assessment of rod photoreceptor function, a strobe white-flash stimulus was presented to the dark-adapted dilated eye with a low flash intensity (24 dB attenuation), and eight responses, recorded in intervals of 8 s, were computer-averaged. Mixed rod and cone responses were obtained using stimulation with white flashes of maximum intensity equal to approximately 1.6 cd^*s/m^2 (Standard Flash, SF, 0 dB attenuation). The retinal responses were measured twice with a 28-s interstimulus interval and averaged. To evaluate the function of cone photoreceptors, animals were light-adapted for 10 min under a white background (32 cd/m^2). After that, a strobe white-flash stimulus was presented to the dilated eye in the Ganzfeld bowl using maximum flash intensity (0 dB attenuation), and responses to eight flashes with intervals of 1 s were recorded and averaged. The amplitude of the b-wave was measured from the a-wave trough to the peak of the b-wave or, if an a-wave was not present, from the prestimulus baseline to the peak of the b-wave.

2.6. Statistical methods

The significance of differences between experimental groups was assessed with the Kruskal–Wallis test followed by the Mann–Whitney test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Morphological restoration of the retinal pigment epithelium in long-term observation

Histopathologic examinations of the retina were conducted at the 7th, 18th, and 28th day and 3rd month after $NaIO_3$ administration. Our previous observations in the short-term post-injury period revealed that in response to a low dose of sodium iodate (15 mg/kg), RPE damage and photoreceptor degeneration were confined to the central region of the retina, with relative sparing of its peripheral zone (Machalińska et al., 2010). In this study, by the 7th day after $NaIO_3$ delivery at a low dose, the thickness of the outer nuclear layer (ONL) in the posterior pole decreased significantly, and the photoreceptor outer segments (OS) and inner segments (IS) were markedly disrupted (Fig. 1B), indicating severe damage to the outer layer of the retina. Interestingly, histological assessment of the retinas after 3 months post-injury revealed the regeneration of RPE structure at the sites of injury (Fig. 1C). These results would suggest the occurrence of favorable proliferative activity of the RPE cells, which were primarily destroyed after acute exposure to sodium iodate. Similarly, considerable improvement of the architecture of the outer photoreceptor segments layer can be observed through long-term observation (Fig. 1C). Of note, the outer nuclear layer continued to decrease significantly until the third month of the experiment, and the outer and inner photoreceptor segments remained shortened when compared to saline-treated controls (Fig. 1D and E). These alterations were detected in the whole post-injury period, and in contrast to the RPE, no total recovery of the nuclear layers was observed.

3.2. Functional recovery of the injured retina in long term observation

To follow the kinetics of the recovery process, we monitored the bioelectrical retinal response on the 7th, 18th, and 28th day and at

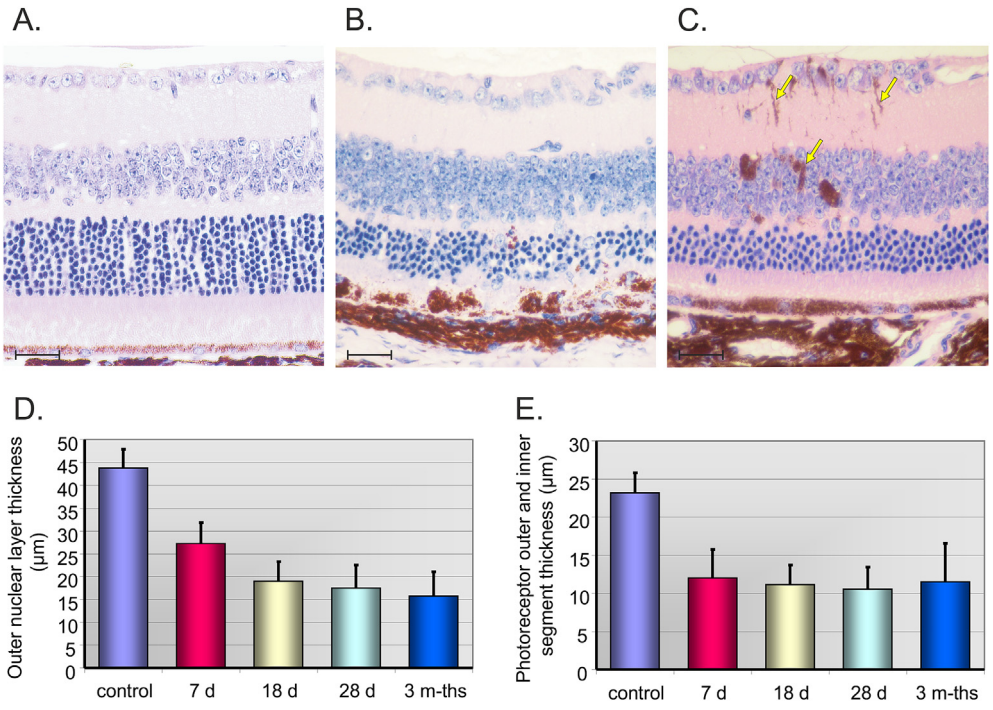

Fig. 1. Effects of sodium iodate (15 mg/kg) on retinal morphology. (A), control healthy retina with no sign of injury. 7 days post $NaIO_3$ injection (B), the RPE is degenerated, ONL is significantly decreased, and the overlying photoreceptor outer (OS) and inner segment (IS) layer is markedly reduced. Histological assessment of the retinas in the 3rd month post-injury (C) revealed regeneration of RPE structure at the sites of injury. Melanin debris spread across the glial cell bodies and their processes (arrows) indicate the direct signs of the local injury to RPE. A comparison of outer nuclear layer thickness (D) and outer and inner photoreceptor segment thickness (E) (μm; mean ± SD) at the different time points after $NaIO_3$ administration. The scale bar is 25 μm.

3rd month after sodium iodate administration. The time course of the changes in the scotopic and photopic ERGs recorded in control animals and at different time points after $NaIO_3$ injection are summarized in Fig. 2. When analyzing the scotopic response, we observed a significant attenuation of the amplitude of b-waves on the 7th day post injection, which persisted through the 18th and 28th days (Fig. 2A). Interestingly, in the 3rd month after $NaIO_3$ administration, the ERG response appeared to be substantially restored, as b-wave amplitude raised significantly ($P < 0.05$) compared to the 28th day. This result may indicate a significant improvement in the rod cell-mediated response. Similar injury-related and recovery trends were observed in the dark-adapted eyes stimulated with white flashes of maximum intensity (Fig. 2B). Here, the b-wave amplitude strongly decreased from day 1 to day 7 post-injection and then increased gradually over all of the time points tested. However, it did not increase significantly at the 3rd month, remaining at approximately 50% of the pre-iodate level. In contrast, when analyzing the cone cell-mediated response, we observed a significant reduction of the b-wave amplitude on the 7th day post-injection, which persisted at a very low level until the end of the experiment, without increasing at the 3rd month (Fig. 2C). This result may suggest a very rapid and profound destruction of cones in the retina following sodium iodate injection.

3.3. *Differential gene expression profile in retinas at sequential time points after injury induction*

In our continuing efforts to characterize the retinal regenerative response to chemical injury, we analyzed the global gene expression pattern in the retinas on the 7th and 28th day and at 3rd month after injury induction. Microarray comparisons of control versus $NaIO_3$-damaged retinas on the 7th day post-injury revealed that 353 genes were at least 2-fold upregulated. For animals on the 28th day post-injection, the respective number was 88. In contrast, for animals at the 3rd month post-injury, 342 genes were detected

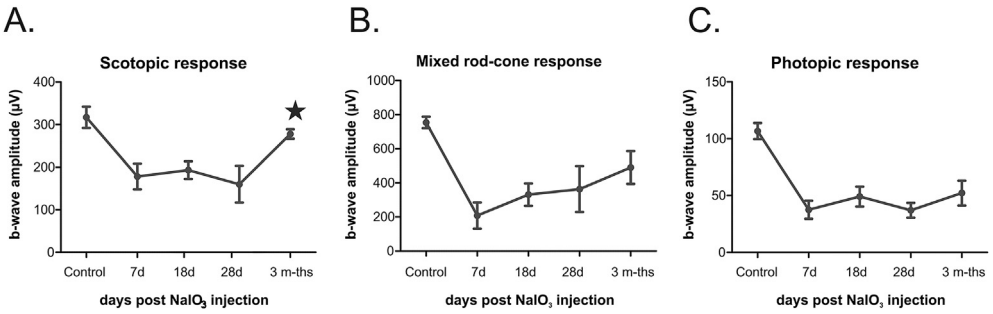

Fig. 2. Effects of sodium iodate (15 mg/kg) on the b-wave ERG amplitudes over time. The scotopic (A), mixed rod-cone (B) and photopic (C) response were analyzed repeatedly on the same individual mice during the time of the experiment. The results are presented as mean ± SD. $^*P < 0.05$ for the 3rd month post injection vs. the 7th day.

as upregulated when compared with control normal animals with no retinal damage. The top 10 upregulated genes with the largest change in expression are presented in Tables S1 for the 7th day post-injection, 28th day post injection and for 3-month post-injury animals, respectively.

Next, all the differentially expressed genes were subsequently classified according to the Gene Ontology (GO) classification of Biological Process. Functional analysis using GO revealed that a number of specific pathways were diversely involved in observed acute retinal damage and the subsequent endogenous regeneration process. On the 7th day post-injury, the bioinformatic analysis of the complex gene dataset identified that the genes involved in the cellular response to chemical stimulus, apoptosis, cellular activation, chemotaxis, immune reactions, inflammatory response to stress, and wounding were among the most upregulated (Table S2a, Supplementary data), including Serpine1, Timp1, Cxcr2, Il1b, Mmp13, Cxcl9, and Ccl17. On the 28th day post-injury, genes such as Nrn1, Scn1a and Scn2a1, Chrna6, Hrh3, Lgi3 or Syngr3 important for the regulation of cellular ion homeostasis, cell signaling and communication, regulation of transport, secretion and neurotransmission, appeared to be switched on. More importantly, expression of a high percentage of genes involved in neuronal regeneration, development processes, neurotransmitter transport and neuron projection development such as neuropilin (Neto1), neurofilament (Nefl), synaptic vesicle glycoprotein 2c (Sv2c) or synapsin 1 (Syn1) were upregulated at 3rd month post-injury (Table S2b, Supplementary data). A summary version of the temporal distribution of genes by Gene Ontology classification of Biological Process is shown in Fig. 3. All together, the analysis of the global gene expression changes revealed that a large number of gene expression alterations can be categorized into two temporal patterns. First, the acute and subacute response associated with retinal cell death, apoptosis induction and acute inflammation were observed within the first seven days post-injury. Second, the late chronic response associated with the endogenous regenerative response can be detected within weeks and months. Noteworthy, the analyzed signaling pathways activated in the early experimental time point and in long-term observation were entirely different.

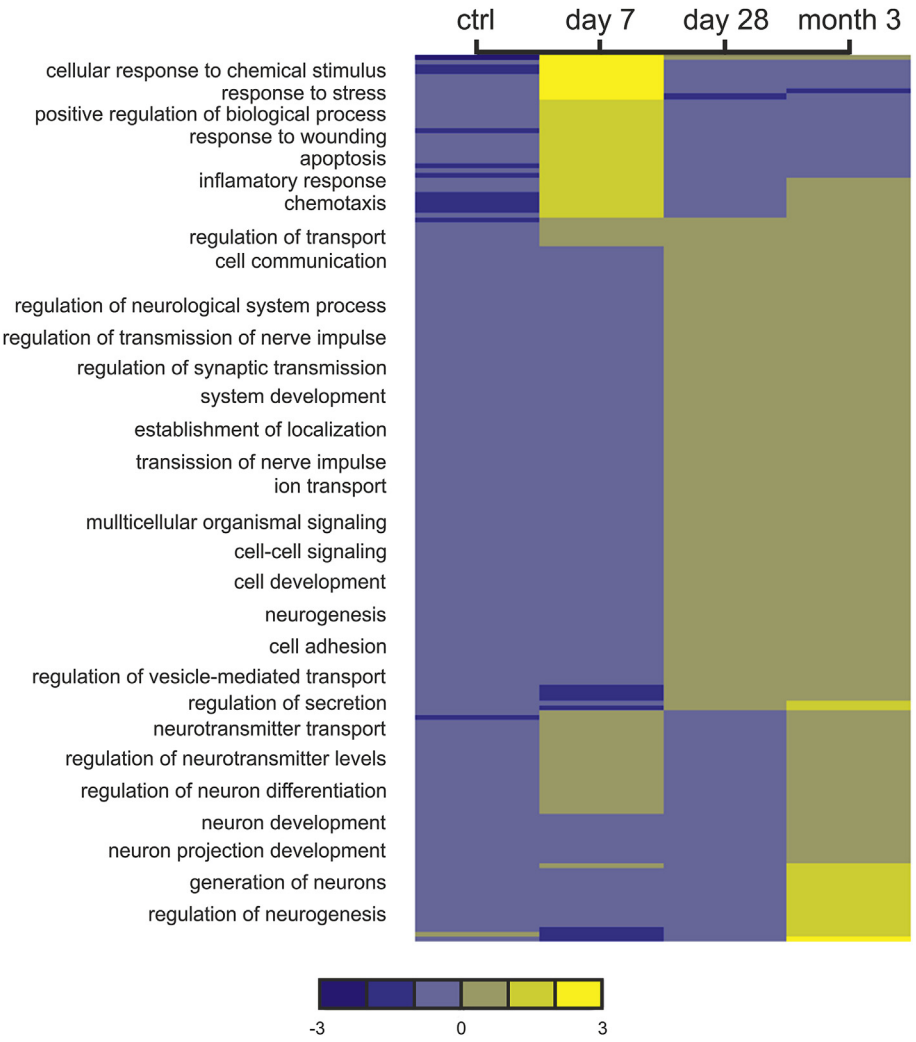

Fig. 3. Global changes in gene expression in murine retinas at selected time points (control, 7th, 28th day and 3rd month) post $NaIO_3$-injury. The heatmap represents expression levels of highly overexpressed genes (FoldChange >4 after 7 days or FoldChange >2 after 28 days or 3 months from injury) in injured animals compared to control animal. For a given gene, an average value was computed and subtracted from each observation. The individual genes are assigned according to Gene Ontology classification to specific biological processes listed on the left side of the graph. Each column comprises a set of horizontal lines, with each line representing a single gene. Levels of gene expression are indicated on a color scale, with yellow corresponding to the highest level of expression and blue corresponding to the lowest level; the range of expression rate of analyzed genes is shown below the graph. The replicates of each sample were averaged to constitute the four samples shown. The diagram shows that retinas collected on the 7th day differ markedly in gene expression profiling from those collected in the 3-month-period post $NaIO_3$-injury.

3.4. Sodium iodate induces glial cell activation, proliferation, and migration within the damaged retina

It is widely accepted that Müller cells are the dominant type of macroglial cells in the retina, playing a number of crucial roles in supporting retinal neurons. Therefore, to characterize their potential role in the post-injury recovery process, we examined the local distribution of glial fibrillary acidic protein (GFAP), which is a retinal glial cell marker and is also used as a specific marker of reactive gliosis. In uninjured retinas, anti-GFAP labels astrocytes and Müller cell end feet (Fig. 4B). In contrast, in the $NaIO_3$-damaged retinas, we observed increased GFAP expression on the 7th day post-injury, which was confined to the area of retinal injury (Fig. 4C). Interestingly, at the site of injury, GFAP labeling became thicker and extended to the photoreceptor layer and even penetrated into the RPE structure (Fig. 4D and E, arrows). To study if glial cells differentiated into the other retinal cell types, immunofluorescence analysis directed against photoreceptor and RPE markers (recoverin, rhodopsin, Otx-2) at all examined time points was performed. In the detected Müller glia we did not observe any positive co-staining with rhodopsin (Fig. 4G) and recoverin at any of the examined time points (Fig. 4H), what indicates lack of intermediate stages of these cells expressing Müller and photoreceptor markers concurrently. Interestingly, we found a few Müller cells expressing the early RPE/photoreceptor transcription factor (Otx2) in their nuclei on the 7th day post-injury (Fig. 4I, arrows). It could imply that some of developmental gene markers are induced in Müller glia while migrating toward the injured RPE.

To determine whether acute chemical injury has a noticeable impact on cell cycle entry and the intensity of proliferation process, we also evaluated the expression of proliferating cell nuclear antigen (PCNA). There were no visible signs of proliferation in the

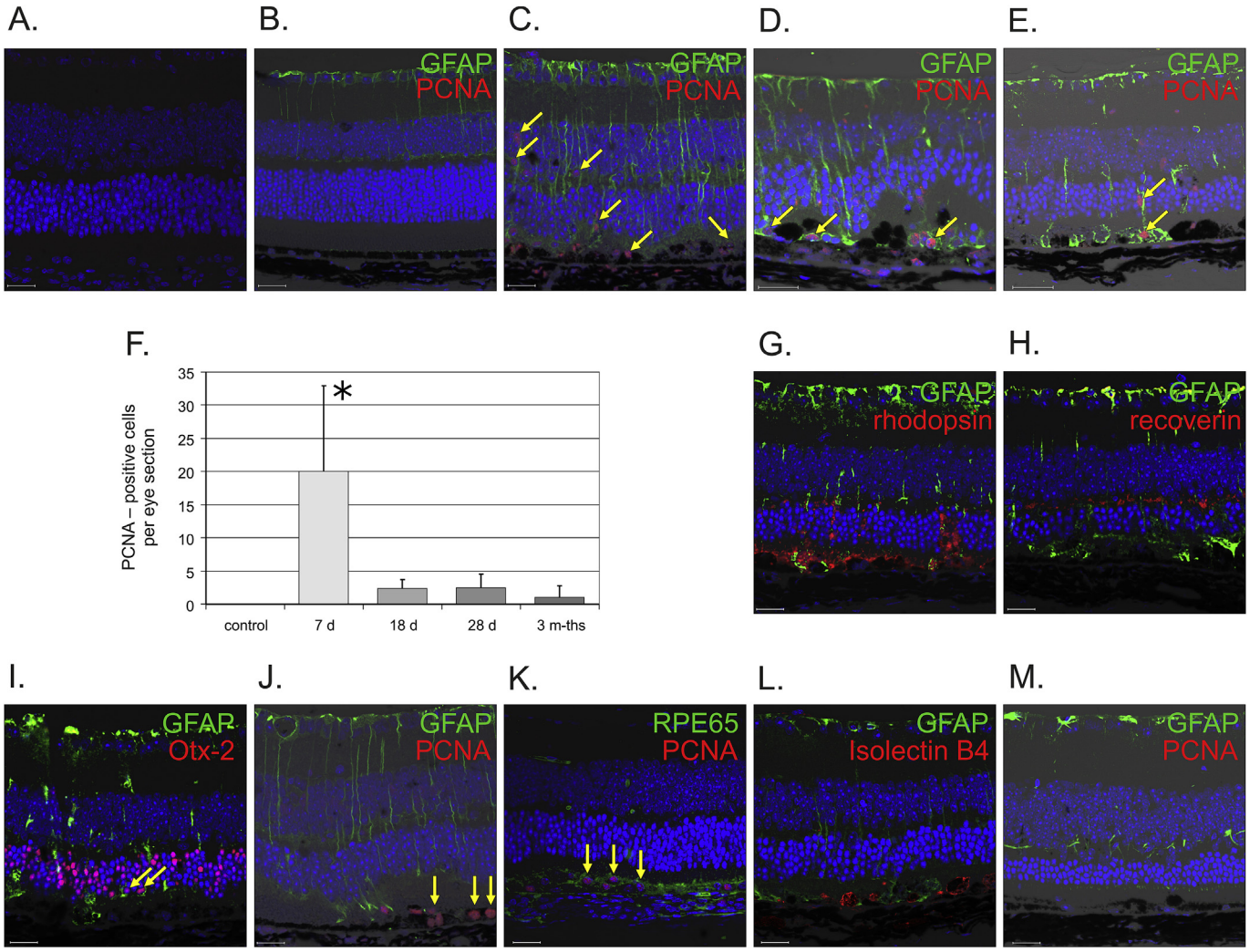

Fig. 4. Immunofluorescence analyses of injured retinas at different time points. Double-stained sections (for GFAP and PCNA) were used to visualize and localize proliferating glial cells. Negative control staining for anti-PCNA antibody (A). Uninjured retina with no sign of proliferation and only slight expression of GFAP in inner retinal layer (B). Retinas on the 7th day post-$NaIO_3$-injury show increased GFAP labeling in addition to intense proliferative responses. PCNA-positive cell nuclei located mainly among GFAP-expressing glial cells are present predominantly at the RPE-photoreceptor junction (C–E, arrows). Immunofluorescence analysis directed against photoreceptor and RPE markers show no positive co-staining of rhodopsin and GFAP (G) and recoverin and GFAP (H). A few Müller cells expressing Otx2 in their nuclei were present in injured retinas on the 7th day after $NaIO_3$ administration (I, arrows). Proliferating RPE cells (arrows) were present at the margin of RPE injury (red, J). Double-staining using antibodies against PCNA and RPE65 confirms the RPE origin of those cells (K). Double-stained sections (for GFAP and IsolectinB4) show that both glial cells and macrophages have an ability to phagocyte cell debris and melanin remnants at the site of RPE damage (L). Retinas on the 3rd month post $NaIO_3$-injury show no signs of proliferation and relatively weak GFAP expression (M). The scale bar is 20 μm. Quantitative analysis of the PCNA-positive cells revealed a significantly higher number of proliferating cells in retinas collected on day 7 post-injury (F). $^*P < 0.05$ for day 7 vs. control and other time points.

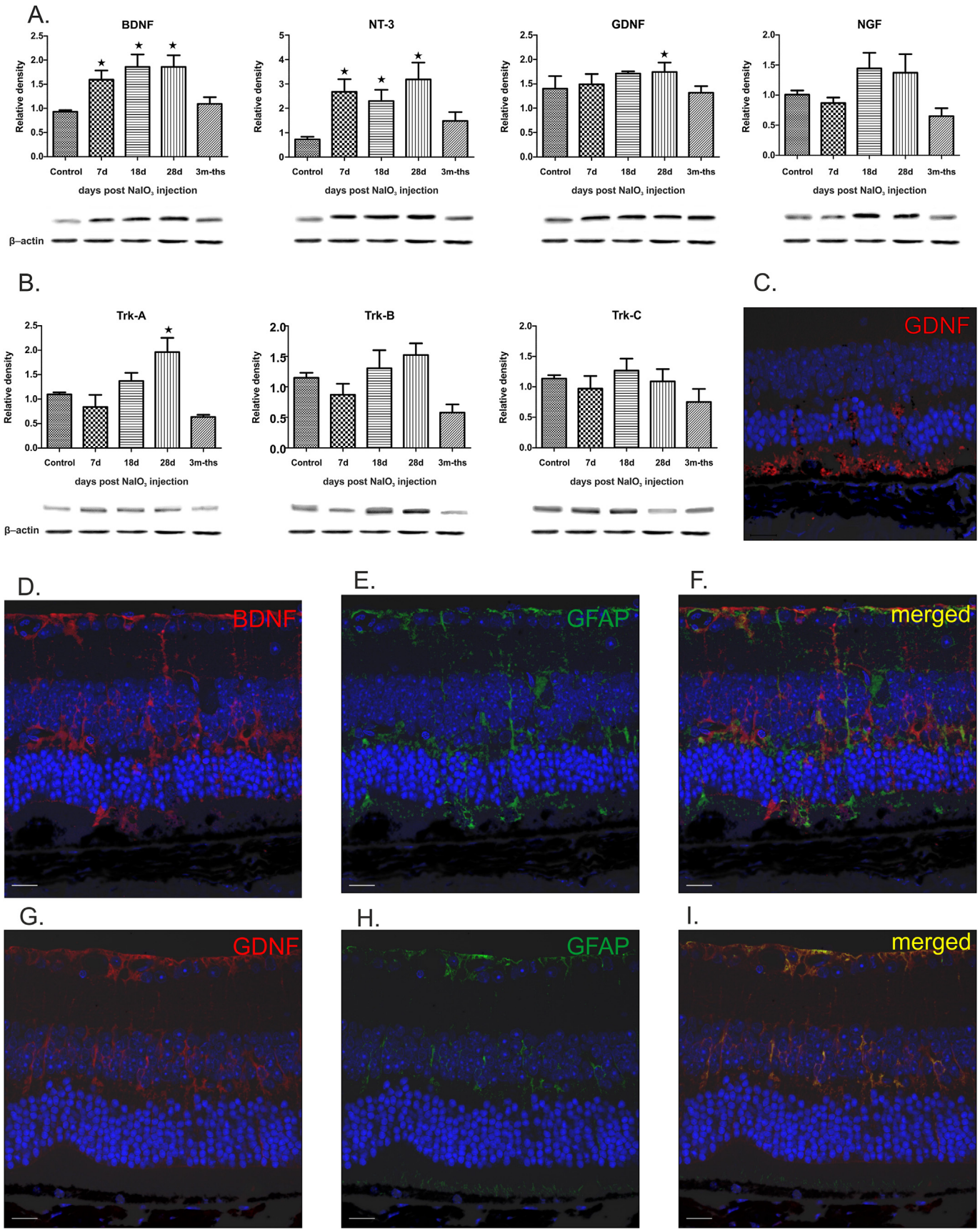

Fig. 5. Western blot time course analyses at selected time points (control, 7th, 18th, 28th day and 3rd month) showing the upregulation of NTs (A) and related NT receptors (B) in the retina exposed to a low dose of sodium iodate. Graphs illustrate densitometric quantification analyses of the normalized protein signals ($n = 5$ animals per time point), which are presented as the mean ratio ± SD of target protein from four independent experiments. $^{*}P \leq 0.05$. vs. healthy control. Representative exposures of enhanced chemiluminescence

control uninjured retina (Fig. 4B). In contrast, within the injured retinal tissue, we detected an intense proliferative response, which was most pronounced on the 7th day after $NaIO_3$ administration (Fig. 4F). Next, to determine the phenotype of the proliferating cells identified in the injured retinas, we performed double immune labeling and found that PCNA-positive cell nuclei were located primarily among GFAP-expressing glial cells. As shown in Panel C, D and E of Fig. 4, proliferating glial cells were found predominantly in the inner and outer nuclear layers as well as at the RPE-photoreceptor junction. Altogether, these data are consistent with the notion that acute retinal injury induces Müller glial cell activation and proliferation and stimulates their subsequent injury-induced migration from the original location in the inner nuclear layer, across the outer plexiform layer, and eventually to the outer nuclear layer and the RPE. Interestingly, we found proliferating cells located in the zones composed of undamaged RPE cells, mostly in the margins of RPE injury regions (Fig. 4J, arrows). To further confirm the RPE origin of those cells we performed double-staining using antibodies against PCNA and RPE65. As shown in Fig. 4K, RPE cell proliferation was markedly demonstrated. This finding indicates that mature RPE cells retain the capacity to enter the cell cycle and complete cellular division to repopulate the lost epithelial cells. In addition, after the 18th day post-injury, we observed melanin granules that were spread across the glial cell bodies and their processes, which might indicate that Müller along with IsolectinB4-positive macrophages have an ability to phagocyte cell debris and melanin remnants at the site of RPE damage (Fig. 4L). Of note, the prominent GFAP up-regulation observed on the 7th day post-injection gradually decreased over the following experimental time points (Fig. 4M), indicating the progressive suppression of reactive gliosis in the late phase of the post-injury period.

3.5. *Sodium iodate stimulates the production of endogenous neurotrophic growth factors and their receptors by glial cells*

To determine whether or not the retinal damage induced by sodium iodate could stimulate local tissue repair through soluble neuroprotective proteins, we analyzed the expression of the selected neurotrophic growth factors and their receptors in sodium iodate-injured retinas. Accordingly, we performed western blotting analysis of lysates isolated from injured retinas harvested on day 7, 18, and 28, as well as 3 months after sodium iodate administration. As shown in Fig. 5, in the analyzed tissues, we observed a significant overproduction of BDNF, GDNF and NT-3, initiated on the 7th day post-injury and continued in a sustained fashion until the 28th day after $NaIO_3$ administration (Fig. 5A). Furthermore, the transient expression of retinal NGF on the 18th and 28th days post-injury was also considerably increased (Fig. 5A). To gain further insight into the biological action of NTs in the local reparative processes, we also investigated the expression of specific receptors for NTs in injured retinal tissue. We found that the expression of Trk-A was significantly up-regulated on the 28th day post-injection ($P < 0.05$) compared to control retinas. Likewise, expression of Trk-B was considerably increased at this time point. In contrast, expression of Trk-C protein levels remained unchanged throughout the experiment (Fig. 5B).

Interestingly, immunofluorescence analysis showed a differential distribution of NTs in the healthy and injured retinas. We found a prominent overexpression of GDNF and BDNF in the RPE-photoreceptor junction that corresponded to the site of injury (Fig. 5C and D), whereas in control healthy retinas, the immunoreactivity of the NTs predominated in inner nuclear layer (Fig. 5G). Furthermore, when double immune labeling was performed, we found that accumulation of the analyzed NTs predominated in GFAP-positive cells (Fig. 5F and I). These data indicate that the Müller glial cell population overproduce neurotrophic factors in response to acute retinal damage. All together, these results demonstrate a distinctive spatial expression of specific NTs and their receptors in glial cells and suggest that Müller glial cells might exert neuroprotective effects in the retina by upregulating the production of neurotrophic factors in response to retinal injury.

4. Discussion

RPE has a wide variety of crucial functions that serve to protect the other cells of the retina and maintain proper retinal physiology. For several decades, RPEs have generally been considered to be terminally differentiated cells, and there has been little or no evidence that the RPE layer regenerates. However, based on several published reports, a modest level of $NaIO_3$-derived injury appears to be critical to enhance RPE cell proliferation and migration, thus inducing the RPE regeneration process in rodents (Kiuchi et al., 2002; Xia et al., 2011). Although published studies are in some respects difficult to compare because of differences in the sodium iodate dose, administration mode, age of animals, and time of analysis after injection, some associations are frequently observed. Complete RPE ablation in mice is often achieved with sodium iodate injected intravenously or intraperitoneally at 40–100 mg/kg body weight. Under these conditions, little (Mizota and Adachi-Usami, 1997) to no (Machalińska et al., 2011; Redfern et al., 2011; Enzmann et al., 2006) regeneration has been found. By contrast, significant regeneration has been suggested to occur at lower doses. For example, intravenously injected $NaIO_3$ at 15 or 25 mg/kg body weight in C57BL/6 mice initially caused profound destruction, with a subsequent partial recovery of visual function as measured by a sensitive optomotor kinetic reflex assay, indicating putative RPE regeneration (Franco et al., 2009). Similarly, a robust RPE regeneration has been found in MRL/MpJ RPE mice injected retro-orbitally with $NaIO_3$ at 20 mg/kg bw (Xia et al., 2011). In addition, it has been demonstrated in rats and humans that the RPE can be subdivided into at least two regions (Al-Hussaini et al., 2008): a peripheral region adjacent to the ciliary margin where there is persistent cell production throughout life, and a senescent central area. Some peripheral RPE appears to divide repeatedly, and there is evidence that some cells migrate centrally (Von Leithner et al., 2010) to compensate for the loss of foveal RPE cells. However, the particular chemotactic signals and the sequence of in situ events such as the elimination of cell debris are still unknown.

Consistent with these studies, we found a complete histological RPE regeneration that resulted in substantial improvement of the ERG response at 3 months after the retinal injury induced by $NaIO_3$ administered at a low dose (15 mg/kg bw). Likewise, the regeneration and recovery of the RPE would then support the surviving photoreceptor cells, leading to their gradual recovery as observed by functional ERG studies. The importance of the incomplete destruction of the retina in this model may reflect a necessity to preserve adult RPE cells or tissue-resident stem cells that would subsequently repopulate the zones of damaged tissue. Consequently, it could lead to sufficient restoration of RPE function, which is essential for photoreceptor viability and activity.

detection of the NTs are shown in the bottom panels. To verify the amount of loaded protein, western blots were incubated with anti-β-actin antibody. Representative immunohistofluorescence analyses of GDNF (C) and BDNF (D) distribution in injured retinas on the 18th day post injection. Immunoreactivity was present at the RPE-photoreceptor junction that corresponds to the site of injury and double-labeled with GFAP (E), as demonstrated in a merged image (F). In uninjured retinas, NTs immunoreactivity was exclusively observed in the inner retinal layer (G) and double-labeled with GFAP (H), as demonstrated in a merged image (I). The scale bar is 20 μm.

Alternatively, high $NaIO_3$ doses may damage retinal stem cells, as previously suggested by Harris et al. (2006) or marginal RPE cells (Kokkinopoulos et al., 2011) required for regeneration as well as alter the tissue microenvironment so that it no longer supports the RPE/photoreceptor regeneration. Furthermore, it may induce secondary damage due to inflammatory responses, as proposed by Longbottom et al. to explain the RPE cell loss in a genetic ablation model (Longbottom et al., 2009). Interestingly, by examining both types of visual reactions, e.g., the scotopic response (of rod activity origin) and photopic response (of cone activity origin) as a function of post-injury retinal revival over the long term, we found that only the scotopic response was restored, indicating that rods are those photoreceptors that highly, although uniquely, recover after injury.

Interestingly, we also observed profound changes in the global gene expression profile using comprehensive RNA microarray analyses. We have shown for the first time that acute chemical retinal injury is associated with specific changes in the patterns of gene expression that trigger diverse signaling pathways within retinal tissue over the long-term post-injury period. We found that genes that were overexpressed on the 7th day post-$NaIO_3$ injection could be broadly clustered into seven categories: cellular response to chemical stimulus, response to stress, response to wounding, positive regulation of biological processes, apoptosis, inflammatory response and chemotaxis (Fig. 3). Several apoptosis and inflammation-related genes became upregulated just after injury, thus indicating that damaged retinas are in an elevated state of acute phase responsiveness. We observed that the most significantly upregulated gene (>3-fold) at this time point was Saa3, a serum amyloid A 3 that belongs to the family of Serum amyloid A (SAA) proteins, which have been identified as prominent acute phase serum reactants synthesized predominately by hepatocytes in response to infection, inflammation and trauma (Larson et al., 2005). A similar high expression (>3-fold) was detected for matrix metallopeptidase 13 (*Mmp13*), which belongs to the metalloproteinase family of highly destructive proteolytic enzymes, enhancing extracellular matrix degradation (Leeman et al., 2002). These results are in agreement with our earlier studies that demonstrated the highest apoptosis rate and cell death on the 7th day post-sodium iodate injection (Kiuchi et al., 2002; Machalińska et al., 2010)).

In contrast, genes that were differentially regulated in the 3-month post-injury period include genes involved in the regulation of cell cycle and cell adhesion, signal transduction and ion transport, cell and tissue development, and the regulation of neurological system processes, including neurogenesis, neuron development and differentiation or neurotransmitter transport and release. All of these genes appear to be essential for the survival and regeneration of injured photoreceptors. However, improvement of RPE integrity is undoubtedly a critical step for both morphological repair as well as functional recovery of outer and inner photoreceptor segments. These results indicate that the above alterations in the gene expression may implicate the multifaceted genomic changes in the retina that are required for the subsequent retinal neuroregeneration and might be responsible for the improvement in visual function. Altogether, our comprehensive microarray studies provide molecular insights into the sequence of the initial acute inflammation and apoptotic processes and the subsequent neural and glial protection that develop due to the changing patterns of gene expression.

To gain further insight into the initial damage and the subsequent regeneration process, we demonstrated here that moderate acute retinal injury is accompanied by a distinctive glial response. Mature Müller glia, which are the dominant type of macroglial cells in the retina, span the retina from the vitreal surface to the RPE layer and maintain biochemical and structural support (Bringmann et al., 2006). These cells have been demonstrated to play essential roles in sustaining homeostasis in the inner retina, including helping neurotransmitter uptake following neuronal excitation, aiding in forming the blood–retinal barrier, protecting retinal neurons against free radicals, providing metabolic support, and even transmitting light through the retina by acting as optical fibers (García and Vecino, 2003). Moreover, many groups have suggested that the activation of glial cells, particularly Müller glia, may be an initial regenerative response to injury (Tackenberg et al., 2009). As with other glial cells of the CNS, Müller glia undergo reactive gliosis, characterized by proliferation, augmented GFAP marker expression, changes in cell shape due to alterations in intermediate filament production, changes in ion transport properties, and secretion of different signaling molecules (Dyer and Cepko, 2000). In fact, gliosis is important for the protection and repair of retinal neurons. Interestingly, Thomas et al. reported that Müller glial cell proliferation significantly correlates with the severity of photoreceptor loss (Thomas et al., 2012).

Our results confirm that the repairing mechanisms within the injured retina involve significant glial reaction marked by glial cell activation and proliferation, followed by their migration from their original location toward the injury site. Next, after cell debris is removed by phagocytosing cells, including both macrophages and Müller glia, the remaining undamaged retinal cells begin to regenerate. We speculate that Müller cells are the dominant type of active macroglial cells in the retina that are involved in supporting the post-injury recovery of neurons and other retinal cells, including enhanced phagocytosis of cellular debris, restoration of the neuronal/retinal homeostasis, biochemical support of neuronal processes (re-) growth, and even, potentially, generation of the new cell types lost in course of the injury. Although Otx2 expression is not observed in the Müller glial cells of the postnatal retina (Glubrecht et al., 2009), we unexpectedly detected Otx2 expression in the limited number of GFAP-positive cells. Otx2 is a transcription factor that is essential for neural retina development, including photoreceptor fate determination; therefore, its expression in the retinal glia could indicate that certain developmental gene markers are induced in these cells during the course of acute degeneration and regeneration. This may indicate the existence of heterogeneous MC cell populations located in separated biological niches that might terminate in different cell fates due to the specific cellular microenvironment. However, further research at different time points is certainly required to fully understand this phenomenon.

Furthermore, our observation supports the view that Müller glial cells might well play an active role not only in retinal cell reorganization following injury, but potentially also in RPE regeneration, which appears to be the key event in the retinal reparative process. Following this concept, we assessed the expression of several neurotrophic factors and their receptors in damaged retinas. Neurotrophins are low abundance, high potency growth factors that modulate the development, differentiation, and maintenance of mature phenotypes in many neuronal populations. As neurotrophins regulate the survival, development, and function of nervous tissue, the increase in the local endogenous NTs expression appears to be fundamental for the reparative potential of damaged retinal tissue. These factors have also been shown to robustly protect photoreceptors from chemically or physically induced damage when injected intravitreally (Ejstrup et al., 2010; Johnson et al., 2011; LaVail et al., 1992). Moreover, recent studies have revealed a diversity of roles for these factors outside the nervous system, most notably in endothelial/epithelial cell function (Meuchel et al., 2011). Consistent with previous studies that demonstrated that NTs promote photoreceptor and RPE cell survival in different models of eye injuries (Mukherjee et al., 2007), we showed, for the first time, that the expression of NTs in glial cells increased in response to chemically induced, sodium iodate-mediated acute retinal injury. Using a

sensitive and specific western-blot analysis, we quantified the levels of proteins such as BDNF, NT-3, GDNF, and NGF within the injured retinas, and, as observed, levels of BDNF, NT-3 and GDNF dramatically increased between days 7 and 28 post-injury. Furthermore, local expression of specific receptors for NTs such as Trk-A, -B and -C in retina was also found at the protein level, thereby allowing retinal cells to respond to locally secreted neurotrophins in a paracrine fashion. It is worthy to mention that based on few reports, injured RPE cells are not able to produce NTs in considerable amount. Accordingly, Bronzetti et al. investigating the effects of neurogenic inflammation on the retina, specifically on the immunostaining of neurotransmitters and neurotrophins at different retinal layers, did not observed any substantial changes in the expression of neurotrophins in RPE cells (Bronzetti et al., 2007). Nevertheless, NTs' receptors, including TrkA, TrkB, TrkC, and p75, have been proved to be expressed in RPE cells, thus indicating that locally released neurotrophins may influence several physiological aspects of the RPE, such as cell growth, proliferation and differentiation (Rohrer et al., 1999; Liu et al., 1997). Altogether, our data indicate that the partial neuroprotection of photoreceptors and RPE cells may be largely attributable to the positive regulation of neurotrophin-dependent signaling pathways.

In conclusion, our findings, in addition to being relevant for the elucidation of the endogenous RPE regeneration process in response to acute retinal injury, bring important new insights into the role of resident non-neuronal cells such glial cells in this process. We also provided novel compelling evidence of the essential role of neurotrophins in the pathophysiology of retinal repair, and we identified the signaling pathways activated during this process. Thus, these findings increase our understanding of the mechanisms induced in the acutely injured retina and the subsequent regeneration process and might facilitate further investigations focused on identifying factors that may improve regenerative approaches for conditions such as age-related macular degeneration and related human diseases. Our results raise the possibility that simultaneous molecular targeting therapies that block apoptotic/cell death pathways in addition to neuroprotective treatments such as the use of neurotrophin-based therapeutics might enable the effective maintenance of visual functions over the course of several degenerating diseases.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.exer.2013.04.004.

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