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Technical Brief

A method for analysis of gene expression in isolated mouse photoreceptor and Müller cells

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Purpose: Molecular analysis of complex phenomena, such as selective death of photoreceptors and their rescue by neuroprotective agents, has been hindered by limitations of techniques for investigating gene expression in individual cells within a heterogeneous tissue such as the retina. The purpose of this study was to develop methods to assess gene expression in single retinal cells.
Methods: Individual cells from papain-dissociated mouse retinae were captured with micropipettes and identified by morphology and by immunocytochemistry. Single cell cDNA libraries were generated by poly-d(T)-primed reverse transcription, poly-d(A) tailing of first strand cDNA, and en masse PCR-amplification using a custom made oligo-d(T). PCR was used to investigate gene expression in cDNAs from individual cells.
Results: Dissociated rod and Müller glia cells maintained their morphology, which correlated with their immunocytochemical properties. RPE cells were recognized by their pigmentation. With the exception of bipolar cells, non-photoreceptor neurons were only identifiable by immunocytochemistry. Abundant cDNA could be synthesized from each individual cell. Cell-specific "markers" were detected by PCR almost exclusively in the predicted cell types. The expression of neurotrophic factor receptors was consistent with previous biological studies.
Conclusions: These studies establish a method to compare, investigate, and analyze gene expression in individual cells of the retina.

Despite considerable progress in the investigation of cellular and molecular mechanisms of retinal development, physiology, and pathology, there currently is only limited understanding of complex phenomena such as cell differentiation, selective cell death in degenerative diseases such as glaucoma and retinitis pigmentosa, or the rescue of these cells by neuroprotective agents. Further investigation of such complex phenomena has been hindered by the limitations of many of the techniques available for the analysis of gene expression in individual cells within heterogeneous tissues such as the retina.

While methods such as in situ hybridization and immunocytochemistry provide good resolution at the single cell level, they are not quantitative and they allow simultaneous assessment of only one or two gene products per sample. Throughput is markedly increased by analyzing mRNA species in whole tissue extracts, and there has been considerable progress in expression profiling studies of the retina [1-5]. Such approaches, however, do not allow determination of the relative contributions of each cell type to the mRNA pool present in the extracts. This issue is particularly significant in the case of cells that represent only a small fraction of the total retinal population, such as ganglion and Müller cells.

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Methods developed in recent years allow high throughput analysis of mRNA species expressed in isolated single cells, using PCR-based cDNA synthesis and global amplification [6-8] or amplified RNA (aRNA) [9,10]. While these methods are not devoid of potential limitations (see Discussion), they have been successfully used for a variety of applications, including characterization of patterns of gene expression in neural and nonneural cells [7,11-14], and for the cloning of genes selectively expressed in particular cell types [4,15-17].

The availability of suitable methods for isolating individual cells from tissues is critical for single cell gene expression analysis. Laser capture microdissection (LCM) is an efficient method for homogeneous tissues, as well as for heterogeneous tissues or tissue layers in which individual cells are not packed tightly together [18,19]. In the case of the retina, such conditions apply to the retinal pigment epithelium (RPE) and the ganglion cell layer, but LCM is not well suited for other retinal cell types that have cell bodies tightly packed amongst other cell types. Isolation of individual Müller cells and photoreceptors is further complicated by their narrow and elongated structure, which makes it practically impossible to capture them without contamination with neighboring cells. An alternative approach is to enzymatically dissociate retinas, identify cell types based upon their morphology [20-23], and isolate them with a micropipette.

We have adapted the enzymatic digestion approach to isolate single cells from adult mouse retinas with the long-term goal of investigating at the molecular level the contributions of different retinal cell types to photoreceptor rescue by intraocular injection of neurotrophic factors. Studies in retinal degeneration animal models have suggested that this complex phenomenon occurs through indirect mechanisms, possibly mediated by activation of Müller glial cells [24-27]. In the present study, single cells isolated from adult mouse retinas were identified by a combination of morphology and immunohistochemistry, and used for the construction of single cell cDNA libraries. PCR with primers for cell type-specific "markers" demonstrated the usefulness of the libraries for investigation of gene expression in single cells. A similar approach was used to characterize the expression of selected neurotrophic factor receptors in photoreceptor and Müller cells. These studies establish a foundation for the analysis of gene expression in individual retinal cells.

METHODS

Animals: Experimental procedures were designed to conform to the guidelines published by the US Public Heath Service (Public Health Service Policy on Human Care and Use of Laboratory Animals). C57BL/6J mice, obtained from the Jackson Laboratory (Bar Harbor, ME), were maintained in a 14 h light/10 h dark cycle. One month to three month old mice were used for the experiments.

Materials: Halothane was purchased from Halocarbon Laboratories (Riveredge, NJ), EDTA, L-cysteine, papain, and Triton X-100 (TX-100) from Sigma-Aldrich (St. Louis, MO), borosilicate glass needles from World Precision Instruments (Sarasota, FL; catalog number TW100-4), 5X MMLV First Strand Buffer, fetal calf serum, AMV (15 U/μl) and MMLV (200 U/μl) reverse transcriptases, 5X TdT Buffer, oligo(dT) 12-18 primer and AL-1 custom primer from Invitrogen (Carlsbad, CA), Nonidet P-40 (NP-40) from USB (Cleveland, Ohio), PrimeRNase inhibitor (Eppendorf, Westbury, NY), RNAguard (Amersham, Piscataway, NJ), Terminal transferase (25 U/μl) and, 100 mM ultrapure dATP, dCTP, dGTP, dTTP (Roche; Indianapolis, IN), AmpliTaq Taq polymerase™ with 10X PCR Buffer II (5.0 U/μl) and 25 mM $MgCl_2$ from Perkin Elmer (Boston, MA), and acetylated BSA from Roche (Indianapolis, IN). The Concert Rapid-PCR Purification System from Invitrogen was initially used for cDNA purification but, when it became unavailable, it was replaced with Qiagen's QIAquick PCR cleanup kit (Valencia, CA). Falcon tissue culture dishes (60 mm; Becton Dickenson; Franklin Lakes, NJ) were used for dissections, 35 mm dishes for cell capture, and 35 mm dishes or Superfrost Plus™ slides (Fisher Scientific; Springfield, NJ) for immunohistochemistry.

Antibodies kindly provided by colleagues were anti-cellular retinaldehyde binding protein (anti-CRALBP; Jack Saari, Seattle, WA), anti-blue and anti-red/green cone opsins (Jeremy Nathans, Baltimore, MD) and anti-rhodopsin (rho4D2; David Hicks, Strasbourg, France). Commercial antibodies were anti-protein kinase C-α (anti-PKCα; Amersham, Piscataway, NJ), anti-calbindin D28 (Sigma; St. Louis, MO), and Alexafluor-488-labeled goat anti-mouse secondary IgG antibodies (Molecular Probes Inc., Eugene, OR). Vectashield® mounting media containing DAPI was from Vector Labs (Burlingame, CA). Images were captured with a Diagnostic Instruments SPOT-RT digital camera (Sterling Heights, MI) using a NIKON microscope equipped with epifluorescence (Nikon, Fluophot).

Tissue dissociation: Animals were euthanized with an overdose of Halothane, followed by cervical dislocation. After eye enucleation, neural retinae were isolated from the retinal pigment epithelium (RPE) in Ca^{2+} and Mg^{2+} free HBSS

Table 1. Oligonucleotides sequences for PCR analysis

Gene	Genebank accession number	mRNA length	Forward primer	Reverse primer	Amplicon region	Amplicon size (bp)
β-PDE	X55968	2743	GCCCTGGAGGAAGAAAAGAA	AGGCAGAGTCCGTATGCAGT	2457 to 2630	174
GCAP1-α	NM_008189	644	TAATCCCTGGAGCGACTCAT	GTTCGCCGTTCTGGAGTCT	366 to 562	197
Phosducin	NM_024458	1263	AGCAGAGGAGGTGGGTAATG	GCCCAAATAAAAATGCCAAG	1079 to 1185	107
Recoverin	NM_009038	1062	GCAAAAATGAAGTGCTGGAGA	CAAACTGGATCAGTCGCAGA	451 to 663	213
Rhodopsin	NM_145383	3249	GATGGAGCCTCAGATGTGGAGT	ATCTAGCCAGCCTGAAGTGGAG	2971 to 3217	247
Rom-1	NM_009073	1349	GCTGAGGCCTAGAAACCTGA	TCTCAACCTTGGTGGTTTCC	1081 to 1283	203
S-antigen	NM_009118	1505	ATCCAGTGAAGTGGCTACCG	GGAGGAATGCTCATGCTTG	1150 to 1439	290
CAC	K00811	1490	TTAGTGACTTTTGATTTCTAGAGGTG	TCCATTAGGTTATGGTAAGTTATCTG	1348 to 1451	104
CRALBP	NM_020599	1869	ACTCTGTCCAGGGTGGAGGT	CTGAGAGTGTTCGGGGAAGA	1698 to 1847	150
GS	NM_008131	2731	AGTAAACACACCCCCACCTC	GCCTGGCAGTTACAGTCTGTTT	2495 to 2665	171
Vimentin	X56397	1748	CCTCTGGTTGACACCCACT	CTGCAGTAAAGGCACTTGAAA	1378 to 1581	204
β-actin	X03672	1892	AGGTGACAGCATTGCTTCTG	GCTGCCTCAACACCTCAAC	1660 to 1856	197
GAPDH	M32599	1228	TGGCCTCCAAGGAGTAAGAA	GTGGGTGCAGCGAACTTTAT	1032 to 1228	197
Ubiquitin C	NM_019639	498	AGAAAGAGTCCACCCTGCAC	TCACACCCAAGAACAAGCAC	185 to 381	197
Trk B	X17647	4351	GTGATGGGACTTGTGCCTTT	ACCTGCCCTAGGCTGCTT	4183 to 4342	160
CNTFRα	NM_016673	1975	CACCAAAGACCCCTCTCATC	GCAAAGGTGGAAGGACTGAA	1511 to 1755	245
FGFR1	BC033447	2877	GCCGTGAGGTTTCTGTTTAGG	CAGTCTCTGCCTCCCTGTCT	2618 to 2759	142

Oligonucleotide sequences corresponding to genes preferentially expressed in photoreceptors and Müllercells, to housekeeping genes, and to neurotrophic factor receptors. The oligonucleotides were designed against 3' regions of these genes.

(CMF), pH 7.4 at room temperature, cut into 8-10 pieces using sharp tungsten needles, and transferred to CMF containing 1 mM EDTA and 5 U/ml papain, preactivated with the reducing agent L-cysteine (2.7 mM) for 30 min at 37 °C. Retinas were incubated at room temperature for 5 min for photoreceptor isolation and for 15 min for Müller cell isolation, rinsed several times with an excess of fresh CMF, followed by a brief incubation in 5 ml Dulbecco's Modified Eagle Medium (DMEM), containing 10% heat-inactivated fetal calf serum (DMEM/serum). The tissue was then dissociated by sequential trituration with wide and narrow-bore Pasteur pipettes and resuspended in 5 ml of DMEM/serum.

Cell capture: Glass micropipettes were pulled with a Model P-80 Micropipette Puller (Sutter Instruments Company; Novato, CA) and beveled to an approximate bore size of 10 to 20 μm (25° angle) with a BV-10 K. T. Brown type beveller (Sutter Instruments). Plastic dishes (35 mm), containing diluted cell suspension aliquots in 2 ml DMEM/serum, were examined with an inverted microscope, under phase contrast optics. Cells were identified on the basis of morphological criteria, verified immunocytochemically (see Results). Two fresh pipettes, presoaked in DMEM/serum, were sequentially used for each cell. The first one was lowered towards the cell of interest using a micromanipulator (Narishige; East Meadow, NJ). The cell was captured under slight negative pressure applied with a nitrogen-pressurized picoinjector (Harvard Apparatus; South Natick, MA), and transferred to a dish containing 1X PBS. After a brief rinse, the cell was re-captured with a fresh needle into a small volume (<0.5 μl) of PBS and transferred to a 0.2 ml PCR tube containing 4 μl of freshly prepared lysis buffer (1X MMLV First Strand Buffer, 0.5% Nonidet-P40, 30 U PrimeRNase inhibitor, 1.27 U RNAguard, 10 μM each dATP, dCTP, dGTP, dTTP, 160 ng/ml oligo(dT) 12-18).

Table 2. Expression of molecular markers and housekeeping genes in photoreceptor and Müller cells

	PhR					Müller				
Genes	-	+/-	+	++	+++	-	+/-	+	++	+++
PhR markers										
β-PDE	41%	0%	3%	17%	38%	100%	0%	0%	0%	0%
GCAP1-α	41%	0%	0%	7%	52%	100%	0%	0%	0%	0%
Phosducin	3%	0%	11%	86%	0%	90%	0%	5%	5%	0%
Recoverin	69%	0%	14%	7%	10%	100%	0%	0%	0%	0%
Rhodopsin	0%	0%	0%	14%	86%	100%	0%	0%	0%	0%
Rom-1	21%	0%	3%	45%	31%	76%	0%	19%	5%	0%
S-antigen	11%	0%	22%	30%	38%	86%	0%	10%	0%	5%
Müller markers										
CAC	100%	0%	0%	0%	0%	24%	5%	71%	0%	0%
CRALBP	95%	3%	3%	0%	0%	16%	0%	0%	0%	84%
GS	97%	0%	3%	0%	0%	6%	0%	0%	0%	94%
Vimentin	93%	0%	3%	3%	0%	35%	0%	12%	0%	53%
Housekeeping genes										
β-actin	69%	0%	10%	10%	10%	40%	0%	0%	10%	50%
GAPDH	3%	0%	10%	83%	3%	5%	0%	19%	14%	62%
Ubiquitin C	62%	0%	5%	33%	0%	53%	0%	6%	41%	0%

Relative intensity scale for PCR bands:

The relative intensity of the PCR products from cells selected by morphological criteria were evaluated using a scale ranging from (-) for absence of product to (+++) when the bands had maximum brightness in gels. Individual cells expressed cell markers corresponding to transcripts typical of those cell types. Housekeeping genes were also detected in these cells.

cDNA synthesis: cDNA synthesis and amplification were carried out essentially as described by Dulac and Axel [15]. Tubes containing cells in lysis buffer were incubated for 1 min at 65 °C, cooled to room temperature for 2 min, placed on ice, and briefly spun at 4 °C. Reverse transcription, polyA tailing, and PCR amplification were carried out with a MRJ Research Thermal Cycler (Watertown, MA). Poly-d(T)-primed reverse transcription was initiated by adding 0.5 µl of a 1:1 (vol:vol) mixture of the reverse transcriptases AMV (15 U/µl) and MMLV (200 U/µl), followed by incubation at 37 °C for 15 min. The reaction was terminated by incubation at 65 °C for 10 min, cooling-down on ice, and brief centrifugation. The cDNA was then extended to generate a 3' poly-d(A) tail by incubation at 37 °C for 15 min in a mixture of 4.5 µl of a 2X terminal deoxytransferase (TdT) reaction mix (2X TdT Buffer [200 mM K Cacodylate (pH 7.2), 4 mM $CoCl_2$, 0.4 mM DTT] and 1.5 mM dATP) and 10 U of TdT (25 U/µl), followed by incubation for 10 min at 65 °C and brief centrifugation at 4 °C.

cDNA amplification: Freshly prepared, ice-cold PCR reaction mixture (90 µl; 1X PCR buffer II, 2.5 mM $MgCl_2$, 10 U AmpliTaq Taq polymerase™ (5 U/µl), 10 µg acetylated BSA, 1 mM each dATP, dCTP, dGTP, dTTP, 0.5% TX-100, and 5 µg AL-1 custom primer [5'-ATT GGA TCC AGG CCG CTC TGG ACA AAA TAT GAA TTC (T) 24-3']) were added to each cDNA sample. First strand cDNAs were amplified en masse in a thermal cycler with an initial denaturation step at 94 °C, followed by 25 cycles of 94 °C for 1 min, 42 °C for 2 min, 72 °C for 6 min, with a 10 s auto-extension per cycle. Fresh AmpliTaq (1 µl) was then added to each tube and PCR was carried out for 25 additional cycles without autoextension. cDNAs were purified with the Concert Rapid-PCR Purification System or the QIAquick PCR cleanup kit, resuspended in 65 µl of ddH_20, and analyzed by electrophoresis on 1.5% agarose gels containing ethidium bromide. Samples with bright smears ranging between 0.2 and 1.2 KB in size were selected for for further analysis. cDNA concentration was determined with an Aglient Technology 2100 Bioanalyzer and DNA 7500 Labchip Kit.

PCR analysis: Oligonucleotide primers used in these experiments are shown in Table 1. Given that cDNA synthesis with this technique is 3' prime-biased, PCR primers were designed to amplify regions relatively close to the polyA tail of each mRNA; they spanned introns whenever possible. For each PCR reaction, 1 µl of cDNA amplified from an individual cell was added to a 0.2 ml PCR tube together with 24 µl of a reaction cocktail comprised of 1X PCR buffer with $MgCl_2$ concentration optimized to each specific primer (1.5 to 3 mM $MgCl_2$), plus 200 to 400 nM of each oligonucleotide primer, 50 µM each of dATP, dCTP, dGTP and dTTP, and 1 U Taq Polymerase. Reactions were carried with an initial 3 min-long, 94 °C denaturation step, 38 cycles of 94 °C for 45 s, 59 °C for

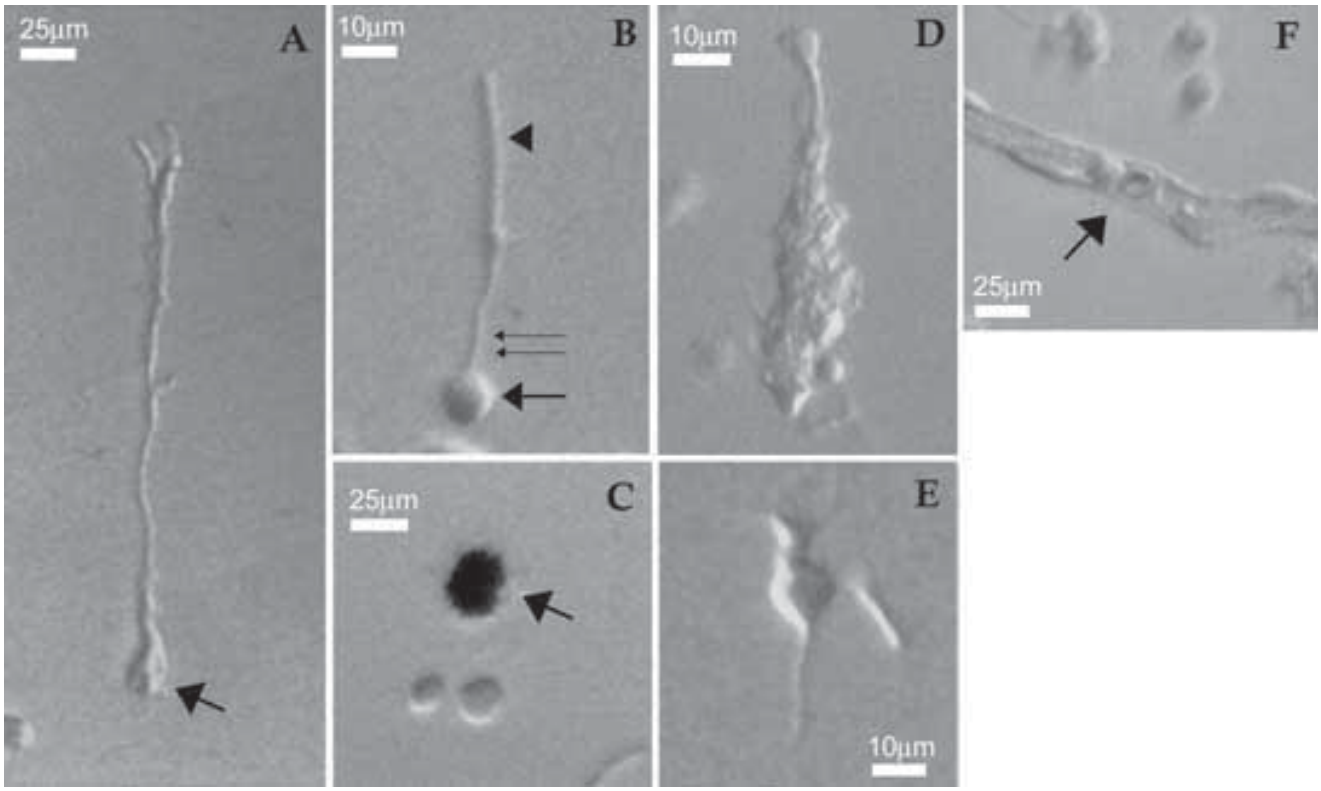

Figure 1. Morphological characterization of dissociated cells from adult mouse retinas. **A**: Müller glial cell; arrow indicates putative end feet. **B**: Rod photoreceptor cell with a round cell body (arrow), inner segment (double arrow) and outer segment (arrowhead). **C**: Heavily pigmented retinal epithelial cell (arrow). Two process free cells are also displayed in this panel. **D**,**E**: Putative neuronal cells whose identity is difficult to determine; the cell in **D** appeared to have several retracted processes. **F**: Small capillary type blood vessel, containing a red blood cell (arrow).

30 s and 72 °C for 1 min 30 s, and a final elongation step of 7 min at 72 °C. Amplification products were separated by electrophoresis on 0.9% agarose gels in 1X TAE buffer and then visualized by ethidium bromide staining. In some cases (see Table 2) the relative intensity of the PCR products was evaluated by an investigator unaware of the identity of each sample, using a scale ranging from (-) for absence of product, to (+++) when the bands had maximum brightness in gels.

Fixation and immunocytochemical analysis of cell suspensions: Exploratory experiments showed that absolute methanol, ethanol, and RNAlater™ tissue stabilizing solution were inferior fixatives compared to paraformaldehyde, which was adopted due to its superior retention of cell morphology and compatibility with both immunocytochemistry and cDNA synthesis (see below). Cells, dissociated as described above, were rinsed in calcium- and magnesium-free Hank's, and 50-100 µl aliquots were dropped into either untreated 60 mm Falcon tissue culture dishes, or Superfrost Plus™ slides containing 4% PFA in 1X HBSS containing 2% HEPES buffer. PAP hydrophobic pen markings were used to prevent fluid spills from slides. Cells appeared attached to the substratum after a few minutes, but fluid changes during subsequent processing nevertheless had to be done very gently to reduce cell losses. Superfrost Plus™ slides offered better cell adhesion, but petri dishes were preferable when the immunostained cells were to be captured for cDNA synthesis. Antibody incubations were carried out at 4 °C. Cells were gently washed in PBS, permeabilized for 20 min in PBS containing 0.25% Triton X-100, blocked for 20 min with 3.0% BSA in 0.1% Triton X-100 PBS, and incubated overnight with primary antibody in blocking solution. Antibody dilutions were: anti-CRALBP and anti-PKC, 1:50, anti-calbindin D, 1:100, anti-blue and anti-red/green cone opsins, 1:2000, and Rho4D2 anti-rhodopsin, 1:1000. Antibody binding was detected with Alexafluor-488-labeled goat anti-mouse IgG at 1:1000. Coverslips were mounted with Vectashield™, containing the nuclear stain DAPI (4',6 diamidino-2-phenylindole), and sealed with nailpolish.

Capturing fixed and fixed-immunostained cells: Immunostained cells were captured as described above, using an inverted microscope equipped with epifluorescence; cDNA synthesis and analysis were as described.

RESULTS

Dissociation of mature mouse retinae into single cell suspensions: Several previously reported protocols for mouse dissociation were compared [20-22,28-30]. Papain yielded better morphological preservation of cellular structures than trypsin. Cell suspension quality was also affected by the duration of enzymatic digestion, the bore diameter of the pipettes used for trituration, and the number of tissue passages through the pipette. Five to ten minute long incubations were best to obtain photoreceptors with intact outer segments, but 15 min

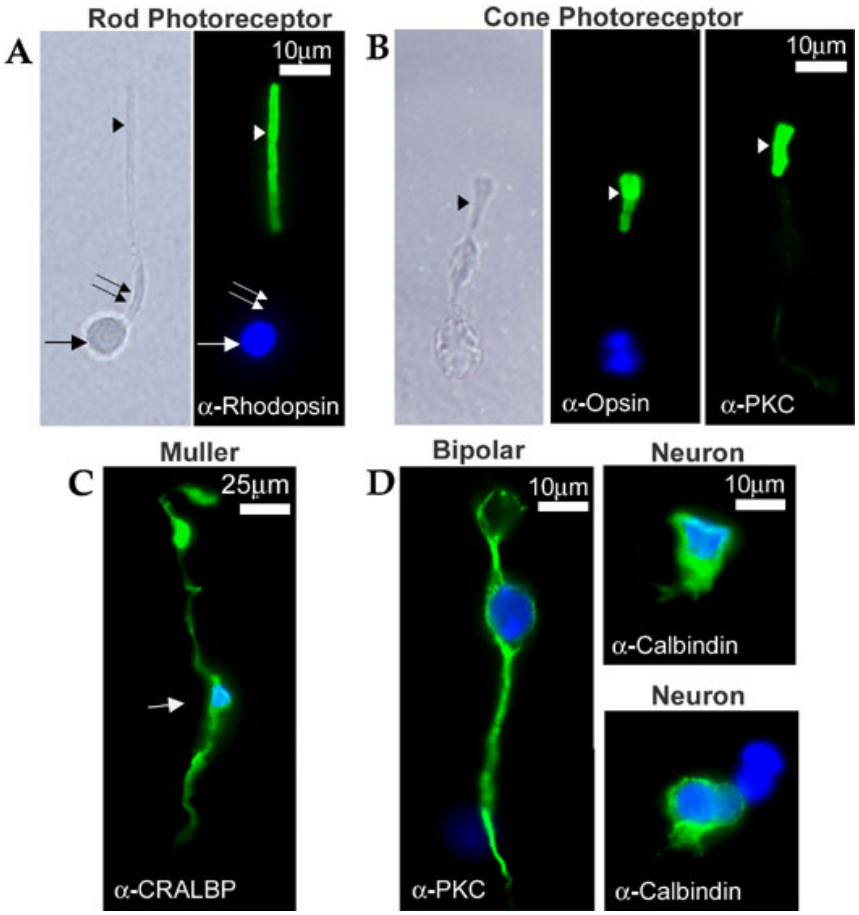

Figure 2. Immunocytochemical characterization of dissociated cells from adult mouse retina. Primary antibody binding was detected in all cases with secondary antibodies labeled with Alexafluor-488, which fluoresces green. DAPI-stained cell nuclei were visualized in the blue channel. **A**: Rod photoreceptor cell shown by phase contrast microscopy (left) and fluorescence microscopy (right). Opsin immunoreactivity is exclusively detected in the cell's outer segment (arrowhead). The inner segment (double arrow) is devoid of fluorescence; the cell nucleus is indicated with an arrow. **B**: Cone photoreceptor cells visualized by phase contrast microscopy (left panel) and by immunofluorescence with an anti-cone opsin antibody (middle panel) and with an anti-PKC antibody (right panel), which stains a subpopulation of cones. Both opsin and PKC immunoreactivities are localized to the cone outer segment (arrowheads). **C**: Müller glial cell immunoreacted with an antibody against cellular retinaldehyde binding protein (CRALBP). The nucleus is localized near the center of the cell (arrow); CRALBP immunoreactive materials are detected throughout the cell, including its processes. **D**: Neuron-like cells immunoreacted with antibodies against PKC (left panel) and calbindin (right panel). The PKC-positive cell displayed on the left has a morphology consistent with a bipolar identity, quite different from the PKC-positive cone cell shown in **B**. The calbindin-positive cells on the right lack characteristic neuronal features, but their immunoreactivity suggests that they could be either horizontal or amacrine cells.

were needed for full Müller cell release. Digestion times shorter than 5 min yielded few isolated cells and many multicellular clumps, whereas longer treatments (e.g., 30-45 min) facilitated tissue dissociation but yielded fragile, morphologically distorted cells. With the exception of bipolar cells, non-photoreceptor neurons did not show good morphological preservation with any of the protocols, but were identifiable by immunocytochemistry (see below).

Morphological identification of isolated cells: Müller glial cells appeared very thin and elongated, often with terminal expansions resembling endfeet (Figure 1A, arrow). In some preparations, particularly those stained with a nuclear dye (Figure 2C), a nucleus-containing cell body was seen between two long projections. Dissociated rod photoreceptors also appeared thin and elongated, but were shorter than Müller cells (Figure 1B), and were polarized, with a spherical cell body (arrow), a rod-like structure likely to represent the outer segment (arrowhead), and an inner segment (double arrow). A short axonal process with a spherule-like terminal could occasionally be seen. Non-photoreceptor neurons generally had a larger cell body than the photoreceptors, and irregular processes of variable length and appearance (Figure 1D,E). Retinal pigment epithelial (RPE) cells could be readily identified due to their unique pigmentation (Figure 1C). Retinal capillaries appeared as long tubes containing red blood cells (Figure 1F). Numerous morphologically undifferentiated, phase-bright cells, whose identity could not be determined based on morphological criteria, were also present in the dissociates (Figure 1C).

Immunocytochemical identification of cell types: The above-mentioned morphological criteria for cell identification were corroborated immunocytochemically using antibodies directed against antigens preferentially expressed in particular cell types (Figure 2). Rod photoreceptors, as identified by structural criteria, showed rhodopsin immunoreactivity restricted to their outer segment (Figure 2A, arrowhead). Likewise, cone outer segments were stained with antibodies directed against cone opsins (Figure 2B, middle panel, arrow) or PKC-α (right panel), which has been reported to identify a cone subpopulation [31]. Müller glial cells were immunoreactive with antibodies directed against cellular retinaldehyde binding protein (CRALBP), with staining throughout their radial processes (Figure 2C). Bipolar cells were also stained with antibodies directed against PKC-α (Figure 2D); they were easily distinguished from cone photoreceptors by their morphology and lack of staining for cone opsins. Other neuron-like elements did not have distinctive morphologies (e.g., cells immunoreactive for calbindin; Figure 2D).

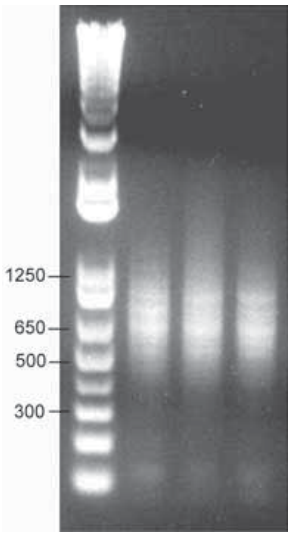

Figure 4. Ethidium bromide-stained agarose gel showing cDNA from three individual cells. The cDNAs appeared as a smear with maximum intensity around 700 base pairs. A DNA ladder is shown on the left.

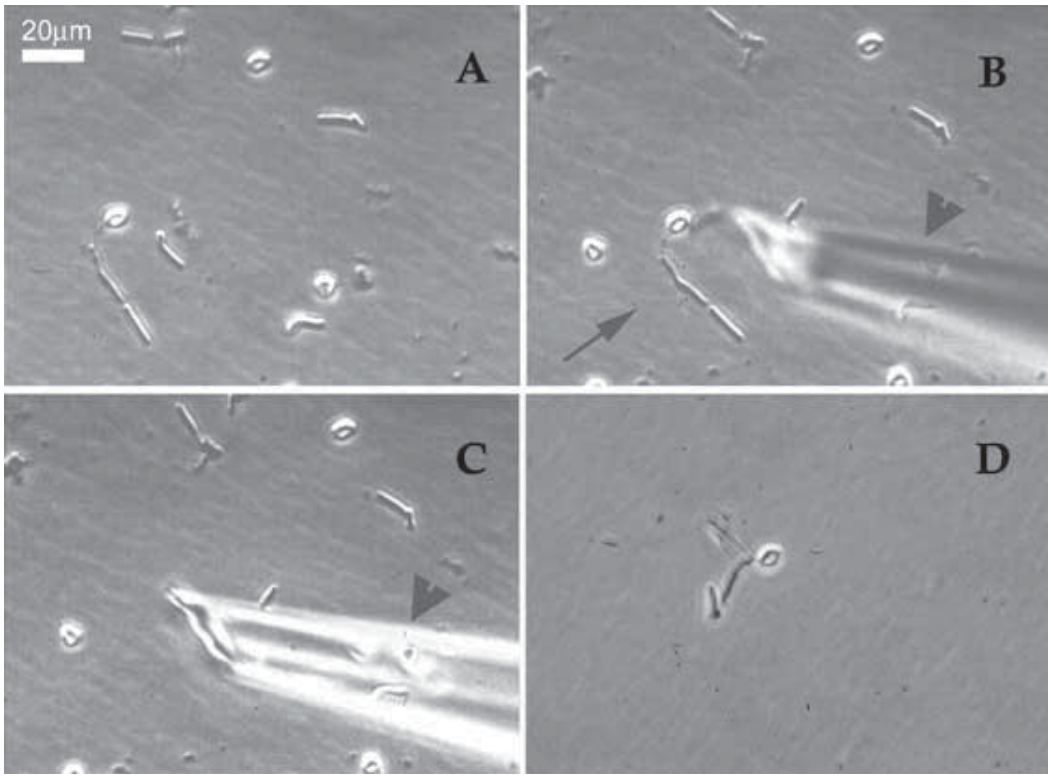

Figure 3. Capture of single cells for cDNA synthesis. **A**: Retinal dissociates contained a mixture of cells with defined morphologies, round cells, and cell debris. **B**: shows a rod photoreceptor cell (arrow) being approached by a thin microcapillary pipette (arrowhead) which is lowered with a micromanipulator. **C**: The rod photoreceptor cell has been aspirated into the capillary micropipette through the application of slight negative pressure (arrowhead). **D**: The cell captured in **C** has been released into a fresh, buffer-containing dish, from which it will be recaptured with a fresh micropipette.

cDNA synthesis from single cells: Individual cells were captured as described in Methods (Figure 3). Contamination with other cells or visible debris could be avoided (or at least minimized) due to the low density of the cell suspension, the small diameter of the micropipettes, and the use of very slight negative pressure. To further reduce possible contamination, particularly from nucleic acids that can be released from broken/damaged cells (our own observations and [15]), each cell was transferred to a rinse dish, from which it was re-captured with a fresh pipette (Figure 3C,D). Given their relative abundance, 3 to 6 Müller cells or 5 to 10 rod photoreceptors could be successfully captured within 45 min.

The range of cDNA lengths generated from individual cells was approximately 200 bp to 1,500 bp, with the majority in the 500 to 700 bp range (Figure 4). This is the expected product size distribution under the reaction conditions used, in which the extent of cDNA elongation was limited by subsaturating nucleotide concentrations and reverse transcription times in order to minimize non-linearities resulting from the reamplification process [15]. Similar results were obtained with photoreceptors, Müller glia, and RPE cells. Global amplification of single cell cDNA as described in Methods [15] routinely yielded 0.45-0.60 μg/cell. Failed cDNA synthesis/amplification was occasionally observed. Smears with similar size distribution (but somewhat lower brightness) were seen with cDNAs from paraformaldehyde-fixed cells; PCR amplification of specific genes from these samples gave inconsistent results and, therefore, their characterization was not further pursued (not shown).

Expression of molecular "markers" in photoreceptor and Müller cells: Isolated cells were characterized at the molecular level by PCR, using primers encoding segments of genes that are preferentially expressed in particular cell types ("cell markers"). Genes preferentially expressed in photoreceptors included in the analysis were β-phosphodiesterase (β-PDE), guanylate cyclase activating protein 1-α (GCAP1-α), phosducin, recoverin, rhodopsin, rod outer membrane protein (Rom-1), and arrestin (S-antigen). Genes preferentially expressed in Müller cells were carbonic anhydrase (CA), cellular retinaldehyde binding protein (CRALBP), glutamine synthetase (GS) and vimentin. Several housekeeping genes (β-actin, GADPH and ubiquitinC) were also included. Data combining the results obtained with all these genes are summarized in histogram form (Figure 5). Six of seven photoreceptor markers were expressed in at least 60% of the photoreceptors, with four of them being present in over 80% of these

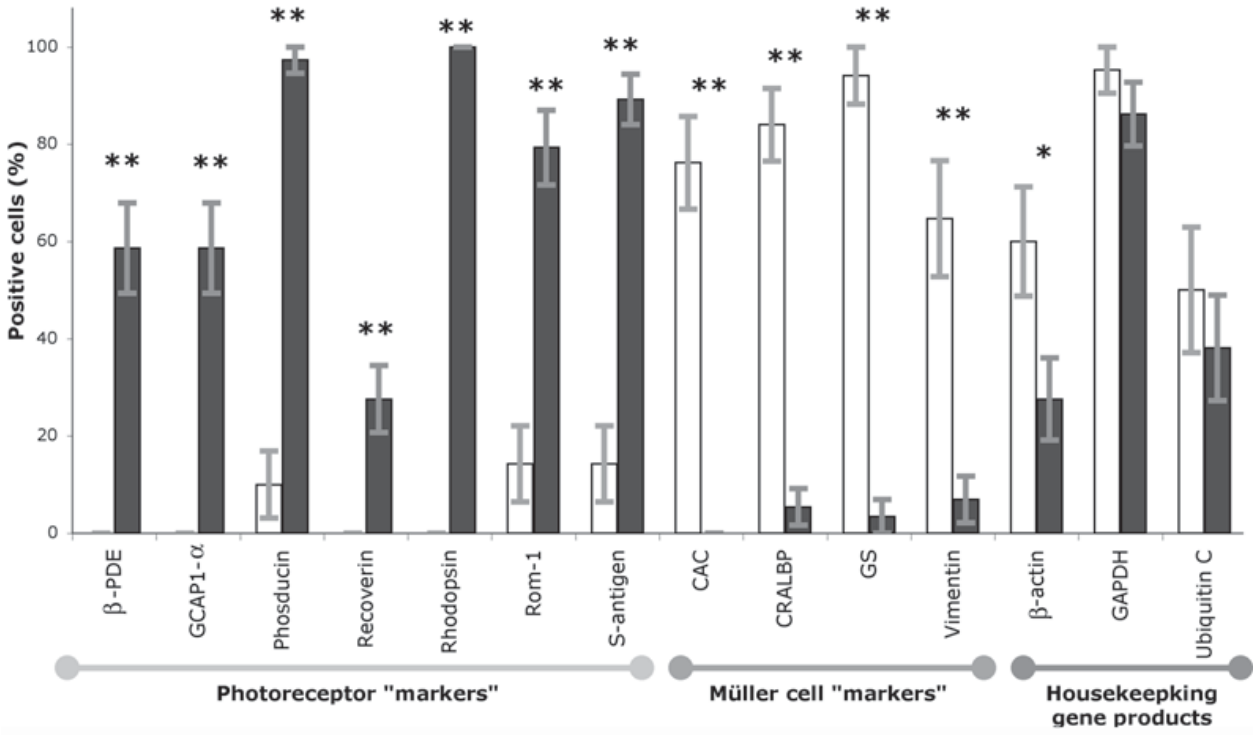

Figure 5. Comparison of the expression of photoreceptor and Müller "markers" and housekeeping gene products. Photoreceptors are shown as closed bars, and Müller cells as open bars. Genes known to be expressed predominantly or exclusively in photoreceptor cells in vivo were detected in photoreceptors in all cases, but with different frequencies (e.g., phosducin, rhodopsin, ROM-1 and S-antigen were present in 80-100% of the rods, β-PDE and GCAP1-α in about 60% of the cells, and recoverin in fewer than 30% of the cells). Several of the molecules were never detected in Müller cells (β-PDE, GCAP1-α, recoverin, and rhodopsin), but phosducin, ROM-1 and S-antigen could be detected in 10-15% of the Müller cells studied. Several Müller cell markers were observed in 65-95% of the Müller cells; carbonic anhydrase was never observed in rod photoreceptors, while CRALPB, glutamine synthetase and vimentin were observed in approximately 5% of the rods. Housekeeping genes were detected in both Müller and photoreceptor cells. β-actin, however, was detectable in twice as many Müller cells as photoreceptors. GAPDH was detectable in over 80% of the cells in both cases, whereas ubiquitinC was detected in 40-50% of the cells, with the differences between cell types not being statistically significant. An asterisk (*) is used to indicate $p<0.05$, and a double asterisk (**) to indicate $p<0.001$. Error bars represent standard error of the mean (SEM).

cells. These photoreceptor markers were observed in 10% or fewer Müller cells, with differences between cell types being highly statistically significant. Housekeeping gene products were detected in approximately 50% of the photoreceptors and nearly 70% of the Müller cells, but these differences were only statistically significant for β-actin.

To further analyze the data, each gene product was analyzed separately; bands generated by PCR products in ethidium bromide gels were classified, by an observer who was masked as to the identity of the samples, as -, +, ++, or +++ according to a relative scale illustrated in Table 2. This semi-quantitative screening allowed evaluation of a large number of samples, which would have been impractical to analyze with more quantitative techniques such as real-time PCR. Despite this limitation, the data (Table 2) showed that: markers were expressed predominantly or exclusively in the "correct" cell type, that not all markers for a particular cell type were detected with the same frequency in cells of that type, that with rare exceptions (such as rhodopsin) individual markers, and even housekeeping genes, were detected in fewer than 100% of the expected cells, and frequently generated PCR bands of different intensities in various cells of the same type, and PCR detection of particular gene products was highly primer-dependent, since rhodopsin was detected in 100% of rod photoreceptors with the primers used for the data included in Table 2, but in only 19% of rods when a different set of primers was used (data not shown). Taken together, the results suggest that the molecular phenotype of individual cells should be investigated using several "markers" rather than just one, and that cDNA from individual cells should be selected for further analysis after careful evaluation of the expression of particular genes, as previously suggested by studies with other cell types [13,15].

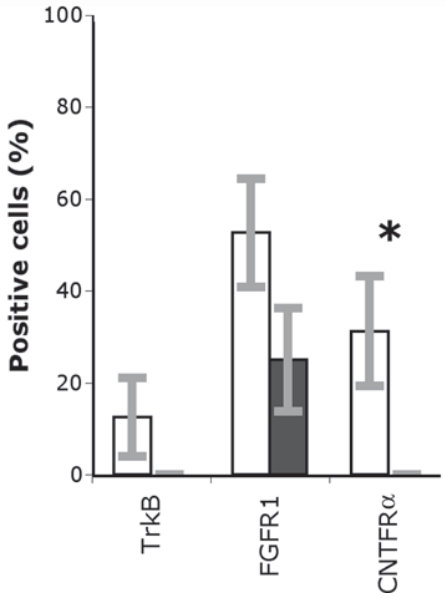

Figure 6. Detection of neurotrophic factor receptors in Müller and photoreceptor cells. TrkB and CNTF-α were undetectable in photoreceptor cells; however, while TrkB was detected in only 10% of Müller cells, CNTF receptor α was observed in approximately 1/3 of the Müller cells. FGF receptor 1 was observed in approximately half of the Müller cells and 25% of the rod photoreceptors, but these differences were not statistically significant. An asterisk (*) is used to indicate $p<0.05$. Filled bars represent photoreceptors while unfilled bars represent Müller cells. Error bars represent standard error of the mean (SEM).

Expression of receptors for neurotrophic factors: PCR analysis with primers for the receptors CNTFR-α, TrkB, and FGFR1 produced results that, although somewhat limited in scope, were generally consistent with predictions based on previous observations (Figure 6). FGFR1 was detected in both Müller and photoreceptor cells, in agreement with previous reports [32-34]; its detection was more frequent in Müller cells than in photoreceptors ($p<0.05$). CNTFR-α was never seen in photoreceptors, but was detected in approximately one third of the Müller cells that were analyzed. TrkB was never seen in photoreceptors either, but in this case only 10% of the Müller cells had detectable PCR products, which appeared very faint in agarose gels. It is noteworthy that these results could not be improved by testing different sets of primers (10 sets for FGFR1, 9 for CNTFRα, 7 for TrkB) under a variety of PCR conditions, including systematic optimization of Mg concentrations at 0.5 mM increments between 1.0 and 4.0 mM.

DISCUSSION

In this study we have shown that cells isolated from the adult mouse retina can be individually captured and processed for cDNA synthesis and global amplification, which in turn allows their molecular characterization by candidate gene PCR. The method is particularly suitable for Müller, photoreceptor, and pigment epithelial cells, which can be readily identified by morphological and/or immunocytochemical criteria. Structural preservation is particularly remarkable in the case of rod photoreceptors, known to be generally quite fragile. Broken photoreceptor fragments are not infrequent in the cell suspensions, but a substantial number of intact rods are obtained, with an outer segment that shows highly polarized accumulation of immunoreactive visual pigment. It is somewhat disappointing that, with the occasional exception of bipolar cells, other neuronal elements fail to retain a structure reminiscent of their in situ appearance, although they can nonetheless be identified by immunocytochemical analysis of cell specific "markers".

As mentioned in the Introduction, individual cells have been extensively used for the characterization of patterns of gene expression in neural and nonneural cells [7,11-14], and for the cloning of genes selectively expressed in particular cell types [15-17]. Permutations of two alternative methods have been successfully used. T7 RNA polymerase based aRNA synthesis, first introduced by Eberwine's group [35] is a linear method that preserves well the relative abundance of various mRNA species in the original source [9,10]. PCR-based exponential methods for "global" cDNA amplification [7], including the protocol used in our studies [15], are less time-consuming and laborious than aRNA synthesis. A concern that has been raised, however, is that exponential amplification may degrade abundance relationships of different messages [12,36]. This concern has been addressed by recent studies

that concluded that, if reverse transcription is limited to a few hundred base pairs by limited reaction time and oligonucleotide concentration, accurate representation of the relative transcript abundances present in the original sample can be preserved through exponential amplifications as high as $3x10^{11}$ fold [13,37].

While gene expression analysis of isolated cells is gaining increasing recognition as a powerful and very useful approach, it is not devoid of limitations. It has been reported that rare messages (e.g., <25 transcripts/cell) cannot be detected consistently [14]. Variability between samples can also be expected, and it has been recommended that, before further analysis, cDNAs from individual cells must be selected by PCR or Southern analysis to determine the presence of particular cell-specific and/or housekeeping gene products [15]. In a recent study, for example, it was reported that only 7/45 and 9/45 cells of two particular types were chosen for microarray analysis [13]. It is therefore not surprising that in our study, in which cells were not pre-selected, most of the photoreceptor or Müller cell-specific transcripts that we investigated were detected in fewer than 100% of the cells of the correct type, as were housekeeping genes which are expected to be expressed by (and detected in) both Müller and photoreceptor cells. It is nonetheless reassuring that cell-specific "markers" were detected predominantly, or even exclusively, in the correct cells. An important consideration for studies of this type is that PCR amplification of many cDNAs was highly primer-dependent. In the case of rhodopsin, for example, we tested 20 different pairs of primers (data not shown), and found that they varied broadly in their capacity to amplify rhodopsin cDNA, with only one of the pairs (shown in Table 1) yielding a strong band in 100% of the photoreceptors tested.

The occasional detection of a putative cell-type specific gene product in unexpected cells could be due to contamination of the samples. The likelihood that this may occur is considerably reduced, but cannot be completely eliminated, by precautions such as washing each cell in a separate dish with fresh medium and recapturing it with a fresh micropipette. It is also conceivable that microscopically undetectable fragments from neighboring cell(s) may remain attached to the cell being captured; this could happen with photoreceptor and Müller cells, which have close attachments at the outer limiting membrane. It would probably be unwise, however, to dismiss every case of gene transcript detection in unexpected cells as contamination not only because it could reflect leaky expression, but also because cell-specific patterns of expression, as described in the literature based on techniques such as immunocytochemistry or in situ hybridization, frequently involve subjective distinctions between "background" and bona fide low level expression.

Neurotrophic receptor expression analysis in isolated cell cDNA yielded results that, although somewhat limited (see below), were generally consistent with biological studies of the effects of neurotrophic factors on photoreceptor rescue. CNTFR-α, for example, was detected in Müller cells but not in photoreceptors, in agreement with findings from this laboratory [24,25] and others [26,27], suggesting that photoreceptor rescue by CNTF occurs thorough indirect mechanisms, possibly mediated by Müller cells. FGFR1 was detected in both photoreceptors and Müller cells, in agreement with immunocytochemical, in situ hybridization and functional data [32-34]. It is noteworthy, however, that FGFR1 was only detected in a fraction of the Müller and photoreceptor cells tested, as was also the case with CNTFR-α and Müller cells. TrkB, moreover, was not detectable in either cell type, a result inconsistent with previous reports of second messenger activation in BDNF-treated Müller cells [24]. It is possible that these results could be due to a low number of transcripts for these receptors (see above, and [14]). It is noteworthy in this regard that, despite the known responsiveness of ganglion cells to BDNF, another study found TrkB only in two out of eight isolated ganglion cells [38]. Alternative interpretations are also possible, however, including differential susceptibility of particular mRNA species to degradation, and/or physiological variations in mRNA levels due to circadian or light-dependent cyclic mechanisms.

Among other applications, the availability of cDNA libraries from individual mouse Müller and photoreceptor cells opens new avenues for the investigation of changes in gene expression associated with mutation-induced photoreceptor degeneration, and with photoreceptor rescue by intraocular neurotrophic factor injection. As mentioned in the Introduction, it has been postulated that photoreceptor rescue by factors such as CNTF, BDNF or FGF2 are likely to occur through indirect mechanisms, probably mediated by Müller cells [24-27]. It appears therefore important to determine the changes in gene expression that occur in both Müller and photoreceptor cells when these neurotrophic factors are injected into the eye, since this could lead to the identification of molecules involved in photoreceptor rescue. These investigations should be facilitated by high throughput microarray analysis of single cell cDNAs, an approach that has recently been shown to be feasible (references [8,9] and our own unpublished observations).

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