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Immunocytochemical Profiling of Cultured Mouse Primary Retinal Cells

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Summary
Primary retinal cell cultures and immunocytochemistry are important experimental platforms in ophthalmic research. Translation of retinal cells from their native environment to the in vitro milieu leads to cellular stress, jeopardizing their in vivo phenotype features. Moreover, the specificity and stability of many retinal immunochemical markers are poorly evaluated in retinal cell cultures. Hence, we here evaluated the expression profile of 17 retinal markers, that is, recoverin, rhodopsin, arrestin, Chx10, PKC, DCX, CRALBP, GS, vimentin, TPRV4, RBPMS, Brn3a, β-tubulin III, NeuN, MAP2, GFAP, and synaptophysin. At 7 and 18 days of culture, the marker expression profiles of mouse postnatal retinal cells were compared with their age-matched in vivo retinas. We demonstrate stable in vitro expression of all markers, except for arrestin and CRALBP. Differences in cellular expression and location of some markers were observed, both over time in culture and compared with the age-matched retina. We hypothesize that these differences are likely culture condition dependent. Taken together, we suggest a thorough evaluation of the antibodies in specific culture settings, before extrapolating the in vitro results to an in vivo setting. Moreover, the identification of specific cell types may require a combination of different genes expressed or markers with structural information. (J Histochem Cytochem 65:223–239, 2017)

Keywords
glia, immunocytochemistry, immunohistochemistry, photoreceptors, primary retinal cell cultures, retinal markers, RGCs

Introduction

For decades, rodents have served as a powerful model in retinal research, in the investigation of retinal structure and function, retinal development, pathology of retinal disorders, and novel treatment approaches. The close similarity to the human eye and retina, especially with regard to retinal structure, makes rodent retinas a relevant model.[1,2] In addition, use of rodents is cost-effective compared with using larger species, such as pigs and non-human primates.

Numerous studies have proven the efficient use of cultured dissociated primary retinal cells as in vitro models to investigate cellular physiology and pathological conditions in ocular diseases, and also used to identify neurotrophic, differentiation, and neurotoxic factors, in drug discovery and investigation of intracellular signaling pathways.[3–5] Retinal cell cultures have gained massive attention in cell replacement therapeutic approaches as a treatment paradigm for different retinal neurodegenerations. To date, several donor cell candidates have been assessed for their transplantation efficacy, ranging from primary retinal tissue to retinal cells derived from different stem cell sources.[6–10] Lately, crucial chemical and physical factors (including bioscaffolds) for optimal donor cell development and administration have been studied by others and us.[11–13]

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The in vitro culture system offers a simplified and isolated milieu where different issues can be explored in a controlled manner.[14] Primary cells are preferably used over immortalized cell lines as the latter are transformed, which alters their characteristics, such as abnormal or uncontrolled metabolic function. In addition, extensive passaging causes genotypic drift, which causes heterogeneity and therefore is not consistent or fully reliable. Generally, primary retinal cells are considered to retain in vitro normal morphology, physiological functions, growth characteristics, and cellular markers of the original tissue and, therefore, can be useful models for early studies of cell biology, gene discovery, target validation, drug testing, and in vitro toxicology.[15,16] However, it has been shown that retinal cells in culture undergo drastic changes due to dissociation stress and while adapting to the in vitro milieu, which varies greatly from the native organism. For example, Liu et al.[17] have compared the global gene expression profile between the development of retina in vivo and retinal explants at ages between postnatal day 2 (PN2) and postnatal day 15 (PN15), and verified that 75% of the retina explants kept the same expression volume and pattern as the retina in vivo studying 8880 expressed genes.[17] They proposed that the changed expression of specific genes is due to the ischemic environment in culture, the change in cell proliferation, and the lack of factors stimulating the later stages of cell maturation. It may also be due to nutrient depletion and accumulation of waste products. Likewise, Hauck et al.[18] reported that adult porcine retinal Müller glial cells when cultured in vitro lose their in vivo features and that cellular retinaldehyde-binding protein (CRALBP), a protein typically expressed by Müller cells in vivo, is downregulated within 2 weeks and that de novo expression of α-smooth muscle actin is induced at this time.[18]

In vitro studies often rely on the detection of stable and specific markers, often antibodies, and visualization through immunochemical techniques for the identification and analysis of retinal cell types and structures, as well as the cell behavior. To further validate the use of primary cell culture systems for retinal studies, it is important to thoroughly evaluate the expression of a large number of markers in retinal cells in in vitro cultures as the majority of markers used have predominantly been described using in vivo retinal tissue. Hence, here, we profile a battery of previously well-described markers in in vivo applications to cultured primary dissociated mouse retinal postnatal cells (RPNCs), describe marker expression maintenance over time in culture, and discuss its suitability for retinal cell identification.

Materials and Methods

Animals

All experimental procedures performed on the animals were performed in accordance with approved guidelines of the Ethics Committee of Lund University, the Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals), and the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research. Animals were housed under a standard 12-hr day/night rhythm.

The eyes of PN4, PN11, and PN22 C3H/HeA mice were used in this study. PN4 mice were decapitated, whereas PN11 and PN22 mice were sacrificed by CO_2 asphyxiation and eyes enucleated.

Tissue Preparation, Embedding, and Cryosectioning

Whole eyes, of PN11- and PN22-old mice, were fixed in a solution of 4% paraformaldehyde (PFA) in 200-mM Sørensen's buffer (200-mM NaH_2PO_4 and 200-mM Na_2HPO_4) for 2 hr and then sequentially transferred to 1× PBS containing 10% and 25% sucrose. The eyes were stored in the 25% sucrose until embedded in Yazulla medium (30% egg albumen and 3% gelatin in water). Twelve-µm-thick sections were cut on a cryostat (MicroM HM 560; Cellab, Radeberg, Germany) and mounted on glass slides coated with a solution of gelatin and chromium potassium sulfate dodecahydrate. Transverse retina cryosections were subsequently stored at –20C until used.

Poly-L-Lysine (PLL) and Laminin Coating of Glass Chamber Slides

All glass chamber slides (Sarstedt, Nümbrecht, Germany) were coated with a 10 µg/ml PLL (Sigma-Aldrich Sweden AB, Stockholm, Sweden) solution for 1 hr at room temperature and subsequently washed with distilled sterile water and air-dried. Then, an overnight incubation at room temperature on a shaker was after performed with 10 ng/ml laminin solution (Sigma-Aldrich Sweden AB). Succeeding washing steps in TBS were performed.

Isolation and Dissociation of Postnatal Day 4 Mouse Retinal Tissue

Retinas were isolated from PN4 mice. For each dissociation session, a single cell suspension of the

retinal tissue was prepared by incubating eight retinas in a 1 mg/ml (18 units/ml) papain solution (DMEM/F-12, 5 ng/ml DNase I [Sigma-Aldrich, St. Louis, MO], 0.3 g/ml L-cysteine [Sigma-Aldrich]) for 30 min at 37C and further mechanically dissociated with a burned-tip glass Pasteur pipette. Single cell suspension was centrifuged for 5 min at 480 × *g* and resuspended in Full-SATO culture medium (neurobasal medium supplemented with insulin [5 µg/ml; Sigma-Aldrich], sodium pyruvate [1 mM; Sigma-Aldrich], L-glutamine [1 mM; Sigma-Aldrich], penicillin/streptomycin [Life Technologies, Waltham, MA], N-acetyl cysteine [5 µg/ml; Sigma-Aldrich], triiodothyronine [40 ng/ml; Sigma-Aldrich], SATO supplement [1:100], B27 and N2 supplement, forskolin [5 mM; Sigma-Aldrich], brain-derived neurotrophic factor [BDNF; 50 ng/ml; Sigma-Aldrich], and ciliary neurotrophic factor [CNTF; 10 ng/ml; Sigma-Aldrich]).[19–21] Cells were counted with an automated cell counter TC20 from Bio-Rad (Hercules, CA) and then seeded onto PLL and laminin-coated flat glass chamber slides at cell densities of 5.3×10^4 viable cells/cm^2 and incubated for 7 or 18 days at 37C in a humidified atmosphere of 5% CO_2. Medium was changed every 2 to 3 days throughout the experiments.

Immunocytochemistry

Retinal cell cultures were fixed in a solution of 4% PFA for 10 min at room temperature and washed three times with 1× PBS, pH 7.2. Retina transverse cryosections and cell cultures were blocked and permeabilized for 30 min using a blocking solution of PBS, 1% BSA (Sigma-Aldrich), and 0.25% Triton X-100 and 5% serum. Blocking was followed by overnight incubation at 4C with primary antibodies diluted in blocking solution. Washing steps were performed before and after 1-hr incubation with the secondary antibodies at room temperature in the dark. Whole mount sections and cell cultures were then mounted using Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA) for nuclei counterstaining. Full lists of the primary and secondary antibodies used are presented in Tables 1 and 2, respectively. Negative control experiments included the omission of primary antibodies and resulted in nonspecific background staining.

Analysis

Microscopy was performed using a fluorescence microscope Axio Imager M2 (Carl Zeiss, Oberkochen, Germany). Images of the stained specimens were obtained using ZEN software from Zeiss. Image enhancements, color balance, contrast, and brightness of the images were adjusted using Adobe Photoshop software (v.CC 2014; Adobe Systems, Mountain View, CA).

Cell-type and structure identification was performed on 7 days in vitro (DIV) cultures and 18 DIV and compared with stained age-matched cryosectioned whole mouse retinas, that is, PN11 and PN22, respectively. Majority of staining sections were not done in parallel between the in vivo eyes and in vitro cultures but done in parallel when necessary to confirm results. Each antibody was tested on a minimum of two and three independent staining sessions to validate the reproducibility of the staining results for intact retinal sections and cell cultures, respectively. In addition, each antibody was validated on three different independent cell culture seedings. No quantitative measurements were made.

Results and Discussion

We cultured the retinal cells on a laminin-coated substrate and in a serum-free medium supplemented with survival and neurotrophic factors, conditions recently reported by us as favorable for survival, phenotypic differentiation, and neurite extension.[13] Retinal cells when dissociated suffer mechanical stress such as breakage of their neurites and axon. Central nervous system neurons, including retinal neurons, lack regeneration capabilities but retain their neuritogenic potential under certain conditions in vivo and in vitro. Axon and neurite regeneration is a complex process that involves neuronal and non-neuronal changes, which commonly activate signaling pathways, and subsequently alter gene expression and protein expression within the neuron.[22] Hence, here, primary retinal cells were cultured on the extracellular matrix protein laminin, a substrate aiding the promotion of axonal regrowth (neuritogenesis),[23] and supplemented with BDNF and CNTF[24] present in the serum-free medium used. This medium was formulated to culture retinal ganglion cells (RGCs) after immunopanning[20,25] and also recently used by us for the efficient culture of the major retinal cell types at fibrous substrates.[13]

It is well known that the expression profile of primary mixed retinal cell cultures is different from their in vivo counterparts.[17,18] When cultured out of their native environment and after mechanical stress, cell morphology, protein expression profile, and cell behavior may change. In this study, we performed an extensive expression profile evaluation of previously in vivo well-described biomarkers (Table 1) on cultured dissociated RPNCs cultured for 7 and 18 days and compared it with their expression in age-matched retina transverse

Table 1. Primary Antibody List.

Antigen	Host	Target Cell	Dilution	Source	Cat. No.
Brn3a	Goat	Retinal ganglion cells	1:50	Santa Cruz Biotechnology, Inc., Santa Cruz, CA	Sc-31984
Chx10	Sheep	Bipolar cells	1:200	Exalpha Biologicals, Inc., Shirley, MA	X1179P
Cone arrestin	Rabbit	Cone photoreceptors	1:5000	Millipore, Temecula, CA	Ab15282
CRALBP	Mouse	Müller cells	1:500	Abcam, Cambridge, UK	Ab15051
DCX	Goat	Immature neurons, horizontal cells	1:200	Santa Cruz Biotechnology, Inc.	SC8066
GFAP	Rabbit	Astrocytes	1:2000	DAKO A/S, Glostrup, Denmark	Z0334
GS	Rabbit	Müller cells	1:2000	Abcam, Cambridge, UK	Ab16802
MAP2	Mouse	Mature neurons	1:200	Sigma-Aldrich	M1406
NeuN	Mouse	Neurons	1:200	Millipore	MAB377
PKC pan	Mouse	Bipolar cells	1:250	BD Biosciences	554207
RBPMS	Rabbit	Retinal ganglion cells	1:500	PhosphoSolutions, Aurora, CO	1830-RBPMS
Recoverin	Rabbit	Photoreceptors	1:15,000	Millipore	AB5585
Rhodopsin	Mouse	Rod photoreceptors	1:600	Millipore	MAB5316
Synaptophysin	Mouse	Neuronal synapses	1:800	DAKO A/S	M0776
TRPV4	Rabbit	Müller cells, retinal ganglion cells	1:500	LifeSpan BioSciences, Inc., Seattle, WA	LS-C94498
Vimentin	Chicken	Müller cells	1:1000	Millipore	AB5733
β-Tubulin III	Mouse	Early neurons	1:1500	Sigma-Aldrich	T8660

Abbreviations: Brn3a, brain-specific homeobox/POU domain protein 3A; Chx10, Ceh-10 homeodomain-containing homolog; CRALBP, cellular retinaldehyde-binding protein; DCX, doublecortin; GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; MAP2, microtubule-associated protein 2; NeuN, neuronal nuclear antigen; PKC, protein kinase C; RBPMS, RNA-binding protein with multiple splicing; TRPV4, transient receptor potential cation channel, subfamily V, member 4.

Table 2. Secondary Antibody List.

Species	Target	Fluorochrome	Dilution	Source	Cat. No.
Donkey	Anti-rabbit	Texas Red	1:200	Abcam, Cambridge, MA	AB6800
Donkey	Anti-sheep	FITC	1:200	Jackson ImmunoResearch Laboratories, Inc., West Grove, PA	713-095-147
Donkey	Anti-goat	Texas Red	1:200	Jackson ImmunoResearch Laboratories, Inc.	705-076-147
Donkey	Anti-goat	Alexa Fluor 488	1:200	Molecular Probes, Inc., Eugene, OR	A-11055
Donkey	Anti-goat	FITC	1:200	Jackson ImmunoResearch Laboratories, Inc.	705-095-147
Donkey	Anti-mouse	Alexa Fluor 488	1:200	Molecular Probes, Inc	A21202
Goat	Anti-mouse	FITC	1:200	Sigma-Aldrich	F8771
Goat	Anti-mouse	Alexa Fluor 594	1:200	Molecular Probes, Inc	A11005
Goat	Anti-rabbit	Alexa Fluor 594	1:400	Molecular Probes, Inc	A-11037
Goat	Anti-rabbit	Alexa Fluor 488	1:200	Molecular Probes, Inc	A11008
Rabbit	Anti-chicken	Alexa Fluor 594	1:500	Abcam, Cambridge, MA	AB6751

sections (PN11 and PN22). We used PN4 retinas as retinogenesis is partially complete and so dissociated cells include a mix of fully differentiated, partially differentiated, and progenitor cells.[26] Also, the use of retinal embryonic and early postnatal cells has been shown to have higher survival rate, and these cells are able to better integrate after transplantation compared with cells collected from adult animals.[7,27] RPNCs were cultured for 7 and 18 days, which represent equivalent in vivo time points before (PN11) and after (PN22) the maturation of most retinal glial and neuronal cells at PN14.[28]

Overall evaluation of the in vitro cultures demonstrated no notable variability on the localization and distribution of the respective antibody-staining pattern in the different seeding sessions nor altered over time; that is, a nuclear marker at 7 DIV remained nuclear at 18 DIV. Only qualitative analysis of the marker expression was made because the cultures formed were very heterogeneous and did not allow for adequate quantification. Representative results from three independent labeling sessions using specimens from three independent cell seedings are here presented. Table 3 presents a global summary of the expression profile of

Table 3. Summary of Cell Markers' Distribution in Intact Retina and in Cultured Retinal Cells.

Antigen	PN11	Immunoreactivity	PN22	Immunoreactivity	7 DIV	18 DIV
Brn3a	+	RGCs	+	RGCs	+	+
Chx10	+	Bipolar cells, Müller cells, amacrine cells	+	Bipolar cells, Müller cells	+	+
Cone arrestin	+	Cone PRs, bipolar cells	+	Cone bipolar cells	−	−
CRALBP	+	Müller cells	+	Müller cells	−	−
DCX	+	Amacrine, horizontal, RGCs	−		+	−
GFAP	+	Astrocytes	+	Astrocytes	+	+
GS	+	Müller cells	+	Müller cells	+	+
MAP2	+	Amacrine, RGCs	+	Amacrine, RGCs	+	+
NeuN	+	Amacrine, RGCs	+	Amacrine, RGCs	+	+
PKC pan	+	Bipolar cells	+	Bipolar cells	+	+
RBPMS	+	RGCs	+	RGCs	+	+
Recoverin	+	Photoreceptors, cone bipolar cells	+	Photoreceptors, cone bipolar cells	+	+
Rhodopsin	+	Rod photoreceptors	+	Rod photoreceptors	+	+
Synaptophysin	+	Synaptic vesicles	+	Synaptic vesicles	Rare	+
TRPV4	+	Müller cells, RGCs	+	Müller cells, RGCs	+	+
Vimentin	+	Müller cells	+	Müller cells	+ (Astrocytes)	+ (Astrocytes)
β-Tubulin III	+	RGCs, cone PRs	+	RGCs, cone PRs	+	+

Abbreviations: PN11, postnatal day 11; PN22, postnatal day 22; DIV, days in vitro; RGCs, retinal ganglion cells; PRs, photoreceptors; Brn3a, brain-specific homeobox/POU domain protein 3A; Chx10, Ceh-10 homeodomain-containing homolog; CRALBP, cellular retinaldehyde-binding protein; DCX, doublecortin; GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; MAP2, microtubule-associated protein 2; NeuN, neuronal nuclear antigen; PKC, protein kinase C; RBPMS, RNA-binding protein with multiple splicing; TRPV4, transient receptor potential cation channel, subfamily V, member 4.

retinal cell markers in intact retina and in cultured retinal cells.

The Retina

The retina is the light-sensitive tissue layer in the eye, where phototransduction, conversion of light into electrical signals, occurs. The vertebrate sensory retina is organized in a layered manner and consists of neurons and glial cells. There are three nuclear layers, the outer nuclear layer (ONL), inner nuclear layer (INL), and the ganglion cell layer (GCL), which are separated by the plexiform layers, the outer plexiform layer (OPL) and the inner plexiform layer (IPL). The retina is also delimited by the outer limiting membrane (OLM), nerve fiber layer (NFL), and the inner limiting membrane (ILM).

Outer Nuclear Layer (Photoreceptor Layer)

The photoreceptors (PRs), together with the RGCs, are responsible for the process of phototransduction.[29] There are two types of PRs, the rods and cones, which are found in the outer part of the retina; their nuclei are located in the ONL, and their inner segments (IS) and outer segments (OS) are oriented toward the retinal pigment epithelium. PRs through their numerous synapses contact the dendrites of bipolar cells and dendrites or axons of horizontal cells at the OPL. The photopigments, which are photosensitive pigments that undergo a chemical change when they absorb light, are localized in the membrane of the OS discs. Rod cells contain a visual pigment called rhodopsin, whereas the cone cells contain the cone opsins, referred to as iodopsins.[29,30]

In this study, we use three different antibodies to detect PRs. Recoverin, a calcium-binding protein that inhibits the rhodopsin kinase,[31] is expressed by rod and cone PRs and a subset of bipolar cells.[32] Rhodopsin, a photopigment of the G-protein-coupled protein superfamily, is present only in rod PRs, thus considered a marker for rod PRs.[33] To identify cone PRs, we use cone arrestin. Arrestins are a superfamily of regulatory proteins involved in G-protein-coupled receptor (GPCR) desensitization, internalization, and GPCR-mediated activation of mitogen-activated protein kinase pathways.[34]

Immunohistochemical analysis of PN11 (Fig. 1A) and PN22 (Fig. 1D) mouse retina shows that recoverin is detected in the somas of all cells located in the ONL (PRs) and some cells in the INL, known to be a subtype of bipolar cells. In addition, recoverin is detected in processes that extend from the OLM to the OPL and some processes at the IPL. However, at PN22, recoverin is highly expressed in cells of the INL, which

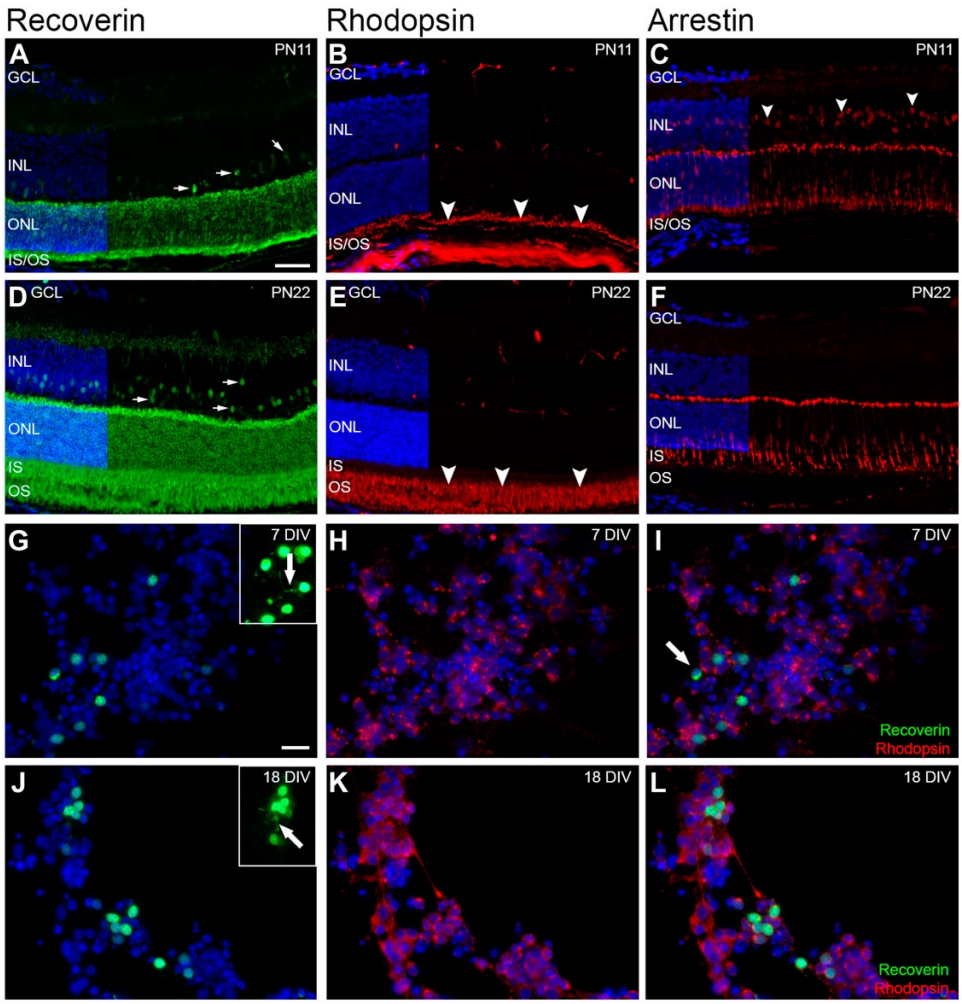

Figure 1. Immunofluorescent staining with anti-recoverin, anti-rhodopsin, and anti-arrestin of postnatal mouse retinas and of in vitro cultured RPNCs. (A–F) Fluorescence photomicrographs of transverse frozen sections through mouse retinae. Retinal layers are indicated as OS, IS, ONL, INL, and GCL. (G–L) Fluorescence photomicrographs of in vitro cultured RPNCs. At PN11 (A) and at PN22 (D), recoverin (green) is labeling the soma of all ONL cells (photoreceptors) and few cells in the INL shown by the arrows. Recoverin labeling is observed in few cells, and its expression is in the soma and the short processes (seen on the enlarged panel) on the cultured RPNCs at both 7 (G) and 18 (J) days in culture (7 and 18 DIV, respectively). Rhodopsin (red) immunoreactivity is observed in the OS at PN11 (B) and PN22 (E) as indicated by the arrowheads in (E). At 7 (H) and 18 DIV (K), rhodopsin immunolabeling is detected in many cells and on the soma and processes in a granular pattern. Double immunolabeling at 7 (I) and 18 DIV (L) shows colocalization of recoverin (green) and rhodopsin (red) but also recoverin-positive but rhodopsin-negative cells (arrow in I). Arrestin (red) is weakly detected on the soma of cone photoreceptors and strongly in the cell processes at both PN11 (C) and PN22 (F). Few cells in the INL corresponding to cone bipolar cells (arrowheads) are also immunoreactive at PN11 but not at PN22. Arrestin was not detected on the in vitro cultures. Abbreviations: RPNCs, retinal postnatal cells; OS, outer segments; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; PN11, postnatal day 11; PN22, postnatal day 22; DIV, days in vitro. Scale bars: A = 50 μm; G = 20 μm.

indicates that recoverin is upregulated in adult retinas in a presumably subtype of bipolar cells. These observations confirm previous studies from Sharma et al.[35]

Rhodopsin is detected in the OS of PRs in retinas at PN11 and PN22 (Fig. 1B and E, respectively), in agreement with observations by Piret et al.[36]

Arrestin-C presented weak and diffuse immunoreactivity in the cell soma and strong immunoreactivity in the processes extending from the IS/OS to the OPL of cone PRs (Fig. 1C and F). In addition, at PN11, positive arrestin-immunolabeling is also observed in dispersed cells in the INL but not at PN22. This might indicate that arrestin is transiently expressed by presumably a subset of bipolar cells, more specifically the cone bipolar cells before full retinal maturation. To our knowledge, this observation has not been shown elsewhere.

In vitro, at both 7 and 18 DIV, recoverin is detected in the soma of few cells (mostly round cell morphology) and in short cell processes (Fig. 1G and J, respectively)

as also observed by Piret et al.[36] at 18 DIV of retina cell cultures on gallium phosphide (GaP) nanowire arrays. Few of the recoverin-positive cells were rhodopsin negative (Fig. 1I and L), and arrestin negative, may be identifying a subtype of bipolar cells. Positive immunoreactivity of anti-rhodopsin was also detected at both time points in the soma and cell processes, mostly in a granular pattern (Fig. 1H and K, respectively).[37] Rhodopsin-positive cells ranged from unipolar to ramified/multipolar morphologies. Arrestin-positive cells could not be detected in our cell culture conditions. Absence of arrestin-positive cells is a surprising finding, as PRs are generated during early embryonic development and maturation completed during the first postnatal week.[38] Also, Piret et al.[36] have observed arrestin-positive cells on GaP nanowires, which indicates that mouse cells do have the ability to retain arrestin expression under certain culture conditions. In addition, McGinnis et al.[4] detected arrestin-expressing cells in primary rat cultures. We hypothesize that in our culture conditions, cone arrestins are downregulated in cone PRs, which may be likely due to the lack of retinoic acid in the present culture medium, known to modulate the expression of arrestin,[39,40] or due to some component in the Full-SATO medium, such as CNTF, resulting in the loss of immunoreactivity.[41] The other hypothesis is that there are no cone PRs in our culture. However, to confirm the presence/absence of cone PRs in our culture, other cone PR markers could be tested in further studies, such as peanut agglutinin lectin, blue (S) or green (M) cone opsin.[42,43]

One intriguing finding was the fact that rhodopsin-positive cells were in higher proportion than recoverin-positive cells at both time points. Similarly, studies by Cao et al.[44] and McGinnis et al.[4] have shown the presence of recoverin-negative PR cells (rhodopsin-positive or arrestin-positive cells) in rat retinal cultures. They suggested that it might be due to an appropriately switched off recoverin gene in PRs. On the contrary, the high number of rhodopsin-positive cells observed in our cultures is in disagreement with studies by Ezzeddine et al.[45] and Kirsch et al.[46], where they have shown that CNTF inhibited rod differentiation in postnatal rat retina, while promoting differentiation of bipolar and Müller cells. In our culture system, the effect of BDNF and insulin may through activation of the insulin growth factor 1[47] compensate the supposed effect of CNTF on rod differentiation.

Inner Nuclear Layer

The INL comprises four cell types: bipolar, horizontal, amacrine, and retinal Müller glia cells. Their cell bodies are located in the INL, but the processes of the neuronal cell types extend from the OPL to the IPL. The INL is bipolar and amacrine cells dominated, making up 41% and 39% of the total INL cells, respectively. Müller cells comprise 16%, whereas horizontal cells make up only 3%.[48]

There are at least 10 types of bipolar cells, which are typically classified as ON and OFF bipolar types, and rod bipolar cells. The ON bipolar cells depolarize at light onset, whereas the OFF bipolar cells depolarize at light offset.[49] Bipolar cells transmit signals through graded potentials from the PRs (rods, cones) or horizontal cells directly to RGCs or indirectly to RGCs via amacrine cells.

The entire bipolar cell population can be identified with the homeodomain-containing transcription factor encoded by the gene *Vsx2*, known as Chx10 (Ceh-10 homeodomain-containing homolog). Chx10 is a regulator of both proliferation of retinal precursor cells and differentiation during retinogenesis.[50] Chx10 is expressed from early retinal development and downregulated in postmitotic retinal cell types, except bipolar cells and a subset of Müller glia cells.[51] Therefore, Chx10 is widely used as a pan bipolar cell marker in adult retina. We observed positive immunolabeling for Chx10 in cell nuclei at both PN11 and PN22 retinas (Fig. 2A and D, respectively) of bipolar cells and probably also a few Müller cells.[52] At PN11, weak Chx10 somatic labeling is also observed in cells at the GCL, most probably corresponding to displaced amacrine cells but not at PN22. We therefore show that Chx10 is downregulated after PN11 in displaced amacrine cells. This is in accordance with previous studies, as the mouse neural retina is considered matured by PN14.[28]

In vitro, very few and weakly labeled Chx10 cells were encountered and its immunoreactivity remained in the cell nuclei (Fig. 2G and J, 7 and 18 DIV, respectively). Rod bipolar cells can be specifically identified with antibodies against protein kinase C alpha (PKCα),[38] although PKCα is also expressed to a lesser extent by amacrine cells and cones.[53] PKC is a member of the family of homologous serine–threonine, calcium-dependent and phospholipid-dependent protein kinases that have a key role in signal transduction and cellular regulation.[54] Immunohistochemical staining with pan anti-PKC antibody (the pan antibody has higher affinity to PKCα) at PN11 and PN22 retinas (Fig. 2B and E, respectively) shows strong reactivity in cell bodies located in the innermost INL (close to OPL) and also in their processes extending to the synaptic terminals at IPL (i.e., synapsing with RGC dendrites). At both time points, positive labeling is observed in the IS/OS region but not observed in cells at the GCL. However, Fan et al.,[28] at PN10, show immunoreactivity

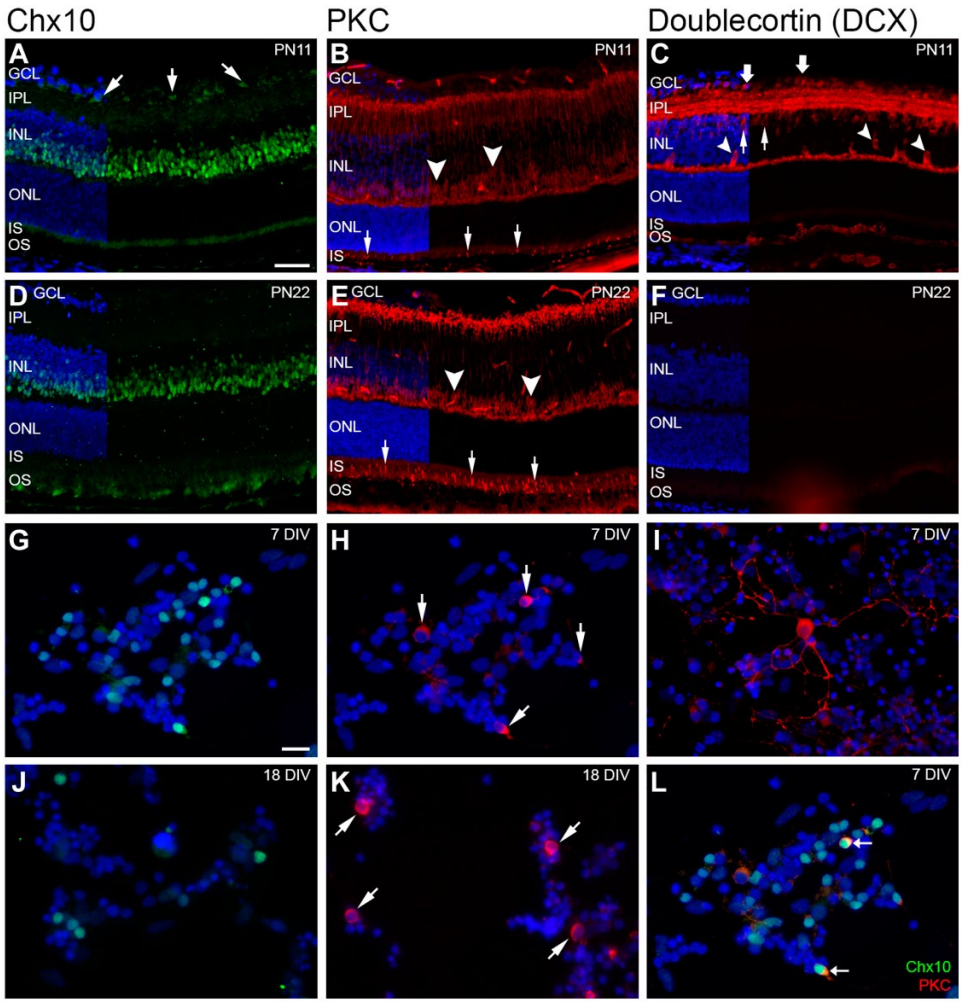

Figure 2. Immunofluorescent staining with anti-Chx10, anti-PKC, and anti-DCX of postnatal mouse retinas and of in vitro cultured RPNCs. (A–F) Fluorescence photomicrographs of transverse frozen sections through mouse retinae. Retinal layers are indicated as OS, IS, ONL, INL, IPL, and GCL. (G–L) Fluorescence photomicrographs of in vitro cultured RPNCs. At PN11 (A) and at PN22 (D), Chx10 (green) is labeling the nuclei of cells in the INL; however, at PN11, Chx10 reactivity is also observed in the cytoplasm of cells at GCL as shown by the arrows. PKC (red) immunoreactivity is observed at both time points (B, E) on the cell bodies (arrowheads) located in the lower INL and processes extending to the synaptic terminals to the IPL and on few processes in the IS/OS region (arrows). DCX (red) strongly immunolabels horizontal cells (arrowheads), amacrine cells in INL (thin arrows), and displaced amacrine cells (thick arrows) and cell processes in the IPL at PN11 (C), but no DCX expression is observed at PN22 (F). At 7 DIV, Chx10 (G) continues to be expressed in the cell nuclei and PKC (H) is detected in the cytoplasm (arrows) and short cell processes. At 18 DIV, the same expression pattern is observed for Chx10 (J) and PKC (K). (L) Double labeling of Chx10 (green) and PKC (red) at 7 DIV, where arrows indicate Chx10+/PKC+ cells. On cultured cells, at 7 DIV, DCX is detected in the cytoplasm and long processes in few large cells with ramified morphology (I). Abbreviations: Chx10, Ceh-10 homeodomain-containing homolog; PKC, protein kinase C; DCX, doublecortin; RPNCs, retinal postnatal cells; OS, outer segments; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; PN11, postnatal day 11; PN22, postnatal day 22; DIV, days in vitro. Scale bars: A = 50 µm; G = 20 µm.

of bipolar cells and no reactivity in the IS/OS region. This difference might be due to the use of distinct anti-PKC antibodies. At 7 and 18 DIV, PKC immunolabeling is observed in very few cells and is located in the cell cytoplasm and cell processes (Fig. 2H and K, 7 and 18 DIV, respectively). In addition, these PKC-positive cells colocalize with Chx10 nuclear staining, confirming the identity of rod bipolar cells and presence of bipolar cells in culture (see Fig. 2L, arrows indicate Chx10+/PKC+ cells).

The low number of bipolar cells found may be due to the fact that genesis of these neurons still occurs at PN4,[38] and hence, the culture protocol used may not support further bipolar cell generation. However, we believe that the used culturing conditions per se should be permissive for bipolar cell survival and genesis. For

example, Wexler et al.[55] examined the effect of different neurotrophic factors and observed that BDNF had a positive and significant effect on the survival of cultured postnatal rat rod bipolar cells.

Horizontal cells together with amacrine cells are interneurons. There are many amacrine cell subtypes, but only one known horizontal cell type is present in the mouse retina.[53] Horizontal cell processes ramify in the OPL and establish connections with PRs and bipolar cells, whereas amacrine cells extend processes laterally within the IPL, establishing postsynaptic connections with bipolar cells and presynaptic connections with RGCs.[56]

Doublecortin (DCX) was here, for the first time, evaluated in mouse retina. DCX is considered as a horizontal cell marker in adult rat retinas.[57] Wakabayashi and colleagues[57] have shown that DCX in the retina is expressed as early as embryonic day 15 and continues to be expressed in horizontal cells in adult rat retinas. DCX is a microtubule-associated protein (MAP) involved in neuronal differentiation, migration, and plasticity. DCX is expressed in neuronal precursor cells, young immature postmitotic neurons, and migrating neurons.[58,59] Here, we show a different expression pattern in the postnatal mouse retina compared with the rat retina. At PN11 (Fig. 2C), strong immunoreactivity is observed in the IPL, OPL, and on few and large cell bodies located in the outer margin of the INL, possibly corresponding to horizontal cells. Weaker immunoreactivity is observed also in cell bodies located in the inner margin of the INL and in a few cells in the GCL, likely to correspond to displaced amacrine cells. At PN22, no DCX immunoreactivity could be detected in any cell or structure, suggesting that DCX may be downregulated in the adult mouse, and further in-depth analysis is needed before it can be suggested as a biomarker for mature mouse horizontal cells (Fig. 2F). Positive and strong DCX immunoreactivity was detected in cell bodies and processes in a few large cells with ramified morphology in cultures at 7 DIV (Fig. 2I) but not at 18 DIV (not shown), in accordance with our observations in corresponding retinal sections.

Müller cells are radial glial cells, with their cell bodies located in the INL, and a bipolar morphology, extending one stem process to the subretinal space and the other to the vitreous surface, where it forms a so-called endfoot.

In the normal postnatal mouse retina, glutamine synthetase (GS) and vimentin are typically used as specific markers for retinal Müller cells.[60–63] GS is an enzyme that converts glutaminate to glutamine,[64] whereas vimentin is an intermediate filament–forming protein.[61] CRALBP, which has an essential function in the visual cycle,[65,66] and the transient receptor potential cation channel, subfamily V, member 4 (TRPV4), a polymodal cation channel known to mediate osmo- and mechanotransduction,[67] are also used to identify Müller cells in the retina; however, they are not specific to Müller cells. Retinal pigment epithelial cells also express CRALBP while TRPV4 is abundantly expressed in RGCs.[66,67] Therefore, CRALBP and TRPV4 cannot be used as specific markers for Müller cells.

At PN11 and PN22, CRALBP specifically and strongly labels the Müller cell, from the processes at the subretinal space to the endfeet, including cell soma (Fig. 3A and E). Thus, the entire cellular structure of the Müller cells is best revealed using CRALBP. At both time points, GS immunolabeling is visualized throughout the entire extent of the transverse retina, including the soma and the fine side branches which appear as "background staining," as seen in Fig. 3B and F. Stronger immunoreactivity is observed in the GCL, and lower immunoreactivity is observed in thin processes in the ONL. In parallel, a very similar GS immunoreactivity pattern was reported by Brand et al.[68] for the mouse retina. However, other studies demonstrate a different distribution pattern of GS immunolabeling that may be explained by different antibodies or tissue processing protocols used.[68–70] At both time points (PN11 and PN22), vimentin is strongly labeling the Müller cell fibers from the NFL to the OPL (Fig. 3C and G, respectively).

The anti-TRPV4 antibody is here shown to intensively label whole Müller cells, their soma, and processes from ILM to OLM and fine side branches but is also observed in the soma of RGCs (Fig. 3D and H). This observation is in accordance with Jo et al.,[71] where they also show TRPV4 immunolabeling in Müller endfeet and proximal processes in the INL as well in the RGCs' soma. However, Piret et al.[36] showed TRPV4 immunoreactivity only in the RGC soma, while using a different batch of the same anti-TRPV4 antibody (Cat No. LS-C94498).

In vitro, at both 7 and 18 DIV, many cells were positively immunolabeled with anti-GS, anti-vimentin, and anti-TRPV4 antibodies. GS immunolabeling is typically strong in the cell nucleus and weaker in cell body and processes, and identifying bi- and multipolar cells (Figs. 3I and L, 5B). Vimentin immunolabeling is observed in cells with other cell morphology, such as polygonal shape (Fig. 3J and M), which we further show being colabeled with anti–glial fibrillary acidic protein (GFAP) antibody (Fig. 5A) but not with GS or TRPV4 (Fig. 5B and C, respectively). TRPV4 immunolabeling is found in cells of different morphology, one being of neuronal type, presenting strong immunolabeling in the large

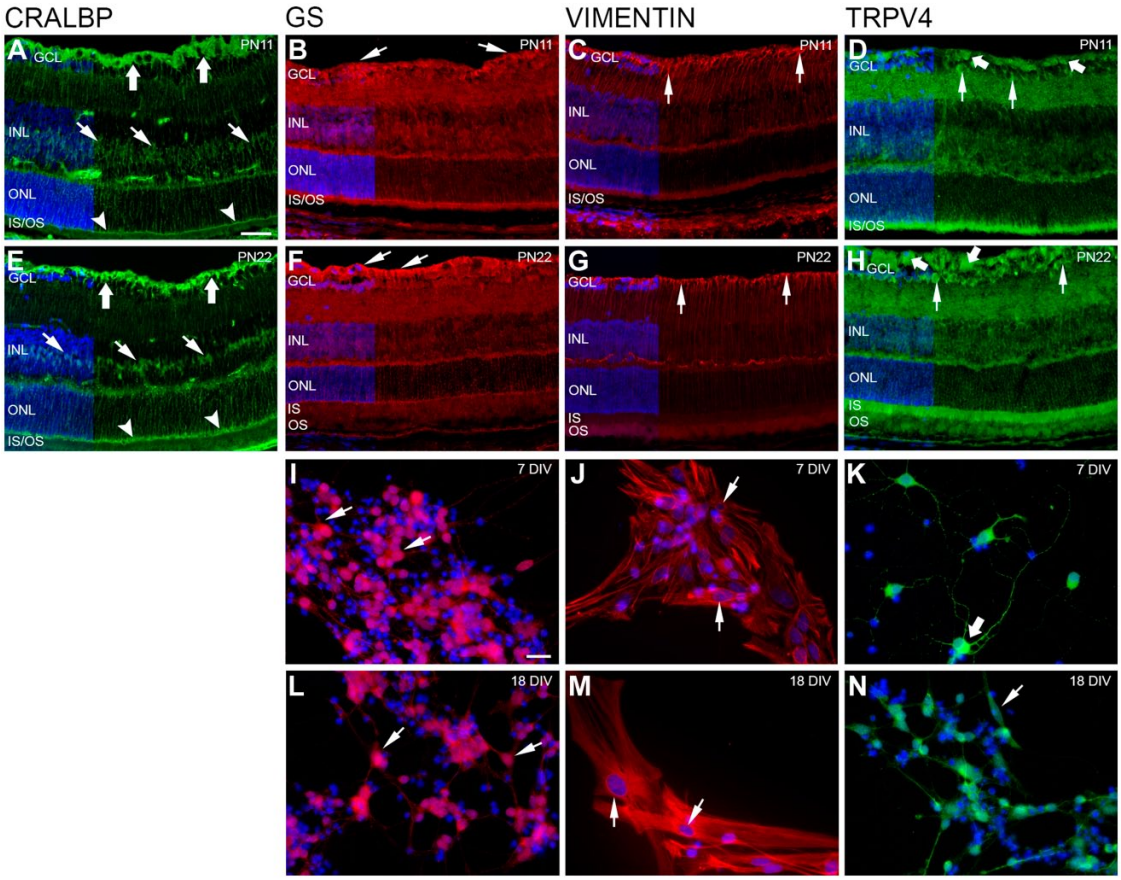

Figure 3. Immunofluorescent staining with anti-CRALBP, anti-GS, and anti-TRPV4 of postnatal mouse retinas and of in vitro cultured RPNCs. (A–H) Fluorescence photomicrographs of transverse frozen sections through mouse retinae. Retinal layers are indicated as OS, IS, ONL, INL, and GCL. (I–N) Fluorescence photomicrographs of in vitro cultured RPNCs. At PN11 (A) and at PN22 (E), CRALBP (green) is specifically labeling the Müller cells. Thin arrows indicate Müller cell soma, whereas arrowheads and thick arrows indicate the endfeet. CRALBP-positive immunoreactivity is not observed in cultured cells. GS (red) immunoreactivity is observed throughout the extent of the retina at both time points (B, F). Müller cells' endfeet are strongly labeled (arrows). At 7 (I) and 18 DIV (L), GS-positive cells are observed with nuclear and cytoplasmic (arrows) and cell processes labeling. At PN11 (C) and at PN22 (G), vimentin (red) is strongly labeling Müller cell fibers from the nerve fiber layer to the outer plexiform layer. Arrows indicate strong labeling of the Müller cell endfeet. At 7 (J) and 18 DIV (M), vimentin labeling shows cells of polygonal shape (arrows). At PN11 (D) and at PN22 (H), TRPV4 (green) is labeling the extent of the retina and is strongly immunoreactive in retinal ganglion cells' soma (thin arrows), axon bundles (thick arrows), and the IS region. TRPV4 immunolabeling on culture cells, at 7 (K) and 18 DIV (N), appears on cells of two different morphologies, one neuronal (thick arrow) and other of Müller cells (thin arrow). Abbreviations: CRALBP, cellular retinaldehyde-binding protein; GS, glutamine synthetase; TRPV4, transient receptor potential cation channel, subfamily V, member 4; RPNCs, retinal postnatal cells; OS, outer segments; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; PN11, postnatal day 11; PN22, postnatal day 22; DIV, days in vitro. Scale bars: A = 50 µm; G = 20 µm.

cell soma and multiple dendrites derived from the soma, probably corresponding to RGCs (as observed in Fig. 3K). The other cell morphology is similar to the cells positively labeled with GS, indicating that they may be Müller cells (as observed in Fig. 3N). Unfortunately, double staining of TRPV4 and GS was not possible, as both antibodies were raised in the same host species.

CRALBP-positive cells were not found in cultured cells; therefore, we hypothesize that it is downregulated in cultured Müller cells using the current culturing protocol. Hauck et al.[18] have shown that in rodent retinas, CRALBP is downregulated after the second week after birth. In addition, CRALBP is important for maintaining normal cone–driven vision and accelerating cone dark adaptation.[65] However, we did not manage to identify cone PRs in our cultures. On the contrary, Piret et al.[36] showed CRALBP-positive cells using the same antibody when cultured on GaP nanoarrays at 18 DIV, probably meaning that CRALBP expression may be dependent on certain chemical and possible physical cues.

Ganglion Cell Layer

In the GCL, RGCs and displaced amacrine cells can be found. The RGCs comprise 3% of the total retinal cells. Multiple RGC types have been identified, and they vary in terms of functions,[72] morphology, and location in the GCL, more specifically relative to the extension of their dendritic arbor in the IPL, but they all possess a long axon that forms the optic nerve.

Many different molecular markers have been identified for RGCs; however, only the RNA-binding protein with multiple splicing (RBPMS) can be used as a pan RGC marker (expressed in whole RGC population).[73,74] RBPMS genes are members of the RNA recognition motif family known to be involved in gene expression regulation.[73] Brain-specific homeobox/POU domain protein 3A (Brn3a), a POU-domain-containing transcription factor, is another widely used RGC specific marker, which identifies about 85% of the mouse RGCs population.[74] Neuronal nuclear antigen (NeuN or Fox3), an RNA-binding protein that is highly specific for postmitotic neurons,[75,76] β-tubulin III, a constituent of neural microtubules and used as a marker for early neuronal lineage and members of MAPs, such as MAP2, are other commonly used markers to identify RGCs. However, they are not exclusively expressed in RGCs. For instance, NeuN and MAP2 are both expressed by amacrine cells,[75,77,78] whereas β-tubulin III, in the adult retina, is mainly found on RGC dendrites in the INL, on the NFL fiber bundles, and in a few processes in the ONL that might correspond to cone processes.[79,80]

In postnatal mouse (and rat) retinas, RBPMS immunolabeling is confined to the cytoplasm of the RGC soma and the dendrites proximal to the perikarya (Fig. 4A and B).[74] Yet, Kwong and colleagues[73] have also observed weak nuclear localization with anti-RBPMS antibodies. Brn3a immunolabeling is located in the nucleus of RGCs (Fig. 4E and F).[81]

At PN11 and PN22, RGC dendrites and axon bundles are strongly immunoreactive for β-tubulin III (Fig. 4I and J). Moreover, some cell processes in the ONL are also labeled with β-tubulin III, which we here show as belonging to cone PRs (Fig. 4U and V). NeuN has the same expression pattern as RBPMS, except that NeuN also weakly labels the soma of amacrine cells in the INL (Fig. 4M and N, PN11 and PN22, respectively).[82] MAP2 at PN11 and PN22 (Fig. 4Q and R, respectively) strongly labels dendrites in the IPL and cell bodies located in the GCL and inner margin of the INL, corresponding to RGCs and amacrine cells, confirming observations from Okabe and colleagues.[77]

In cultured retinal cells, (7 and 18 DIV), RBPMS immunoreactivity is observed in very few cells and displays a strong somatic labeling (Fig. 4C and D, respectively), whereas Brn3a is found in the cell nucleus (Fig. 4G and H, 7 and 18 DIV, respectively). Both antibodies have the expression pattern as in vivo. However, Piret et al.[36] show Brn3a immunolabeling using the same antibody (Sc-31984) in the perinuclear area and cell processes when cells were cultured on GaP nanowire arrays. This difference in expression pattern allows us to assume that its expression is substrate and culture medium dependent.

Strong immunolabeling with anti-β-tubulin III antibody is observed in the cell cytoplasm and in the cell processes (Fig. 4K and L, 7 and 18 DIV, respectively). The same expression pattern is observed with the anti-MAP2 antibody (Fig. 4S and T, 7 and 18 DIV, respectively). NeuN immunolabeling is weakly found in cell nuclei and in a granular pattern in the cell cytoplasm and some processes (Fig. 4O and P, 7 and 18 DIV, respectively).

An intriguing observation is that all NeuN-positive cells are TRPV4 positive (Fig. 5D and E). NeuN is supposed to label cells of neuronal type and not glia. Therefore, our results indicate that NeuN cannot be used as a general neuronal marker for retinal cell cultures as it also seems to label Müller cells.

Optic Nerve Fiber Layer

Astrocytes are the second type of retinal macroglial cells. In the adult mouse, they are found in the innermost part of the retina, below the ILM, where they have pivotal functions in neuronal signaling and maintaining the blood–retina barrier.[63] Glial fibrillary protein (GFAP), an intermediate filament protein, is routinely used to identify astrocytes. Upregulation of GFAP can occur in Müller cells, which is a hallmark of retinal gliosis.[83] As mentioned earlier, CRALBP in young retinas is also expressed by astrocytes but is downregulated after the second week after birth.[66]

At PN11 and PN22, GFAP immunolabeling appears in the OPL and in the NFL (Fig. 6A and B, respectively). In the NFL, positive immunoreactivity identifies astrocytes. Why there is positive immunoreactivity at OPL, even with vimentin (Fig. 3C and G), is not yet understood.

GFAP-positive cells in culture are present at 7 and 18 DIV and are mainly of flat irregular or polygonal shape, typical of astrocytes in culture (Fig. 6C and D, 7 and 18 DIV, respectively).[84,85] In addition, we observed that some GFAP-positive cells extended one or more long processes, resembling an astrocyte toward a radial glia-like phenotype,[86,87] which was also reported by Piret et al.[36] Furthermore, double labeling with anti-vimentin antibody clearly shows colocalization with GFAP (Fig. 5A). This might indicate that vimentin in

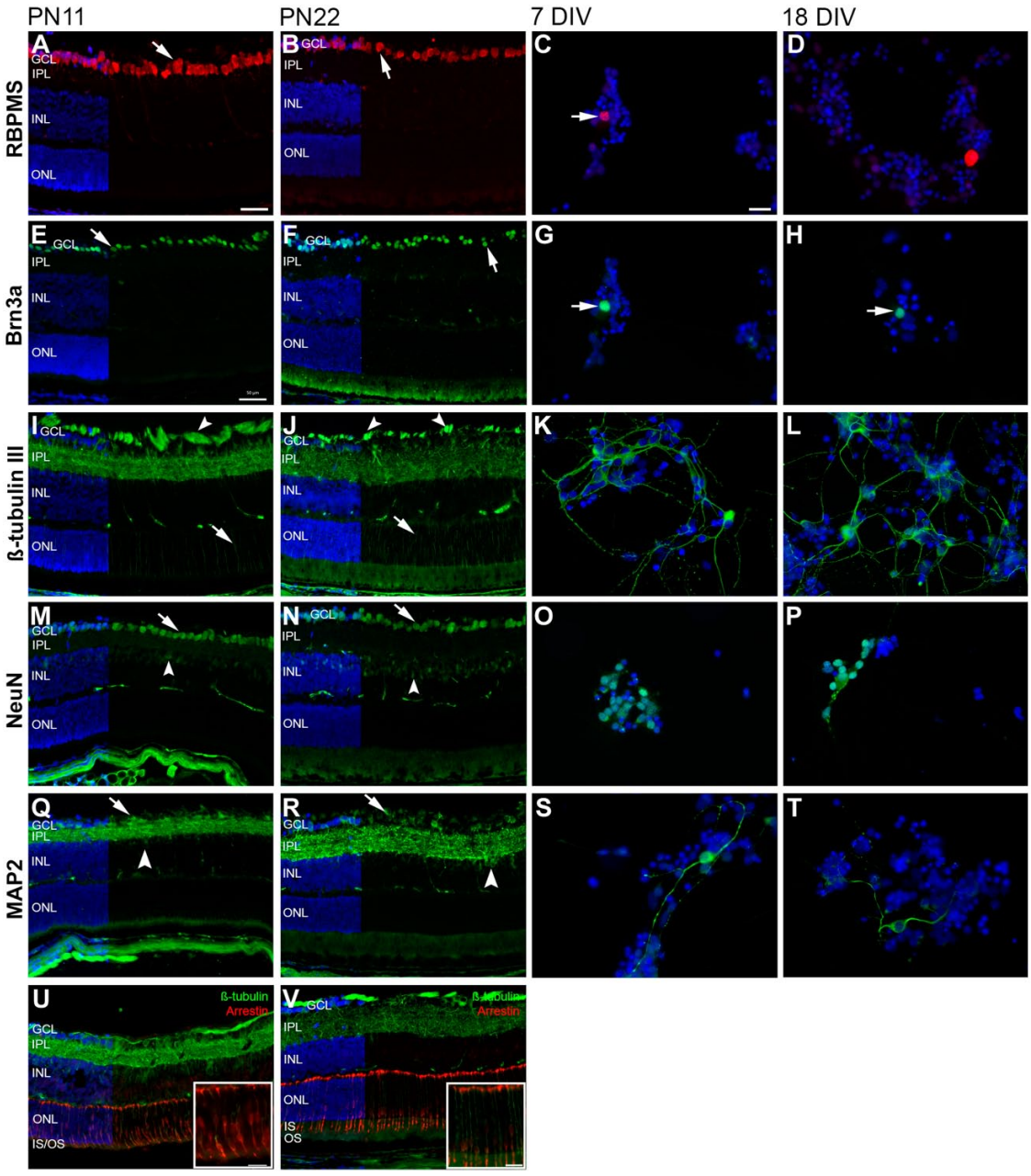

Figure 4. Immunofluorescent staining with commonly used RGC markers on postnatal mouse retinas and on in vitro cultured RPNCs. RBPMS immunolabeling on transverse frozen sections through mouse retinae at PN11 (A) and at PN22 (B) and on in vitro cultured RPNCs for 7 (C) and 18 (D) days. Retinal layers are indicated as OS, IS, ONL, INL, IPL, and GCL. RBPMS is localized in the RGCs' soma on both the retina and on cultured cells. Brn3a nuclear immunolabeling on mouse retinae at PN11 (E), at PN22 (F), and at in vitro cultured RPNCs for 7 (G) and 18 (H) days. β-tubulin III immunolabeling on mouse retinae at PN11 (I) and at PN22 (J) is localized in RGC axon bundles (arrowheads) and dendrites at IPL and some at ONL (thin arrow). (K, L) On cultured cells, β-tubulin III labels neurons with long processes. (M, N) NeuN strongly labels the soma of RGCs (arrows) and weakly the soma of amacrine cells (arrowheads) on the mouse retina at PN11 and PN22. At 7 (O) and 18 DIV (P), NeuN strongly labels cell nuclei and weakly the perinuclear cytoplasm. (Q–T) MAP2 immunolabeling on mouse retina at PN11 (Q) and at PN22 (R) is localized in RGCs and amacrine cells' soma and dendrites at IPL. (S, T) On cultured cells, MAP2 labels neurons with long processes. (U, V) β-Tubulin III (green) and arrestin (red) double labeling, at PN11 and PN22, clearly shows colocalization of β-tubulin III dendrites at ONL with cone photoreceptors. Abbreviations: RGCs, retinal ganglion cells; RPNCs, retinal postnatal cells; RBPMS, RNA-binding protein with multiple splicing; PN11, postnatal day 11; PN22, postnatal day 22; OS, outer segments; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; Brn3a, brain-specific homeobox/POU domain protein 3A; NeuN, neuronal nuclear antigen; DIV, days in vitro; MAP2, microtubule-associated protein 2. Scale bars: A = 50 μm; C = 20 μm.

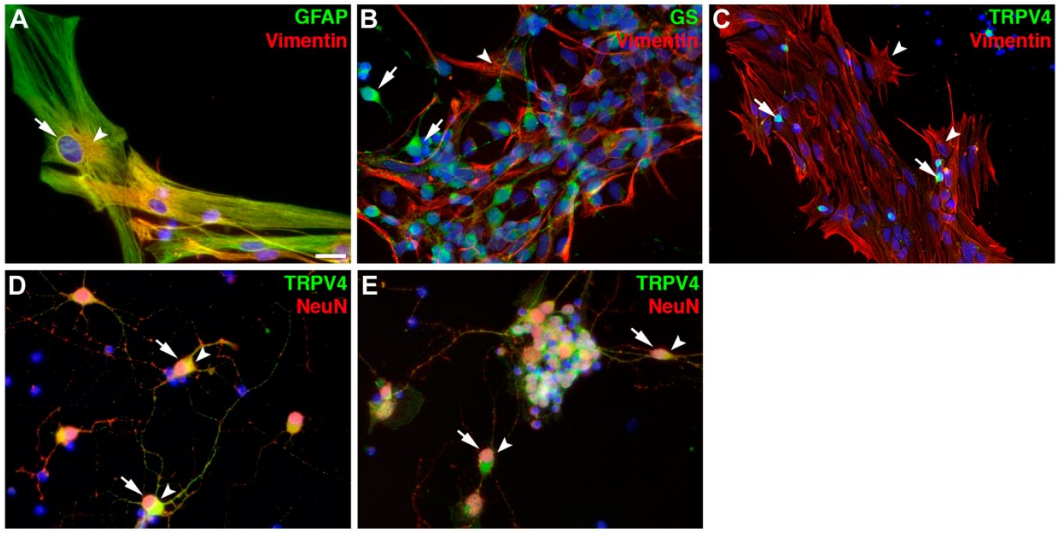

Figure 5. Morphological and immunocytochemical characterization with anti-vimentin, anti-GFAP, anti-TRPV4, and anti-NeuN antibodies of in vitro cultured RPNCs. (A–E) Fluorescence photomicrographs of in vitro cultured RPNCs at 7 (B–E) or 18 DIV (A). Vimentin (red, arrowhead) and GFAP (green, white arrow) expressions are found colocalized (A), but vimentin is not colocalized with GS (green, white arrows; B) or TRPV4 (green, white arrows; C). This indicates expression of vimentin in astrocytes and not in Müller cells nor neurons. (D, E) Colocalization of TRPV4 (green) and NeuN (red) in cells with typical neuronal cell morphology (arrowheads) and also Müller cell morphology (arrows). Abbreviations: GFAP, glial fibrillary acidic protein; TRPV4, transient receptor potential cation channel, subfamily V, member 4; NeuN, neuronal nuclear antigen; RPNCs, retinal postnatal cells; DIV, days in vitro; GS, glutamine synthetase. Scale bar: A = 20 µm.

culture is expressed in astrocytes instead of Müller cells. Moreover, as mentioned previously, vimentin-positive cells do not colocalize with GS (Fig. 5B).

Functional Neuronal Circuitry

In culture, formation of functional neuronal circuits is required and desired; thus, neurons should be actively communicating with surrounding cells to resemble the native phenotype. Consequently, formation of functional synaptic circuits is essential. Neuronal dendrites are filled with synaptic vesicles, which are specialized secretory organelles that release neurotransmitters. Presynaptic vesicles can be identified with synaptophysin, a synaptic vesicle membrane protein, also known as synaptic vesicle protein p38.[88,89]

At PN11 and PN22, synaptophysin immunoreactivity is found in the cell processes in the IPL and OPL (Fig. 7A and B, PN11 and PN22, respectively), which is in accordance to Fan et al.[28]

Our cell culture conditions offered an excellent environment for retinal neurons to grow and extend long neurites. We also observed that cells established connections with other cells and expressed synaptophysin. At 7 DIV (Fig. 7C), very few cells were labeled with the antibody against synaptophysin compared with 18 DIV (Fig. 7D). Synaptophysin labeling presented a punctate appearance most probably representing an aggregation of the vesicles in the cell body and in cell processes. Synaptophysin labeling was observed to be associated with cell clusters, which indeed is in agreement with its neuronal function. We therefore here conclude that there is a progression of neuronal functional maturation over time in culture and that synaptophysin may be used as an indicator of this process. However, whether the retinal neurons have the potential to become electrically active using the current culture protocol needs to be confirmed using electrophysiological recordings.

Conclusion

In this report, we evaluated a battery of retinal markers for their use in a retinal cell culture assay, using routine planar culture substrates, and compared their expression pattern in cultured cells to that in age-matched intact retinas. The use of in vitro primary cell cultures is considered to be a relevant initial approach to investigate cellular physiology and pathological conditions before considering in vivo models. Our results clearly indicate that a few of the markers examined display an altered expression profile in cultured cells and that some commonly used markers are not suitable for the proper identification of a specific retinal cell type population by immunocytochemistry because they are expressed in more than one cell

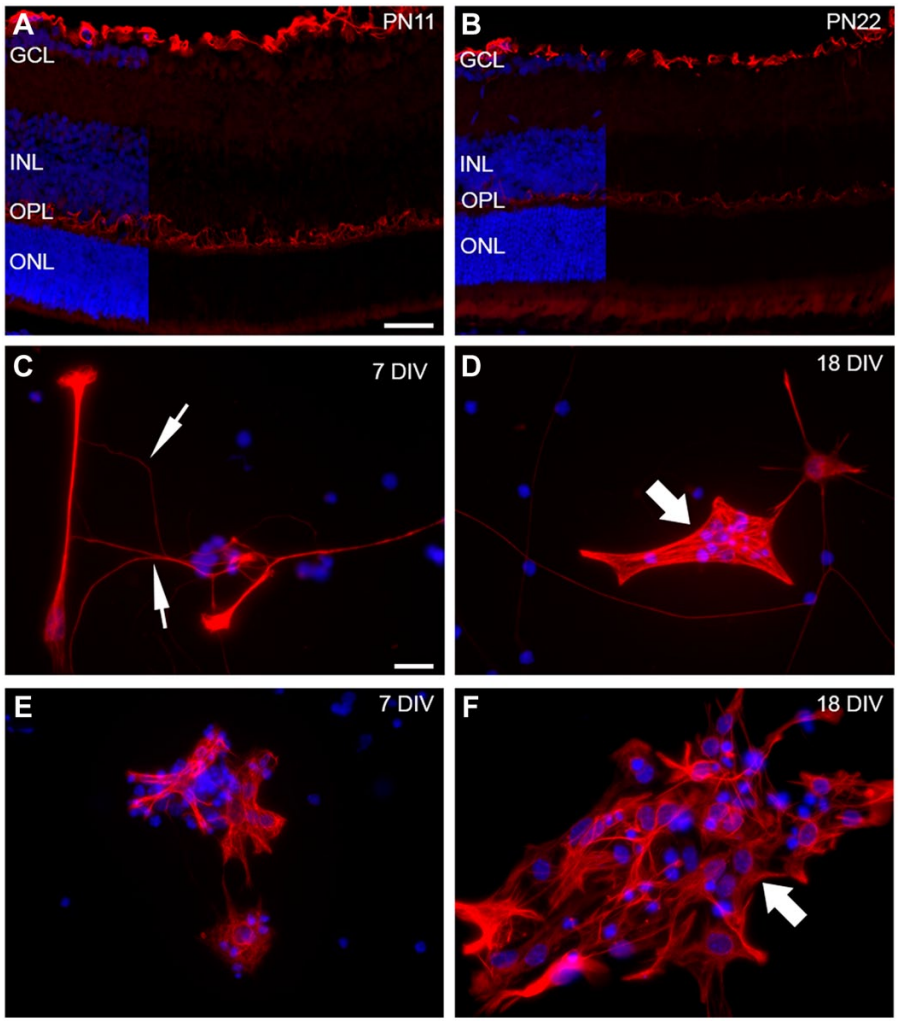

Figure 6. Immunofluorescent staining with anti-GFAP of postnatal mouse retinas and of in vitro cultured RPNCs. (A, B) Fluorescence photomicrographs of transverse frozen sections through mouse retinae. Retinal layers are indicated as ONL, OPL, INL, and GCL. (C–F) Fluorescence photomicrographs of in vitro cultured RPNCs. At PN11 (A) and at PN22 (B), GFAP (red) is specifically labeling the astrocytes. On cultured cells, GFAP labels cells of polygonal morphology (thick arrows) which may possess long processes (thin arrows). Abbreviations: GFAP, glial fibrillary acidic protein; RPNCs, retinal postnatal cells; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer; PN11, postnatal day 11; PN22, postnatal day 22; DIV, days in vitro. Scale bars: A = 50 μm; C = 20 μm.

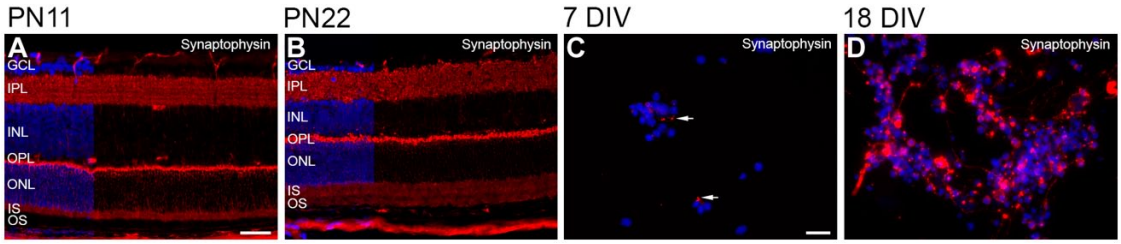

Figure 7. Immunofluorescent staining with anti-synaptophysin of postnatal mouse retinas and of in vitro cultured RPNCs. (A, B) Fluorescence photomicrographs of transverse frozen sections through mouse retinae. Retinal layers are indicated as OS, IS, ONL, OPL, INL, IPL, and GCL. (C–F) Fluorescence photomicrographs of in vitro cultured RPNCs. At PN11 (A) and at PN22 (B), synaptophysin (red) is strongly labeling the OPL and IPL. At 7 DIV (C), sparse immunoreactivity (arrows) is observed, whereas at 18 DIV (D), high synaptophysin immunoreactivity is observed. Abbreviations: RPNCs, retinal postnatal cells; OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; PN11, postnatal day 11; PN22, postnatal day 22; DIV, days in vitro. Scale bars: A = 50 μm; C = 20 μm.

type. Together, our report emphasizes the importance of thoroughly characterizing specific cell culture systems before these can be used to model events of relevance for in vivo applications.

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Competing Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

MCZ performed experimental work and data acquisition and analysis, participated in work design, and drafted the manuscript. SJ performed experimental work and drafted the manuscript. UEJ designed the study and drafted the manuscript. All authors have read and approved the final manuscript.

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