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Thyroid hormone regulatory system in the mouse retina: integrated transcriptional and translational analysis of deiodinase enzymes, thyroid hormone receptors and thyroid hormone transporters

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Abstract

Background Thyroid hormone (TH) signaling is involved in complex processes regulating development and energy balance, especially in organs with high-rate metabolism such as the retina. Despite evidence of TH involvement in regulation of retinal functions and disease susceptibility, a comprehensive analysis of the tissue distribution of the components of the retinal TH regulatory system is lacking. The aims of the present study were (i) to provide a detailed description of the retinal expression sites and localization patterns of deiodinases (DIO2 and DIO3), TH receptors (THRa and THRβ), and TH transporters (monocarboxylate transporter 8, MCT8, and organic anion-transporting polypeptide 1C1, OATP1C1); (ii) to recapitulate the retinal alterations following genetic deletions of specific TH regulatory elements in specific knockout (KO) mice.

Methods We first predicted the cell-specific expression of TH regulatory elements in mouse and human retina *via* bioinformatic analysis of single-cell RNAseq databases. The results were subsequently validated in mouse retinas with RNAscope and protein localization was assessed with immunofluorescence. Cone photoreceptor morphology and retinal layer thicknesses were evaluated in the retinas of KO mice.

Results *Dio2* mRNA was expressed by Müller glial cells and neuronal populations of the inner retina, while prominent DIO2 immunoreactivity was in cone photoreceptors, with an evident mismatch between mRNA and protein localization. DIO3 was expressed by virtually all neural cell populations of the retina. THRa and THRβ were predominantly localized to the inner and outer retina, respectively, with prominent expression of THRa in putative amacrine cells and ganglion cells, and of THRβ in cone photoreceptors. Both MCT8 and OATP1C1 were detected in

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the retinal pigment epithelium, in Müller glia, and in blood vessels. MCT8 was also expressed in some retinal ganglion cells. In KO mice, drastic alterations were observed in cone density and morphology and in retinal layer thickness following either *Dio2* or *Dio3* genetic deletion. In contrast, deletion of *Thrs* or TH transporters resulted in only minor changes, indicating potential compensatory mechanisms.

Conclusions The present mapping of the TH regulatory system in the mouse retina provides a framework for understanding molecular and cellular mechanisms regulating local TH availability and signaling in health and disease.

Keywords RNAscope, Immunofluorescence, Confocal microscopy, DIO2, DIO3, THR α , THR β , MCT8, OATP1C1

Background

Circulating levels of thyroid hormones (THs) depend primarily on the activity of the thyroid gland, which produces both 3,5,3'-triiodothyronine (T3), the biologically active form of TH, and its precursor thyroxine (T4). In the retina, as in other organs, a regional regulation of TH signaling is constantly provided thanks to the activity of local deiodinase enzymes (DIO2 and DIO3). DIO2 is responsible for the activation of T4 into T3, while DIO3 converts T4 and T3 into reverse T3 and 3,5-diiodothyronine (T2), respectively, which have only low biological activity [1]. Through these reactions, deiodinase enzymes regulate local T3 availability and provide a tissue-specific control of TH signaling. Local TH signaling in the retina is further influenced by hormone transport. Indeed, THs reach the retina through both the outer and the inner blood retinal barrier [2] and enter their target cells using specific transporters, such as the monocarboxylate transporter 8 (MCT8) encoded by the *Slc16a2* gene, and the organic anion-transporting polypeptide 1C1 (OATP1C1) encoded by the *Slco1c1* gene [3, 4]. At cell level, THs may exert genomic effects by directly binding specific nuclear receptors (THRs), namely THR α and THR β [5], or modulate non-genomic, regulatory effects mediated by membrane receptors such as integrin $\alpha\beta$ 3 [6].

As demonstrated in a variety of studies, TH signaling in the retina is crucial for multiple aspects of cone photoreceptor biology, including their development, dorso-ventral patterning, and survival in the mature retina, (see [7–9] for review). Since THs affect cellular metabolism, their regulation is likely to be of fundamental importance not only for photoreceptor development / survival but also for a variety of retinal functions both in health and in disease. For instance, TH deficiency during fetal or post-natal development may affect retinal neuronal migration and differentiation, optic nerve myelination, eye growth, and alteration of retinal and visual cortex processing of visual stimuli [10–12]. Recent data have also revealed a role of DIO2 expressed in Müller glial cells for the regulation of T3 in a mechanism of light adaptation [13]. In addition, TH signaling is involved in retinal diseases such as diabetic retinopathy and age-related macular degeneration [7, 9]. In this respect, local modulation of DIO2 and DIO3 expression in the diabetic retina has been reported

to establish a retinal low T3 state, which is likely to represent an adaptive mechanism aimed at preserving cellular energy and mitigating the metabolic stress induced by hyperglycemia [14].

Despite the likely involvement of TH signaling in a variety of retinal functions and diseases, a comprehensive analysis of the tissue distribution of the components of the retinal TH regulatory system is lacking. The expression of TH signaling elements has been reported in retinas of amphibians [15, 16], zebrafish (see [17] for references), salmonids [18, 19], and chickens [20–22]. In mammals, the only well-established evidence is the presence of DIO2 expression in Müller cells [8, 13, 23–26], while there are reports of the presence of both THR β 1 [27] and THR β 2 [28, 29] in cone photoreceptors.

The goals of the present work were: (i) to verify the translational relevance of the observations in the mouse tissue through a comparative prediction of cell-specific expression of the main regulators of TH signaling in murine and human retina; (ii) to provide a validation of the expression predictions through a detailed description of the expression sites and localization patterns of deiodinases, TH transporters, and TH receptors in the mouse retina; (iii) to recapitulate the effects of genetic deletion of specific components of TH signaling on the maturation of the retinal structure and of selected retinal cell populations such as cone photoreceptors and retinal ganglion cells. A thorough mapping of the retinal TH regulatory system in the mouse model would provide a crucial framework for understanding how TH availability and signaling are locally controlled within the retina. Identifying the cell type- and layer-specific distribution of deiodinases, TH transporters, and TH receptors would clarify the circuits and developmental processes directly governed by THs and highlight cell populations vulnerable to TH imbalance. Such an atlas would not only refine mechanistic models of TH-dependent retinal development and function but also guide targeted therapeutic strategies for TH-related visual disorders.

Methods

Bioinformatic analysis

A predictive analysis of deiodinase, TH receptor and TH transporter expression in different cell populations of

mouse and human retina was performed using the PLatform for Analysis of scEiad (PLAE) dataset (<https://plae.nei.nih.gov/>). scEiaD (single-cell Eye in a Disk) meta-atlas includes around 1.1 million single-cell eye transcriptomes across 44 different datasets, provided by 35 different publications. A total of 1.1 million single-cell eye and body tissue transcriptomes underwent deep metadata mining, rigorous quality control analysis and deep learning-based batch effect correction to obtain single-cell differential gene expression. The predicted expression of *Dio2*, *Dio3*, *Thra*, *Thrb*, *Slc16a2* (encoding the MCT8 protein), *Slco1c1* (encoding the OATP1C1 protein), *Slc16a10* (encoding the MCT10 protein), *Slc7a5* (encoding the L-type amino acid transporter 1, LAT1), and *Lat2* mRNAs in different retinal cell populations of both mouse and human retina were retrieved applying “Cell type Predict” and “Organism” (*Homo sapiens* and *Mus musculus*) as two sorted-filters. Data was plotted as the mean $\pm$ SEM of the percentage of cells expressing a given marker within a specific retinal cell type. Only study combinations with more than 50 cells were included in this study, displayed as Expression Plot and In Situ Projection diagrams. The analysis did not consider the range of ages as a variable as this information was not available in the meta-atlas.

Animals and tissue preparation

Two month-old C57BL/6J wild type (WT) mice were purchased from Charles River Laboratories Italy (Calco, Italy). *Thra* 0/0 (hereinafter referred to as *Thra* knockout [KO]) and *Thrb* -/- (hereinafter referred to as *Thrb* KO) mice were bred in the animal core facility of the Dresden University of Technology. Mice with a homozygous mutation of *Thra* 0/0 and *Thrb* -/- do not express any THR α isoforms or functional THR β 1 or THR β 2 variants, respectively [30, 31]. All the animals used in these studies were treated in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and with the European Communities Council Directive (2010/63/UE). Animal procedures and organ collection were approved by the Italian Ministry of Health (decree number 256/2024-PR) and by the institutional animal care committee of the Technische Universität Dresden and the Landesdirektion Sachsen (TVT 03/2023), according to the 3Rs principles for ethical use of animals and ARRIVE guidelines. The animals were maintained in a regulated environment (23 ± 1 °C, $50 \pm 5\%$ humidity) with 12/12 h light/dark cycles and fed with a standard diet and water ad libitum. Eyes from KO mice for *Dio2* [32], *Dio3* [33], *Slc16a2* [34], or *Slco1c1* [35] as well as eyes from *Dio2/Slc16a2* KO mice [36] were donated by the colleagues listed in the Acknowledgments section.

Mouse euthanasia and tissue collection were all performed between 10:00 and 12:00 pm in order to limit

possible influences of the light/dark cycle. The mice were deeply anesthetized with Avertin (1.2% tribromoethanol and 2.4% amylene hydrate in distilled water, 0.02 mL/g body weight, i.p.), they were perfused with 4% paraformaldehyde/0.1 M phosphate-buffered saline (PBS), and their eyeballs were enucleated and postfixed in the same solution for 2 h at room temperature. Fixed eyeballs of WT mice were transferred to sucrose/0.1 M PBS at increasing concentrations (10% – 30% every 1 h) and stored at 4 °C overnight. These eyes were embedded immediately into cryo-gel medium and stored at -80 °C to preserve mRNA stability for RNAscope or RNAscope / immunofluorescence double labeling. Fixed KO eyes, instead, were received in 30% sucrose and they were used only for immunofluorescence. All eyes were cut into 10 μ m thick coronal sections and mounted onto superfrost slides. Alternatively, for whole mount retinal preparations, dissected retinas were immersion-fixed in 4% paraformaldehyde in 0.1 M PBS for 2 h at 4 °C and then stored in 25% sucrose in 0.1 M PBS at 4 °C until use.

RNAscope

All the materials used for the RNAscope experiments were purchased from Advanced Cell Diagnostics, Inc., Newark, CA, USA. Single or duplex in situ hybridizations were performed using RNAscope probes for *Dio2* (Cat. No: 479331-C1), *Dio3* (Cat. No: 561641-C2), *Thra* (Cat. No: 519431-C3), *Thrb* (Cat. No: 451441-C1), *Slc16a2* (Cat. No: 545291-C2), and *Slco1c1* (Cat. No: 491821-C3). The hybridization was performed using the RNAscope Multiplex Fluorescent Reagent Kit v2 according to the protocol provided by the manufacturer. Retinal sections were pretreated with heat and protease (30 min at 40 °C) using the HybEZ Oven before hybridization with the appropriate mRNA probes listed above. After incubation with the primary amplification probes (AMP1 and AMP2 for 15 min, AMP3 for 30 min, at 40 °C), retinal sections were washed with RNAscope wash buffer and incubated with TSA vivid fluorophores 520, 570, or 650 for 2 h at room temperature. Additional retinal sections were incubated with triplex positive and negative probes (provided by the manufacturer) as positive or negative controls. The slides were coverslipped with Fluoroshield mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Cat. No: F6057) and stored overnight at 4 °C before microscope examination. The observations were confirmed in at least 3 retinas deriving from different animals.

Immunofluorescence

Retinal sections were incubated with primary antibodies (listed in Table 1) diluted in 0.1% Triton X-100 in 0.1 M PBS overnight at 4 °C. The specificity of the primary antibodies directed to DIO2, DIO3, THR α , THR β , SLC16A2,

Table 1 Primary and secondary antibodies

Antigen	Host	Source	Code	Fluorophore	Stock concentration	Dilution
βIII-tubulin	Mouse	R&D Systems ^a	MAB1195		0.5 mg/mL	1:400
Calbindin	Mouse	Sigma Aldrich ^b	C9848		27 mg/mL	1:200
Cone arrestin	Rabbit	Merck Millipore ^c	AB15282		1 mg/mL	1:500
DIO2	Rabbit	Invitrogen ^d	PA5-49631		2 mg/mL	1:200
DIO2	Mouse	Proteintech ^e	66813-1		1 mg/mL	1:200
DIO2	Goat	Invitrogen ^d	PA5-18659		0.5 mg/mL	1:200
DIO2	Goat	Bioss ^f	NBP1-00178		0.5 mg/mL	1:200
DIO3	Rabbit	Sigma Aldrich ^b	SAB1301592		na	1:200
DIO3	Rabbit	Novus biological ^g	NBP1-05767		1 mg/mL	1:200
γ-aminobutyric acid (GABA)	Mouse	Merck ^c	A0310		26 mg/mL	1:100
Glutamine synthetase (GS)	Rabbit	Abcam ^h	ab228590		0.65 mg/mL	1:100
RNA-binding protein with multiple splicing (RBPMS)	Rabbit	Novus Biological ^g	NBP2-20112		0.26 mg/mL	1:500
Rhodopsin	Mouse	Abcam ^h	ab5417		1 mg/mL	1:200
SLC16A2 (MCT8)	Rabbit	Sigma Aldrich ^b	HPA003353		0.2 mg/mL	1:200
SLCO1C1 (OATP1C1)	Rabbit	Abcam ^h	ab234729		5 mg/mL	1:200
THRa	Rabbit	ThermoFisher ⁱ	PA1-211 A		na	1:500
THRa	Rabbit	Abcam ^h	ab53729		1 mg/mL	1:200
THRB (N-term)	Rabbit	Abcam ^h	ab180612		0.21 mg/mL	1:200
Tyrosine hydroxylase	Mouse	Merck ^c	MAB318		2–10 mg/mL	1:100
Mouse immunoglobulins	Donkey	Invitrogen ^d	A10036	Alexa Fluor 546	2 mg/mL	1:200
Rabbit immunoglobulins	Donkey	Invitrogen ^d	A21206	Alexa Fluor 488	2 mg/mL	1:200
Rabbit immunoglobulins	Donkey	Invitrogen ^d	A10040	Alexa Fluor 546	2 mg/mL	1:200
Rabbit immunoglobulins	Goat (Fab Fragment)	Jackson Labs ^j	111-097-003	FITC	1.7 mg/ml	1:400
Rabbit immunoglobulins	Donkey (Fab Fragment)	Jackson Labs ^j	711-007-003		1.3 mg/ml	1:50

^aR&D Systems, Inc., Minneapolis, MN, USA; ^bSigma Aldrich, St. Louis, MO, USA; ^cMerck, Rahway, NJ, USA; ^dInvitrogen, Carlsbad, CA, USA; ^eProteintech, Rosemont, IL, USA; ^fBioss, Woburn, MA; ^gNovus biological, Centennial, CO, USA; ^hAbcam, Cambridge, UK; ⁱThermoFisher, Waltham, MA, USA; ^jJackson ImmunoResearch Europe Ltd., Cambridge, UK; *na* not available

and SLCO1C1 was tested with immunolabeling of retinas from *Dio2* KO, *Dio3* KO, *Thra* KO, and *Thrb* KO, *Slc16a2* KO, and *Slco1c1* KO mice, respectively. The specificity of antibodies directed to DIO2 or to SLC16A2 was also tested using retinas from *Dio2/Slc16a2* double KO mice. After 3 × 5 min washes in 0.1 M PBS, retinal sections were incubated with the appropriate Alexa Fluor-conjugated secondary antibody (Table 1) diluted in 0.1% Triton X-100 in 0.1 M PBS for 2 h at room temperature. Double immunolabeling with primary antibodies from different host species were performed incubating the retinal sections with both primary antibodies overnight at 4 °C, and then with the appropriate Alexa Fluor 546 or Alexa Fluor488-conjugated secondary antibodies for 2 h at room temperature. Conversely, double immunolabeling with both primary antibodies produced in rabbit were conducted by first incubating the sections with one of the primary antibodies diluted in 0.5% Triton X-100 in 0.1 M PBS for 3 h at room temperature, and then with an anti-rabbit fluorescein isothiocyanate (FITC)-conjugated Fab Fragment for 1.5 h at room temperature. After an overnight blocking step at 4 °C with an anti-rabbit

unconjugated/unlabeled Fab Fragment (see Table 1 for details on anti-rabbit Fab fragment), retinal sections were incubated with the second primary antibody for 3 h at room temperature, and then with a secondary anti-rabbit Alexa-Fluor 546-conjugated antibody (1:400) for 1.5 h at room temperature. The slides were coverslipped with Fluoroshield mounting medium containing DAPI and stored overnight at 4 °C before microscope examination.

For immunofluorescence in whole mount retinal preparations, the retinas were incubated with the primary antibody diluted in 2% Triton X-100 in 0.1 M PBS for 72 h at 4 °C. After incubation with secondary anti-rabbit Alexa Fluor 488-conjugated antibody diluted in 2% Triton X-100 in 0.1 M PBS for 48 h at 4 °C, whole mount retinas were flat mounted on polarized glass slides with ganglion cell layer (GCL) up, coverslipped with Fluoroshield mounting medium containing DAPI, and stored at 4 °C before observation with the confocal microscope. The observations were confirmed in at least 3 retinas deriving from different animals (either WT or KO).

Double label RNAscope / immunofluorescence

Combined RNAscope / immunofluorescence analysis was performed using a specific protocol provided by the manufacturer (Manual pre-treatment PRO protocol, Advanced Cell Diagnostics). Retinal sections underwent a pretreatment protocol with heat and pretreatment PRO solution (30 min at 40 °C) with no protease incubation, and the antigen retrieval was performed with a co-detection buffer. At the end of the RNAscope procedure described above, retinal sections were incubated with primary antibodies overnight at 4 °C diluted in 0.1% bovine serum albumin in 0.1 M Tris-buffered saline. The sections were then incubated with appropriate Alexa-Fluor 546-conjugated secondary antibodies for 2 h at room temperature. After 3×5 min washes in 0.1 M PBS, the slides were coverslipped with Fluoroshield mounting medium containing DAPI and stored at 4 °C before microscope examination. The observations were confirmed in at least 3 retinas deriving from different animals.

Labeling of retinal blood capillaries

In some retinal sections, RNAscope labeling was evaluated in retinal blood capillaries. After the RNAscope procedure described above, retinal vessels were visualized using FITC-conjugated Isolectin B4 (Vector Laboratories, Newark, CA, USA, Cat. No: FL-1201-0.5) diluted 1:200 in 0.1% Triton X-100 in 0.1 M PBS overnight at 4 °C. After 3×5 min washes in 0.1 M PBS, the slides were coverslipped with Fluoroshield mounting medium containing DAPI and stored at 4 °C before microscope examination. The observations were confirmed in at least 3 retinas deriving from different animals.

Confocal microscopy and figure preparation

The slides were analyzed using a Nikon A1 confocal inverted microscope (Nikon A1, Eclipse Ti). Confocal images were acquired at similar laser powers and black levels using a 40× objective (numerical aperture: 1.15) at a detector resolution of 1024×1024 pixels, with a z-step of 0.5 µm. Stacks of confocal slices (18–25 steps each) were processed with ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA) to obtain Z Projections using the “Projection type” option “Max Intensity”. The final images were then corrected for brightness and contrast using ImageJ. Adjustments were performed using linear scaling and were applied uniformly to all pixels in each image. Comparable brightness and contrast settings were applied across images within the same experiment, with background signal used as a reference to ensure appropriate comparisons. Images representing the staining patterns across multiple biological replicates were trimmed and arranged in figure panels using Adobe

Photoshop (Adobe Photoshop CS3; Adobe Systems, Mountain View, CA, USA).

Quantitative analyses of retinal ganglion cells, cone photoreceptors, and retinal layers

Retinal sections from C57BL/6J mice and from KO mice were immunolabeled with RNA-binding protein with multiple splicing (RBPMS) [37]. Ganglion cell density was evaluated by counting the number of RBPMS immunopositive cells per section unit length (approximately 300 µm per section) in the peripapillary area of the optic nerve head. The total number of ganglion cells in each retinal section was divided by the section length and then expressed proportionally as ganglion cells / mm. The density of cone photoreceptors was evaluated by counting the number of cone arrestin immunopositive cells per section and it was then expressed as cones /mm. The size of cone pedicles was evaluated by measuring the maximum diameter of cone arrestin immunostained cone pedicles visualized in maximum projection images along 100 µm per section and the diameters of the pedicles from each section were averaged. Quantitative measures of RBPMS immunolabeled ganglion cells, cone density, and cone pedicle diameter were obtained using ImageJ. The thickness of retinal layers was measured in images of DAPI stained retinal sections using the Analysis menu of Adobe Photoshop. Briefly, in each image the measurement scale was set and the outline of the retinal layers was drawn on a separate image layer. Then, the area of each layer was measured and divided by the length of the section to obtain the average layer thickness. Values obtained from different sections belonging to the same retina were averaged. A total of 5 retinas were considered in all quantitative analyses.

Statistical analysis

Statistical analyses were performed using Prism 8.0.2 (GraphPad, Boston, MA, USA). The normal distribution of the data was verified using the Shapiro–Wilk test. One-way analysis of variance (ANOVA) followed by Tukey’s post hoc tests were used to analyze the differences of cone density, cone pedicle diameter, ganglion cells density and retinal layer thickness between WT and KO mouse retinas. Two-way ANOVA followed by Bonferroni’s post hoc test was instead used to compare the cell-specific expression of each TH signaling component between mouse and human. Differences with $P < 0.05$ were considered significant.

Results

Bioinformatic analysis: cell-specific expression of components of the TH regulatory system

The differential cellular expression of the genes encoding the main regulators of retinal TH signaling in the

mouse retina was retrieved via bioinformatic analysis of available single cell RNAseq datasets as specified in the methods section. This analysis was performed to set up a working model summarizing predicted cell-specific mRNA expression in the mouse retina to be used as a reference for the subsequent localization of specific mRNAs and proteins in the tissue. The resulting outputs, with 10% of the total cells used as the threshold value for cell-specific positive/negative expression, are shown in Fig. 1. High *Dio2* mRNA expression was predicted in Müller cells (Fig. 1A), while *Dio3* mRNA expression levels were the lowest among all the genes analyzed and no *Dio3* mRNA expression could be predicted in any of the retinal cell types considered in the analysis (Fig. 1B). *Thra* mRNA was predicted in cells of the inner retina, including horizontal cells, bipolar cells, amacrine cells, ganglion cells, and Müller cells (Fig. 1C). *Thrb* mRNA was likely to be maximally expressed by rod and cone photoreceptors, while lower levels were also expected in all other retinal cell types, except cells of retinal blood vessels (Fig. 1D). Among TH transporters *Slc16a2* expression was predicted in cells of the retinal pigment epithelium (RPE), Müller cells, and blood capillaries (Fig. 1E), while that of *Slco1c1* was mainly in RPE and Müller cells (Fig. 1F). The expected cell-specific expression of the TH regulatory system components in the mouse retina are summarized in Table 2. Bioinformatic data were also retrieved to compare the predicted expression of components of the retinal TH regulatory system in mice with that in humans. The results, summarized in figure S1, showed a high degree of similarity between mice and humans relative to the expression patterns of deiodinase, receptor, and transporter mRNAs, with the only notable difference regarding a prominent predicted expression of *Slc7a5* mRNA (encoding LAT1) in different cell types of the human retina, which was not detected in mice.

mRNA localization using RNAscope *in situ* hybridization

Both RNAscope positive and negative controls were as expected (Fig. S2), indicating specific labeling of the used probes. As shown in Fig. 2A, the *Dio2* mRNA signal was densely packed in cell bodies in the mid-inner nuclear layer (INL) and more sparsely distributed in proximity to the GCL. Interestingly, within the inner plexiform layer (IPL) labeled puncta appeared to be organized along vertically oriented processes (also evident in Fig. 2G and J) seemingly connected with the labeled cell bodies in the mid-INL. Overall, the *Dio2* mRNA localization pattern suggested the presence of the transcript in Müller cell bodies and processes in the inner (but not in the outer) retina. The *Dio2* mRNA labeling near the GCL is consistent with its localization in Müller cell endfeet near the inner limiting membrane or in somata of the GCL (ganglion cells and/or displaced amacrine cells).

Sparse *Dio3* mRNA labeling was observed throughout the nuclear layers of the retina (outer nuclear layer [ONL], INL, and GCL), while some fluorescent puncta were also observed in the outer plexiform layer (OPL) and in the IPL (Fig. 2B). This localization pattern suggested *Dio3* mRNA expression by virtually all cell types of the retina.

Regarding *Thra* mRNA staining (Fig. 2C), we observed densely packed fluorescent clusters in the most proximal portion of the INL and in the GCL, while the outer retina (OPL and ONL) was devoid of any *Thra* transcript signal. This pattern suggested the expression of *Thra* mRNA in a variety of inner retinal cell populations, and in particular in amacrine cells and in ganglion/displaced amacrine cells.

Thrb mRNA appeared to be mainly distributed to the outer retina. As shown in Fig. 2D, the *Thrb* mRNA signal was particularly dense at the level of cone photoreceptor somata and in cone outer segments (OS). Fluorescent puncta were also evident in the ONL, likely indicating *Thrb* mRNA expression in rod photoreceptors, and, more sparsely distributed, in the INL and in the GCL. These observations suggested the presence of *Thrb* mRNA mainly in photoreceptors (particularly in cones) and, in lower amount, in cells of the inner retina.

The *Slc16a2* mRNA signal was mainly localized to the mid-INL (Fig. 2E), in a pattern reminding that of *Dio2* mRNA. Therefore, *Slc16a2* mRNA was likely to be expressed in Müller cells; however, different from *Dio2* mRNA, it did not appear in putative Müller cell processes in the IPL. In addition, sparse *Slc16a2* mRNA labeled cells were observed in the GCL. Considering the possible involvement of MCT8 in the transport of THs through the outer blood retinal barrier, the expression of *Slc16a2* mRNA was evaluated not only in the neural retina but also in the RPE, where it appeared to be extensively distributed, as shown in Fig. 1E.

Slco1c1 transcripts (Fig. 2F) were primarily confined to a narrow band in the mid-INL, in a pattern suggestive of *Slco1c1* mRNA expression in Müller cells. Some sparse *Slco1c1* mRNA labeling was also in the GCL and in elongated profiles localized to the OPL and IPL resembling blood capillaries. In addition, *Slco1c1* mRNA labeling was distributed throughout the RPE.

Components of the TH signaling system have been reported to have differential regional distributions in the retina during development [38]. In particular, a dorso-ventral patterning would contribute to the differentiation of two different cone photoreceptor populations in mice [8, 38, 39], therefore we checked for possible variations in the dorsal and ventral expression patterns of TH component mRNAs. As shown in figure S3, no appreciable differences were observed in the RNAscope patterns of

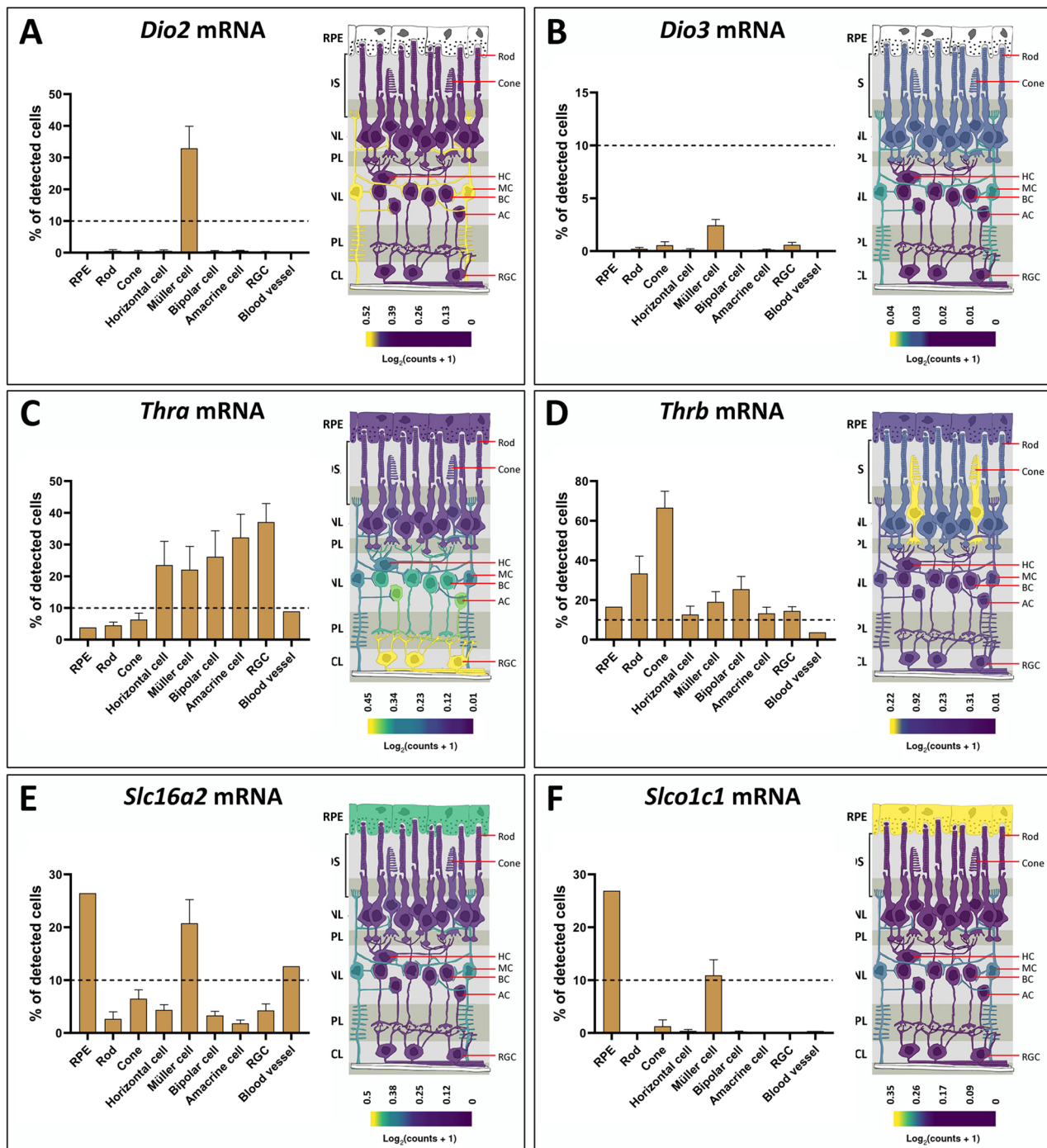


Fig. 1 Predicted mRNA expressions in different retinal cell populations of *Dio2* (A), *Dio3* (B), *Thra* (C), *Thrb* (D), *Slc16a2* (E), and *Slco1c1* (F) mRNAs evaluated using the PPlatform for Analysis of scEiad (PLAE) dataset (<https://plae.nei.nih.gov/>), applying Cell type Predict and Organism (Mus Musculus) as two sorted-filters. Only cell type-study combinations with more than 50 cells were included and displayed as Expression Plot (on the left in each panel) and *In Situ* Projection diagrams (on the right in each panel). The dashed lines indicate 10% of detected cells, used as the threshold value for cell-specific positive/negative expression. AC, amacrine cell; BC, bipolar cell; GCL, ganglion cell layer; HC, horizontal cell; INL, inner nuclear layer; IPL, inner plexiform layer; MC, Müller cell; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, photoreceptor outer segments; RGC, retinal ganglion cells; RPE, retinal pigment epithelium.

Table 2 mRNA localization in mouse retina from bioinformatic prediction (PLAE dataset)

	Dio2	Dio3	Thra	Thrb	Slc16a2	Slco1c1
RPE	-	-	-	+	++	++
Rod	-	-	-	+++	-	-
Cone	-	-	-	+++	-	-
MC	+++	-	+	+	++	+
HC	-	-	++	+	-	-
AC	-	-	+++	+	-	-
INL other	-	-	+	++	-	-
DAC	na	na	na	na	na	na
RGC	-	-	+++	++	-	-
BV	-	-	-	-	+	-

Percentage of positive cells: -, value < 10%; +, 10% < value < 20%; ++, 20% < value < 30%; +++, value > 30%. Symbols are assigned based on the data shown in Fig. 1. ACs amacrine cells, BV Blood vessel, BC bipolar cell, DAC displaced amacrine cell (GABA immunoreactive cell in the GCL), HC horizontal cell, INL other other cells in the INL, MC Müller cell, na not available, RGC retinal ganglion cell, RPE retinal pigment epithelium

deiodinase enzymes, THR_s and TH transporters in dorsal and ventral retinal regions.

Since both *Dio2* and *Thra* mRNAs were localized to the INL and to, or near, the GCL, we compared the two patterns in the same retinal sections using duplex RNAscope. As shown in Fig. 2G, the two fluorescent signals in the INL were clearly distinct from each other, which further supports *Dio2* expression by Müller cells and *Thra* expression by putative amacrine cells. In the GCL, there was a partial overlap of the two signals.

The simultaneous detection of the mRNAs of *Thra* and *Thrb* (Fig. 2H) confirmed that *Thra* was expressed exclusively in the inner retina, while *Thrb* transcript was mostly detected in the outer retina. Interestingly, there was an extensive overlap of the two signals in the INL and in the GCL, indicating that putative amacrine and ganglion cells are likely to express both types of THR_s.

The localization patterns of *Dio2*, *Slc16a2*, and *Slco1c1* mRNAs shared important features. Indeed, in duplex RNAscope experiments the signals of *Dio2* and *Slc16a2* mRNAs (Fig. 2I) showed numerous sites of overlap in the mid-INL, suggesting the expression of both genes in Müller cells. In addition, *Dio2* and *Slc16a2* appeared to be co-expressed in sparse cells of the GCL. Similar to the *Slc16a2* mRNA signal, also that of *Slco1c1* mRNA showed extensive overlap with *Dio2* mRNA in the mid-INL (Fig. 2J), suggesting co-expression in Müller cells of *Dio2*, *Slc16a2*, and *Slco1c1* mRNAs. This possibility was further supported by observations of *Slc16a2* and *Slco1c1* mRNA double labels (Fig. 2K), in which extensive colocalization of the two signals was observed throughout the mid-INL. In addition, sparse cells in the GCL were also double labeled. Profiles resembling blood capillaries were mostly labeled only by the *Slco1c1* mRNA probe, although colocalization with *Slc16a2* mRNA staining was sporadically observed. Finally, both mRNAs appeared to be co-expressed in RPE cells.

DIO2 is expressed in Müller glia and neurons with divergent patterns of *Dio2* mRNA and protein localization

Dio2 mRNA and protein localization patterns were investigated integrating the RNAscope observations with immunofluorescence data. The distribution of the *Dio2* mRNA signal (Fig. 3A) was compared with the DIO2 immunostaining patterns (Fig. 3B–G) obtained using the antibodies directed to DIO2 listed in Table 1. Of them, only antibody Invitrogen PA5-49631 was found to provide specific immunostaining, since it did not produce any immunolabeling in *Dio2* KO retinas (Fig. 3H). Interestingly, comparing the patterns of *Dio2* mRNA signal (Fig. 3A) and of DIO2 immunoreactivity (Fig. 3B–G) revealed a picture with marked differences. In retinal sections, the most notable mismatch was the presence of bright DIO2 immunofluorescence in cone photoreceptor OS, in some of their somata in the ONL, and in their axonal terminals in the OPL (Fig. 3B), while no *Dio2* mRNA signal could be seen in the outer retina (Fig. 3A). The presence of DIO2 immunoreactivity in putative cone photoreceptor somata and axons running through the ONL was confirmed in whole mount retinas (Fig. 3C and D, respectively). DIO2 immunolabeled profiles were also scattered throughout the INL, in particular in its distal portion (Fig. 3E). Similarly, smaller DIO2 immunostained somata were observed in the proximal INL (Fig. 3F). Notably, DIO2 immunostaining could be observed in the mid-INL, where *Dio2* mRNA positive clusters were also observed (Fig. 3A and B). Finally, strong DIO2 immunoreactivity was found in and near the GCL, where a large number of DIO2 immunostained large-sized somata (most likely ganglion cells) and thick axonal bundles could be seen (Fig. 3B and G).

Together, the RNAscope and the immunofluorescence data suggested the possible localization of DIO2 to cone photoreceptors, Müller cells, horizontal cells, amacrine cells, and ganglion cells, although there was an evident mismatch between the retinal distribution of *Dio2* mRNA and DIO2 immunoreactivity. For better characterization,

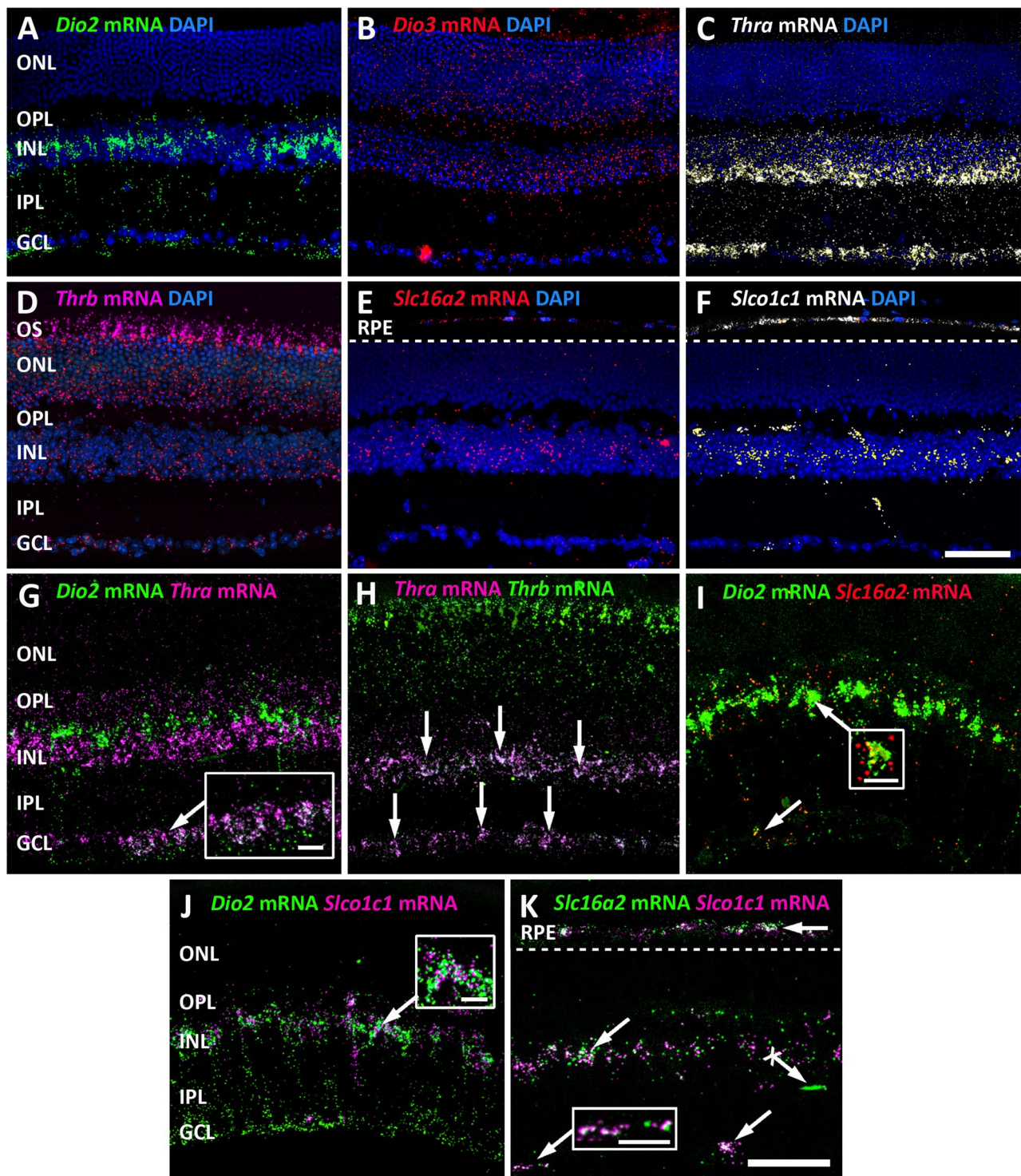


Fig. 2 Expression patterns of *Dio2* (A), *Dio3* (B), *Thra* (C), *Thrb* (D), *Slc16a2* (E), and *Slco1c1* (F) mRNAs detected by RNAscope in situ hybridization and shown on the relative DAPI backgrounds to visualize the retinal layers. Panels (G–K) show double labels using pairs of RNAscope probes to ascertain occurrence/absence of colocalization of *Dio2* and *Thra* mRNAs (G), of *Thra* and *Thrb* mRNAs (H), of *Dio2* and *Slc16a2* mRNAs (I), of *Dio2* and *Slco1c1* mRNAs (J), and of *Slc16a2* and *Slco1c1* mRNAs (K). The arrows indicate sites of co-occurrence of both RNAscope signals. The insets are higher power images of the cells indicated by the relative arrows. The arrow with an “x” on its tail (red arrow) indicates a blood vessel that is not double labeled and displays *Slc16a2* but not *Slco1c1* RNAscope signal (K). The dashed lines in (E), (F), and (K) indicate that the images of the RPE were taken from retinal sections different from those used to show the other retinal layers. The original images used for duplex RNAscope are shown in figure S4. Scale bars in F (referring to A–F) and in K (referring to G–K), 50 μ m (10 μ m in the insets). See Fig. 1 for abbreviations of the retinal layers

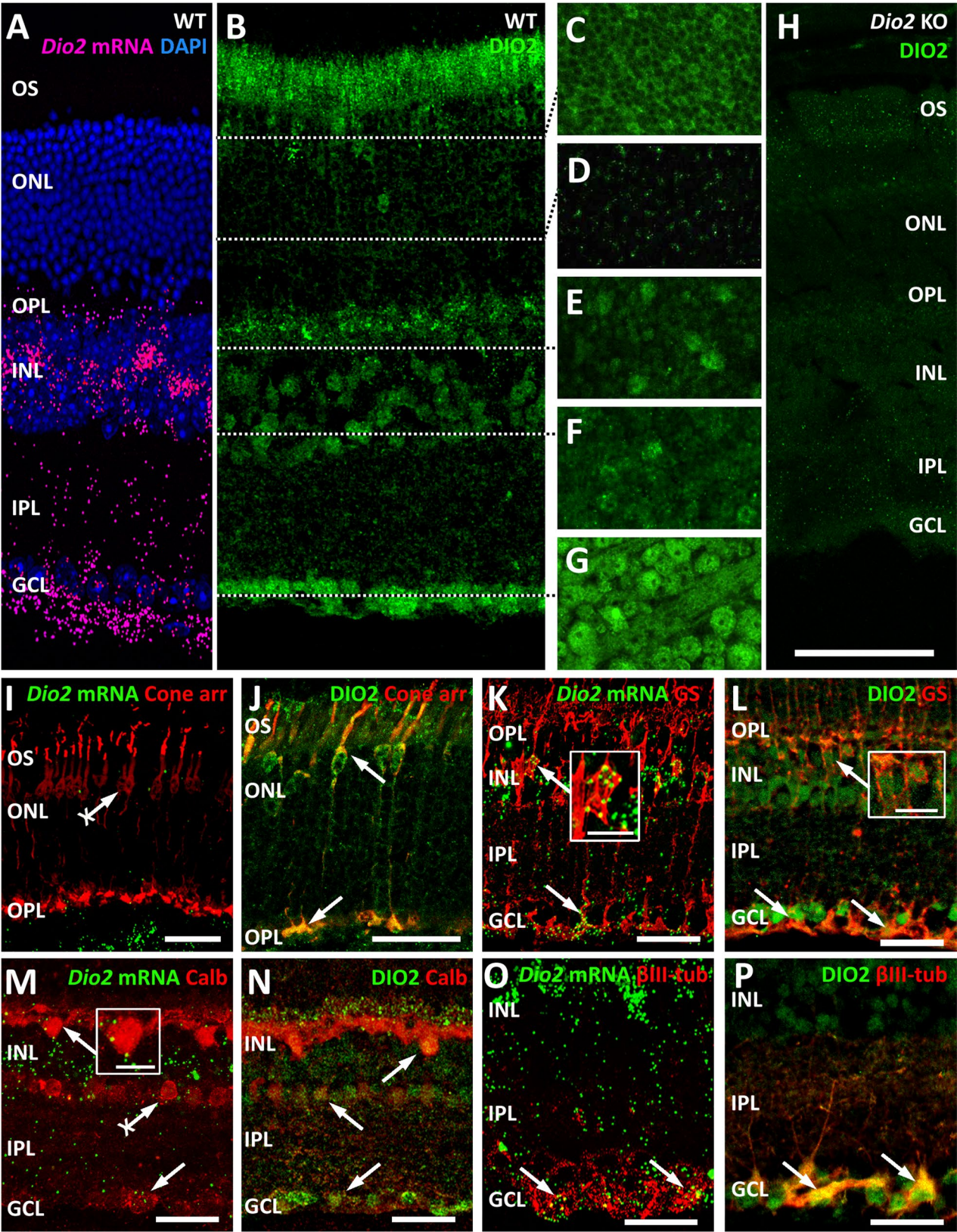


Fig. 3 (See legend on next page.)

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Fig. 3 DIO2 localization patterns. *Dio2* mRNA visualized with RNAscope in situ hybridization (**A**) and DIO2 immunoreactivity (**B**) in mouse retinal sections. (**C–G**) confocal scans at different levels (dashed lines) through the thickness of a retinal whole mount immunolabeled with an antibody directed to DIO2 and showing DIO2 immunoreactive cone somata in the distal ONL (**C**), cone axons through the ONL (**D**), cell bodies in the distal INL (**E**), other immunolabeled somata in the proximal INL (**F**), and DIO2 immunolabeled somata in the GCL (**G**). The specificity of the DIO2 antibody (Invitrogen PA5-49631) was confirmed by absence of immunostaining in retinas from DIO2 KO retinas (**H**). The results of double label experiments using different immunohistochemical markers of retinal cell populations in conjunction with either the *Dio2* mRNA probe or the DIO2 antibody are shown in panels (**I–T**). The arrows indicate double labeled profiles, while xed arrows indicate immunolabeled cells that do not display *Dio2* mRNA staining. The insets are higher power images of the cells indicated by the relative arrows. Cone arrestin immunoreactivity, identifying cone photoreceptors, did not colocalize with the RNAscope signal for *Dio2* mRNA (**I**) but colocalized with DIO2 immunoreactivity (**J**). Glutamine synthetase (GS) immunostaining, identifying Müller cells, colocalized with both *Dio2* mRNA (**K**) and DIO2 immunoreactivity (**L**) in Müller cell somata and endfeet. Calbindin (Calb) immunoreactive horizontal cells and ganglion cells, but not Calb immunoreactive amacrine cells, also displayed *Dio2* mRNA staining (**M**), while all three types of Calb immunostained cells were also DIO2 immunolabeled (**N**). Both *Dio2* mRNA (**O**) and DIO2 immunoreactivity (**P**) were in β -III tubulin immunoreactive ganglion cells. Scale bar in H, 50 μ m (referring to panels **A–H**); Scale bars in (**I–P**), 30 μ m (10 μ m in the insets). See Fig. 2 for abbreviations of the retinal layers

double labels were performed using either *Dio2* mRNA probes or DIO2 antibodies in conjunction with immunohistochemical markers for different retinal cell populations. As expected, no overlap was observed between the *Dio2* mRNA signal and cone arrestin immunoreactivity (Fig. 3I), while extensive colocalization of DIO2 and cone arrestin immunostainings was found in cone photoreceptors (Fig. 3J). The effective colocalization of *Dio2* mRNA and DIO2 protein in Müller cells was demonstrated using antibodies directed to glutamine synthetase (GS), a marker of Müller cells [40], as shown in Fig. 3K and L. In addition, considering the DIO2 immunostaining pattern depicted in Fig. 3B–G, we tested the possible presence of *Dio2* mRNA and / or DIO2 immunoreactivity in horizontal cells, in amacrine cells, and in ganglion cells using an antibody directed to calbindin, a common marker of these cellular types [41]. The results showed a few *Dio2* mRNA positive clusters associated with some calbindin immunolabeled horizontal cells and ganglion cells (Fig. 3M), while DIO2 immunoreactivity could be detected in most of the calbindin immunolabeled horizontal, amacrine, and ganglion cells (Fig. 3N). In addition, γ -aminobutyric acid (GABA)-containing amacrine cells did not show any *Dio2* mRNA labeling, while they displayed evident DIO2 immunoreactivity (Fig. S5A and S5B, respectively). Finally, we used antibodies directed to β III-tubulin to label retinal ganglion cells [42, 43]. A subset of these cells also showed *Dio2* mRNA labeling (Fig. 3O), while virtually all β III-tubulin immunoreactive ganglion cells were also DIO2 immunolabeled (Fig. 3P). A summary of the *Dio2* mRNA distribution and DIO2 immunostaining patterns is reported in Tables 3 and 4, respectively.

DIO3 has a widespread distribution throughout the retina

The distribution of the *Dio3* mRNA signal (Fig. 4A) was compared with the DIO3 immunostaining patterns (Fig. 4B–F) obtained using the antibodies directed to DIO3 listed in Table 1. Of them, we choose to use antibody Sigma Aldrich SAB1301592 since it did not label retinal sections from *Dio3* KO mice except for an intense immunofluorescent band confined to the proximal

portion of the OS (Fig. 4G), which, therefore, was deemed non-specific. In line with the above described sparse distribution of the *Dio3* mRNA signal throughout the entire ONL, INL, and GCL (Fig. 4A), we could expect presence of DIO3 immunoreactivity in any retinal cell type. Indeed, specific, although relatively weak, DIO3 immunostaining was observed in the external portion of the photoreceptor OS and in small cell bodies (most likely of rod photoreceptors) in the ONL, as revealed by confocal scans of retinal whole mounts (Fig. 4C). Similar to DIO2 immunostaining, DIO3 immunoreactivity was localized to sparse somata in the distal INL, reminding horizontal cells (Fig. 4D), and to other cell bodies in the proximal INL, likely corresponding to amacrine cells (Fig. 4E). In addition, numerous large DIO3 immunopositive somata, presumably belonging to ganglion cells, were found throughout the GCL (Fig. 4F).

Together, the RNAscope and the immunofluorescence data suggested the possible localization of DIO3 to rod photoreceptors, horizontal cells, amacrine cells, and ganglion cells. Excluding the non-specific immunoreactive band in the proximal OS, in double label experiments both the *Dio3* mRNA signal and the DIO3 immunostaining overlapped with rhodopsin immunolabeled OS (Fig. 4H and I, respectively) and rod photoreceptor somata (Fig. 4J and K, respectively). In addition, some *Dio3* mRNA positive puncta were observed in association with sparse calbindin immunostained horizontal cells and ganglion cells (Fig. 4L), while DIO3 immunoreactivity was observed in virtually all calbindin immunopositive horizontal, amacrine, and ganglion cells (Fig. 4M). GABA containing amacrine cells did not show any *Dio3* mRNA staining (Fig. S5C), but they contained DIO3 immunoreactivity (Fig. S5D). Finally, virtually all of the retinal ganglion cells identified by β III-tubulin immunoreactivity displayed both *Dio3* mRNA signal (Fig. 4N) and DIO3 immunostaining (Fig. 4O). A summary of the *Dio3* mRNA distribution and DIO3 immunostaining patterns is reported in Tables 3 and 4, respectively.

Table 3 mRNA localization (RNAscope)

	Dio2	Dio3	Thra	Thrb	Slc16a2	Slco1c1
RPE	-	-	-	-	+++	+++
Rod	-	++	-	+	-	-
Cone	-	na	-	+++	-	-
MC	+++	-	na	na	+++	+++
HC	+	+	+	+	na	-
AC	-	-	+++	+	+	-
INL other	-	+	++	+	+	-
DAC	-	-	na	na	na	na
RGC	++	++	+++	++	+	+
BV	-	-	-	-	+	+++

Signal intensity: -, no RNAscope signal; +, low RNAscope signal; ++, moderate RNAscope signal; +++, strong RNAscope signal. Symbols are assigned based on qualitative assessment of the density / extension of the hybridization signal. ACs amacrine cells, BV Blood vessel, BC bipolar cell, DAC displaced amacrine cell (GABA immunoreactive cell in the GCL), HC horizontal cell, INL other other cells in the INL, MC Müller cell, na not available, RGC retinal ganglion cell, RPE retinal pigment epithelium

Table 4 Protein localization (immunofluorescence)

	DIO2	DIO3	THRa	THRβ	MCT8	OATP1C1
RPE	-	-	-	-	+	-
Rod	-	+++	-	-	-	-
Cone	+++	-	-	+++	-	-
MC	++	na	na	na	-	+++
HC	+++	+++	na	+++	-	-
AC	+++	++	na	+++	-	-
INL other	+	+	na	-	-	-
DAC	++	++	na	na	na	na
RGC	+++	++	+++	+++	+	-
BV	-	-	-	-	-	-

Signal intensity: -, no immunofluorescence signal; +, low immunofluorescence signal; ++, moderate immunofluorescence signal; +++, strong immunofluorescence signal. Symbols are assigned based on qualitative assessment of the density / extension of the immunofluorescence signal. ACs amacrine cells, BV Blood vessel, BC bipolar cell, DAC displaced amacrine cell (GABA immunoreactive cell in the GCL), HC horizontal cell, INL other other cells in the INL, MC Müller cell, na not available, RGC retinal ganglion cell RPE retinal pigment epithelium

THRa is preferentially distributed to neuronal populations of the inner retina

The spatial distribution of the *Thra* mRNA signal (Fig. 5A) was compared with the pattern of THRα immunoreactivity in retinal sections (Fig. 5B). Although both the THRα antibodies used in these studies (Table 1) provided a THRα immunostaining pattern in accord with that of *Thra* mRNA, they also extensively labeled the INL in retinal sections from *Thra* KO mice (Fig. 5C). Therefore, we did not investigate the THRα immunostaining in the INL any further. However, double labeling of *Thra* mRNA and calbindin immunostaining showed *Thra* mRNA expression in numerous horizontal cells and in virtually all calbindin immunostained amacrine cells, while other non-calbindin immunopositive cells in the proximal INL were also *Thra* mRNA positive (Fig. 5D). In the GCL, THRα immunostaining appeared to be specific, since this retinal layer was not immunolabeled in sections from *Thra* KO retinas (see arrowheads in Fig. 5B and C). The actual presence of both *Thra* mRNA and THRα protein in virtually all retinal ganglion cells was confirmed in double label experiments using βIII-tubulin

antibodies (Fig. 5E and F). A summary of the *Thra* mRNA and immunostaining patterns is reported in Tables 3 and 4.

THRβ is expressed in cone photoreceptors and in neuronal populations of the inner retina

The distribution of the *Thrb* mRNA signal (Fig. 6A) was compared with the THRβ immunostaining patterns (Fig. 6B–G). The THRβ antibody used in these studies was found to provide specific immunostaining, since it did not produce any immunolabeling in *Thrb* KO retinal sections (Fig. 6H). Consistent with the pattern of *Thrb* mRNA staining, prominent THRβ immunoreactivity was observed in cone OS (Fig. 6C), in cone somata in the distal ONL (Fig. 6D), and in cone pedicles in the OPL (Fig. 6B). Similar to DIO2 and DIO3 immunostainings, also THRβ immunoreactivity was found in faintly immunolabeled cell bodies in the distal INL (Fig. 6E) and in sparse somata in the proximal INL (Fig. 6F). Some THRβ immunostained cell bodies were also detected in the GCL (Fig. 6G).

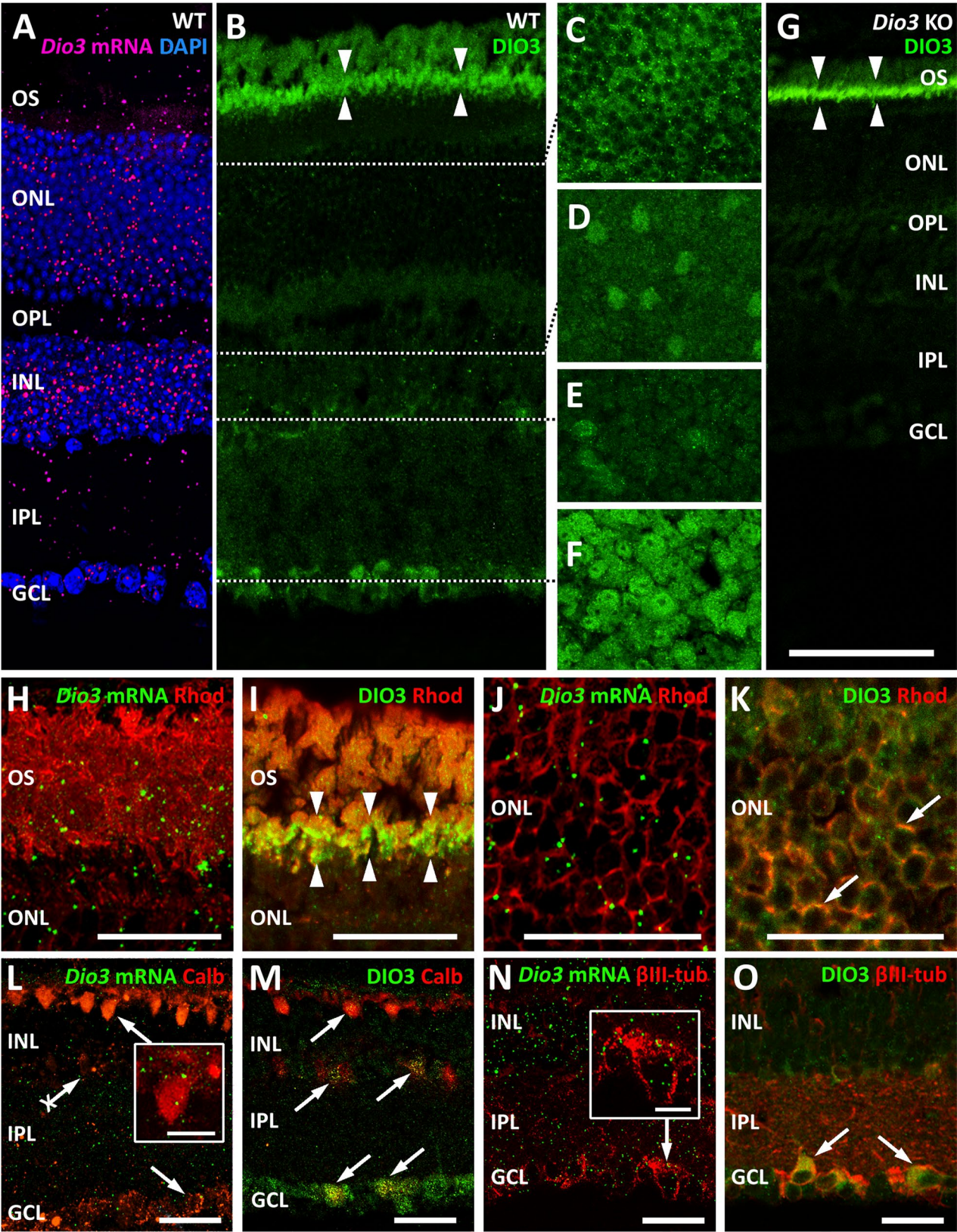


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Fig. 4 DIO3 localization patterns. *Dio3* mRNA visualized with RNAscope in situ hybridization (**A**) and DIO3 immunoreactivity (**B**) in mouse retinal sections. (**C–F**) confocal scans at different levels (dashed lines) through the thickness of a retinal whole mount immunolabeled with an antibody directed to DIO3 and showing DIO3 immunoreactive somata of rod photoreceptors in the ONL (**C**), cell bodies in the distal INL (**D**), other immunolabeled somata in the proximal INL (**E**), and DIO3 immunolabeled somata in the GCL (**F**). The specificity of the DIO3 antibody was verified in retinal sections from *Dio3* KO retinas (**G**). The arrowheads in (**B**) and in (**G**) indicate non-specific DIO3 immunoreactivity in the photoreceptor OS. The results of double label experiments using immunohistochemical markers of retinal cells together with either the *Dio3* mRNA probe or the DIO3 antibody are shown in panels (**H–O**). The arrows indicate double labeled profiles, while the xed arrow indicates an immunolabeled cell without *Dio3* mRNA staining. The insets are higher power images of the cells indicated by the relative arrows. Both rhodopsin immunoreactivity and *Dio3* mRNA fluorescent puncta were in rod OS (**H**), which also displayed both rhodopsin and DIO3 immunoreactivities, with non-specific DIO3 immunoreactivity indicated by the arrowheads (**I**). Both *Dio3* mRNA (**J**) and DIO3 immunoreactivity (**K**) were in rhodopsin immunolabeled rod photoreceptors. Calbindin (Calb) immunoreactive horizontal cells and ganglion cells, but not Calb immunoreactive amacrine cells, also displayed *Dio3* mRNA staining (**L**), while all three types of Calb immunostained cells were also DIO3 immunolabeled (**M**). Both *Dio3* mRNA (**N**) and DIO3 immunoreactivity (**O**) were in β -III tubulin immunoreactive ganglion cells. Scale bar in G, 50 μ m (referring to panels A–G); Scale bars in (H–O), 30 μ m (10 μ m in the insets). See Fig. 1 for abbreviations of the retinal layers

Together, the RNAscope and the immunofluorescence data suggested the possible localization of THR β to cone photoreceptors, horizontal cells, amacrine cells, and ganglion cells. In double label experiments, both the *Thrb* mRNA signal and the THR β immunostaining overlapped with cone arrestin immunoreactivity. In particular, the *Thrb* mRNA signal was in cone somata and OS (Fig. 6I), while the THR β immunostaining was concentrated in the cone OS and in cone pedicles (Fig. 6J). A few *Thrb* mRNA positive clusters were associated with some calbindin immunolabeled horizontal cells and, perhaps, amacrine cells, while only very rare calbindin immunopositive ganglion cells were observed to display *Thrb* mRNA labeling (Fig. 6K). In parallel, THR β immunoreactivity was found in most of the calbindin immunolabeled horizontal, amacrine, and some ganglion cells (Fig. 6L). Among amacrine cells, tyrosine hydroxylase immunoreactive, dopaminergic amacrine cells also displayed both *Thrb* mRNA signal and THR β immunostaining (Fig. 6M and N, respectively). Finally, both *Thrb* mRNA fluorescent puncta and THR β immunostaining were observed in some, but not all, retinal ganglion cells identified by β III-tubulin immunoreactivity (Fig. 6O and P, respectively). A summary of the *Thrb* mRNA and immunostaining patterns is reported in Tables 3 and 4.

MCT8 and OATP1C1 are distributed to Müller glia, retinal ganglion cells, and blood vessels

The distribution of the *Slc16a2* mRNA signal (Fig. 7A) was compared with that obtained with MCT8 immunostaining in retinal sections (Fig. 7B). The antibody used in these studies produced some unspecific, uniformly distributed immunostaining in the photoreceptor OS layer, in a pattern also observed in sections from *Slc16a2* KO retinas (Fig. 7C). Specific MCT8 immunoreactivity was observed at the RPE and in sparse somata in the GCL. The specificity of this immunolabeling was supported by the absence of immunostaining in the RPE and GCL of *Slc16a2* KO retinas. Although the RNAscope data indicated *Slc16a2* expression also in the INL and in some blood capillaries, no detectable immunolabeling was found at these sites. In double label experiments, *Slc16a2*

mRNA signal was observed in GS immunostained Müller cell bodies in the mid-INL (Fig. 7D), while the *Slc16a2* expressing cells in the GCL were identified as ganglion cells by β III-tubulin immunoreactivity (Fig. 7E).

Finally, the spatial localization of the *Slco1c1* mRNA signal (Fig. 7F) was compared with the OATP1C1 immunostaining patterns (Fig. 7G). The OATP1C1 antibody provided specific immunostaining, since no immunolabeling was evident in *Slco1c1* KO retinas (Fig. 7H). Consistent with the localization of *Slco1c1* transcript in the mid-INL, suggesting *Slco1c1* expression in Müller cells, OATP1C1 immunoreactivity was observed in radially oriented fibers spanning the entire retinal thickness, characteristic of Müller cell processes, and in enlarged profiles just ventral to the GCL, resembling Müller cell endfeet. In contrast with the distribution pattern of *Slco1c1* mRNA, no OATP1C1 immunoreactivity was observed in the RPE, in cells of the GCL, or in putative blood capillaries. Double label experiments with the *Slco1c1* mRNA probe in conjunction with an antibody directed to GS (Fig. 7I) or with isolectin B₄ (Fig. 7J) confirmed *Slco1c1* expression in Müller cells and in blood capillaries, respectively.

A summary of the *Slc16a2* and *Slco1c1* mRNA distribution and of the MCT8 and OATP1C1 immunostaining patterns is reported in Tables 3 and 4, respectively.

Effects of genetic deletions of specific components of the retinal TH regulatory system

Cone photoreceptors were immunolabeled using an antibody directed to cone arrestin. As shown in Fig. 8A, in WT retinas strong immunolabeling was observed in cone OS, in cone somata in the distal ONL, in their axons spanning through the ONL, and in cone pedicles in the OPL. In the different KO retinas, the most notable changes were (i) various degrees of fragmentation and disorganization of the OS; (ii) presence of displaced cone somata in the mid-ONL; (iii) reduced number of cones (Fig. 8B); (iv) swelling of cone pedicles (Fig. 8C). In particular, significant decreases in cone numbers were detected in *Dio2* KO, *Dio2/Slc16a2* KO, *Dio3* KO, and *Thrb* KO retinas (23%, 19%, 70%, and 29% vs. WT,

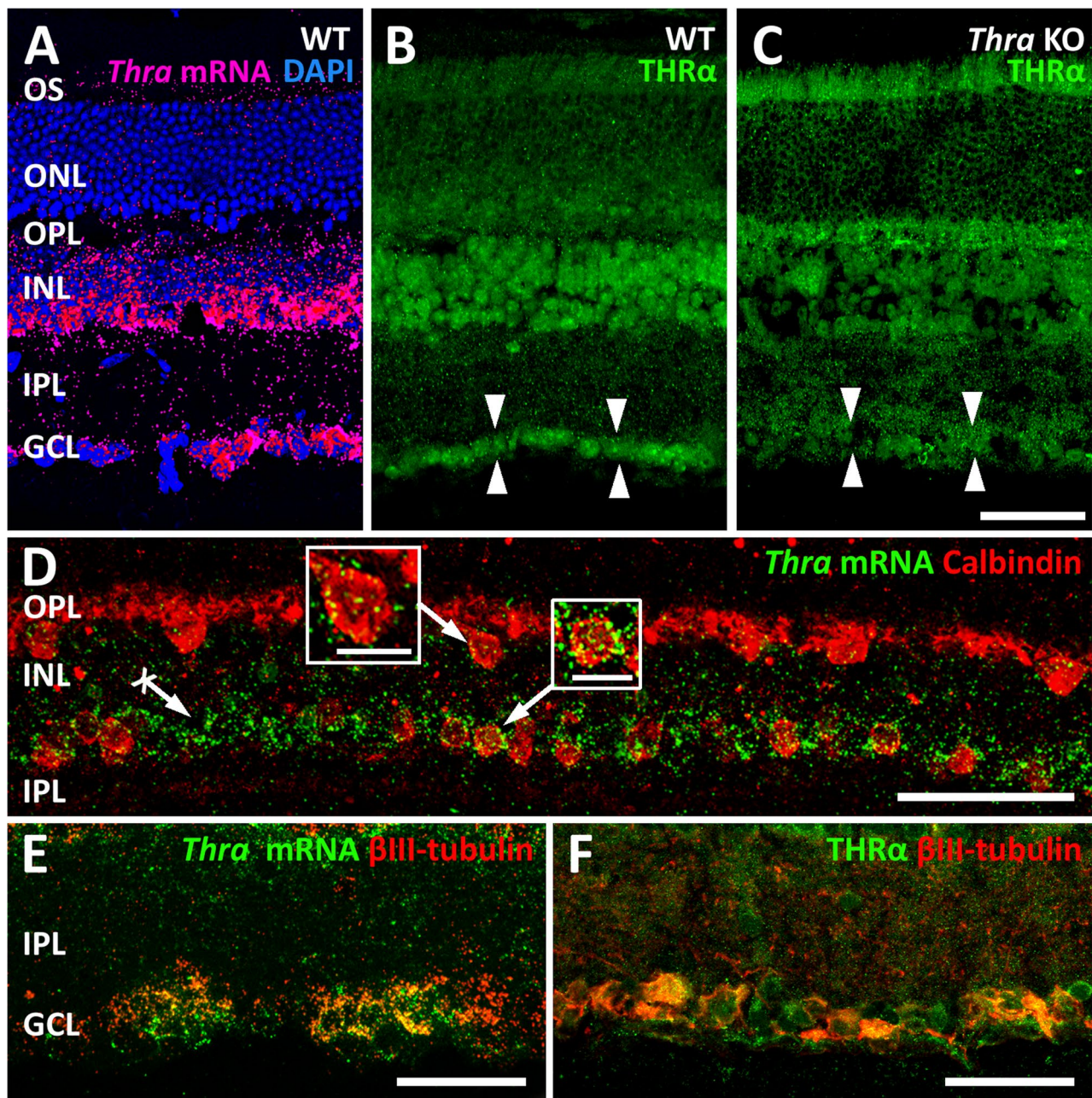


Fig. 5 THRa localization patterns. *Thra* mRNA visualized with RNAscope in situ hybridization (**A**) and THRa immunoreactivity in WT (**B**) and in *Thra* KO (**C**) retinas (the images show immunostaining obtained with antibody ThermoFisher PAI-211 A). The arrowheads indicate specific THRa immunoreactivity in the GCL of WT (**B**) that is not observed in THRa KO retinas (**C**). (**D**) *Thra* mRNA was in calbindin immunoreactive horizontal cells and amacrine cells (insets) as well as in other non-calbindin immunoreactive amacrine cells (xed arrow). Both *Thra* mRNA (**E**) and THRa immunoreactivity (**F**) were in β -III tubulin immunoreactive ganglion cells. Scale bar in C, 50 μ m (referring to panels A–C); Scale bars in (D–F), 30 μ m (10 μ m in the insets). See Fig. 1 for abbreviations of the retinal layers

respectively), while significant swelling of cone pedicles was observed in *Dio2* KO, *Dio2/Slc16a2* KO, *Dio3* KO, *Slc16a2* KO, and *Slco1c1* KO retinas, as evidenced by increases in cone pedicle diameters of about 48%, 30%, 42%, 37% and 36%, respectively, as compared to those of WT retinas.

Since most, if not all, of the players related to the retinal TH system considered in the present studies were likely to be expressed in retinal ganglion cells, a quantitative analysis was performed to ascertain whether the genetic deletion of any of these factors could influence ganglion cell survival. RBPMS immunoreactive ganglion cells were visualized in retinal sections from WT and KO retinas

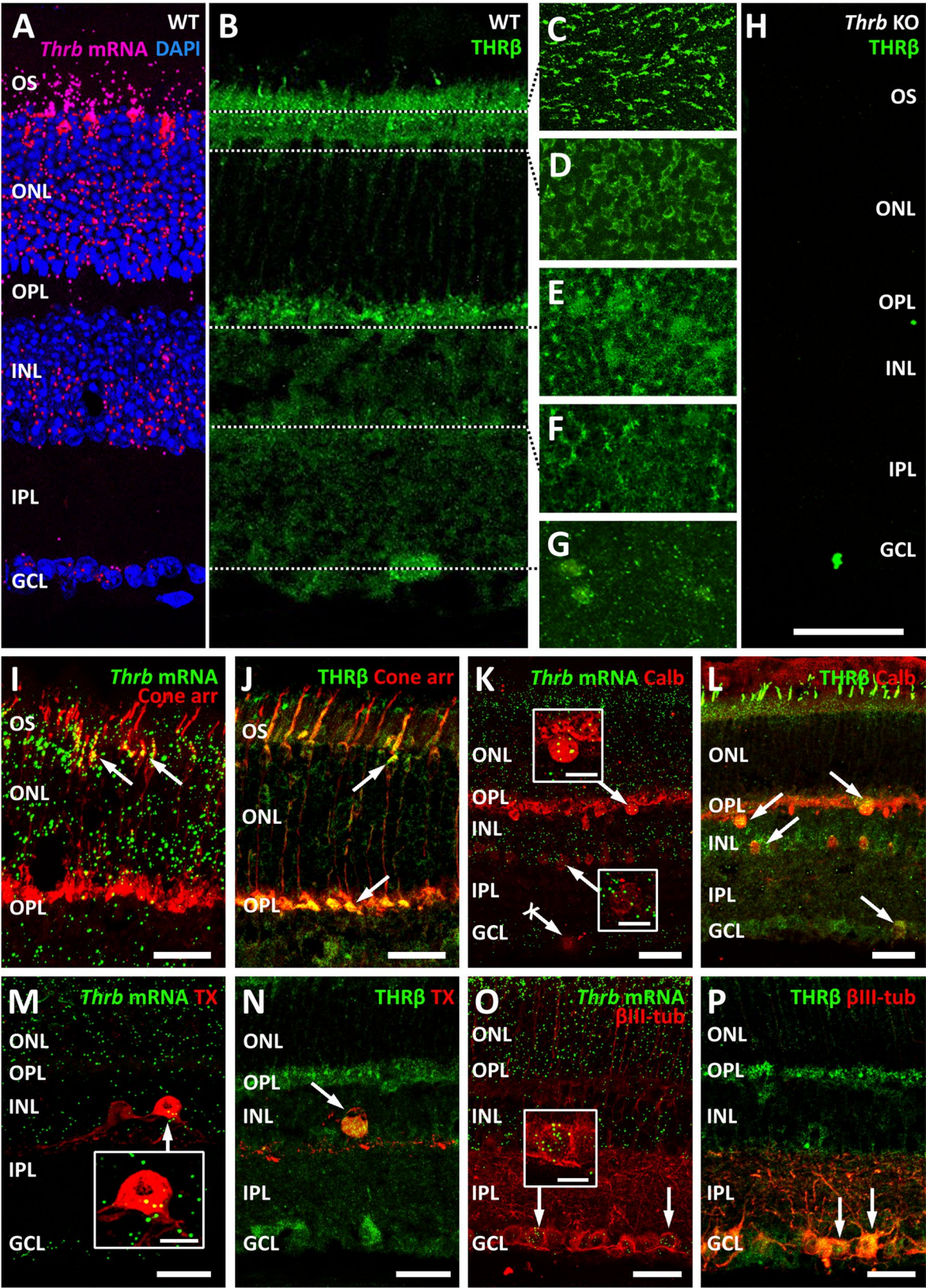


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Fig. 6 THR β localization patterns. *Thrb* mRNA visualized with RNAscope in situ hybridization (**A**) and THR β immunoreactivity (**B**) in mouse retinal sections. (**C–G**) confocal scans at different levels (dashed lines) through the thickness of a retinal whole mount immunolabeled with an antibody directed to THR β and showing THR β immunoreactive cone OS (**C**), cone somata in the distal ONL (**D**), cell bodies in the distal INL (**E**), other immunolabeled somata in the proximal INL (**F**), and THR β immunolabeled somata in the GCL (**G**). The specificity of the THR β antibody used in these studies was confirmed by absence of immunostaining in retinal sections from *Thrb* KO retinas (**H**). The results of double label experiments using different immunohistochemical markers of retinal cell populations in conjunction with either the *Thrb* mRNA probe or the THR β antibody are shown in panels (**I–P**). The arrows indicate double labeled profiles, while the xed arrow indicates an immunolabeled cell that does not display *Thrb* mRNA staining. The insets are higher power images of the cells indicated by the relative arrows. Cone arrestin colocalized with *Thrb* RNAscope signal in cone photoreceptor somata (**I**) and with THR β immunoreactivity both in cone somata and pedicles (**J**). Calbindin (Calb) immunoreactive horizontal cells and amacrine cells also displayed *Thrb* mRNA staining, while ganglion cells did not (**K**). All three types of Calb immunostained cells were also THR β immunolabeled (**L**). Both *Thrb* mRNA and THR β immunoreactivity were in tyrosine hydroxylase (TX) immunolabeled amacrine cells (**M** and **N**, respectively) and in β -III tubulin immunoreactive ganglion cells (**O** and **P**, respectively). Scale bar in **H**, 50 μ m (referring to panels **A–H**); Scale bars in (**I–P**), 30 μ m (10 μ m in the insets). See Fig. 1 for abbreviations of the retinal layers

and their densities did not appear to differ significantly in any of the experimental groups (Fig. S6).

As shown by the representative images of Fig. 8D, retinas from WT and from KO mice displayed variable thicknesses of retinal layers. According to quantitative analysis, summarized in Fig. 8E, both *Dio2* KO and *Dio3* KO retinas exhibited significant reductions in the thickness of GCL+IPL (25% and 35%, respectively), INL (36% and 37%, respectively), OPL (43% and 71%, respectively) and ONL (37% and 50%, respectively) as compared to WT. Both *Dio2* / *Slc16a2* KO and *Slc16a2* KO retinas instead showed significant thinning limited to the INL (32% and 24% vs. WT, respectively) and the ONL (24% and 17% vs. WT, respectively). In addition, the ONL of *Slco1c1* KO retinas was reduced by 18% compared to WT, while *Thra* KO and *Thrb* KO retinas did not show appreciable alterations in any of the layers. Figure 8F represents an analysis of the relative layer thickness in each experimental group and indicates that, despite changes in absolute thickness, the general pattern of retinal coverage by each layer was similar to that of WT in most of the KO retinas. Notable exceptions were *Dio2* / *Slc16a2* KO retinas, which displayed a 23% increase in the relative thickness of the GCL+IPL and a 20% decrease of that of the INL. Similarly, *Dio3* KO retinas showed a 18% increase in the relative thickness of the GCL+IPL in conjunction with a 50% reduction of the coverage of the OPL. Full quantitative details and statistical analyses are provided in figure S7.

Discussion

The studies reported here provide a detailed and extensive description of the localization patterns of the main elements controlling TH availability in retinal neurons. To our knowledge, this is the first comprehensive description of the retinal TH signaling system, which includes a predictive bioinformatic analysis of scRNA-seq data followed by validation through evaluations of both the sites of mRNA expression and those of protein localization. In addition, the observations in KO retinas, which confirm and expand those of previous studies, contribute to the understanding of the involvement of the

retinal TH system in retinal cell development and survival. The translational value of the findings reported in the present study is supported by the bioinformatic data indicating important similarities between mouse and human expression patterns.

Comparison with previous studies

The largest availability of localization data on the retinal TH system is about *Dio2* expression in Müller cells. Indeed, *Dio2* mRNA has been reported in Müller cells of mouse retinas using a variety of techniques, including microarray [24], conventional in situ hybridization [25], RNAscope and scRNA-seq [13] or cre drivers for *Dio2* [7]. In addition, *Dio2* expression in Müller cells has been reported also in human and monkey retinas using RNAscope and scRNA-seq [23, 26]. Our results expand these observations providing convergent data from bioinformatic analysis, in situ hybridization (RNAscope), and immunofluorescence. In addition, our findings also indicate DIO2 expression (both mRNA and protein) in ganglion cells. As noted above, the immunofluorescence patterns of DIO2 display impressive mismatches with the localization of *Dio2* mRNA. In particular, DIO2 immunoreactivity, but not *Dio2* mRNA, was observed in cone photoreceptors, in horizontal cells, and in populations of amacrine cells. These mismatches are discussed below, but it is interesting to note that another immunofluorescence study in rat retinas using a different DIO2 antibody reported a similar pattern of DIO2 immunostaining [44]. Different from DIO2, very scarce evidence of DIO3 expression / localization in retinas is available in the literature. To our knowledge, only one immunofluorescence study reported DIO3 immunoreactivity in the OS of cone photoreceptors [45], in a pattern similar to that observed in the present study also in *Dio3* KO retinas. Therefore, these results should be interpreted with caution. Although the bioinformatic data indicate very low expression of *Dio3* mRNA in mouse retinas, our experimental observations establish that virtually all cells in the retina may express low levels of *Dio3* mRNA and the immunofluorescence findings confirm the presence of

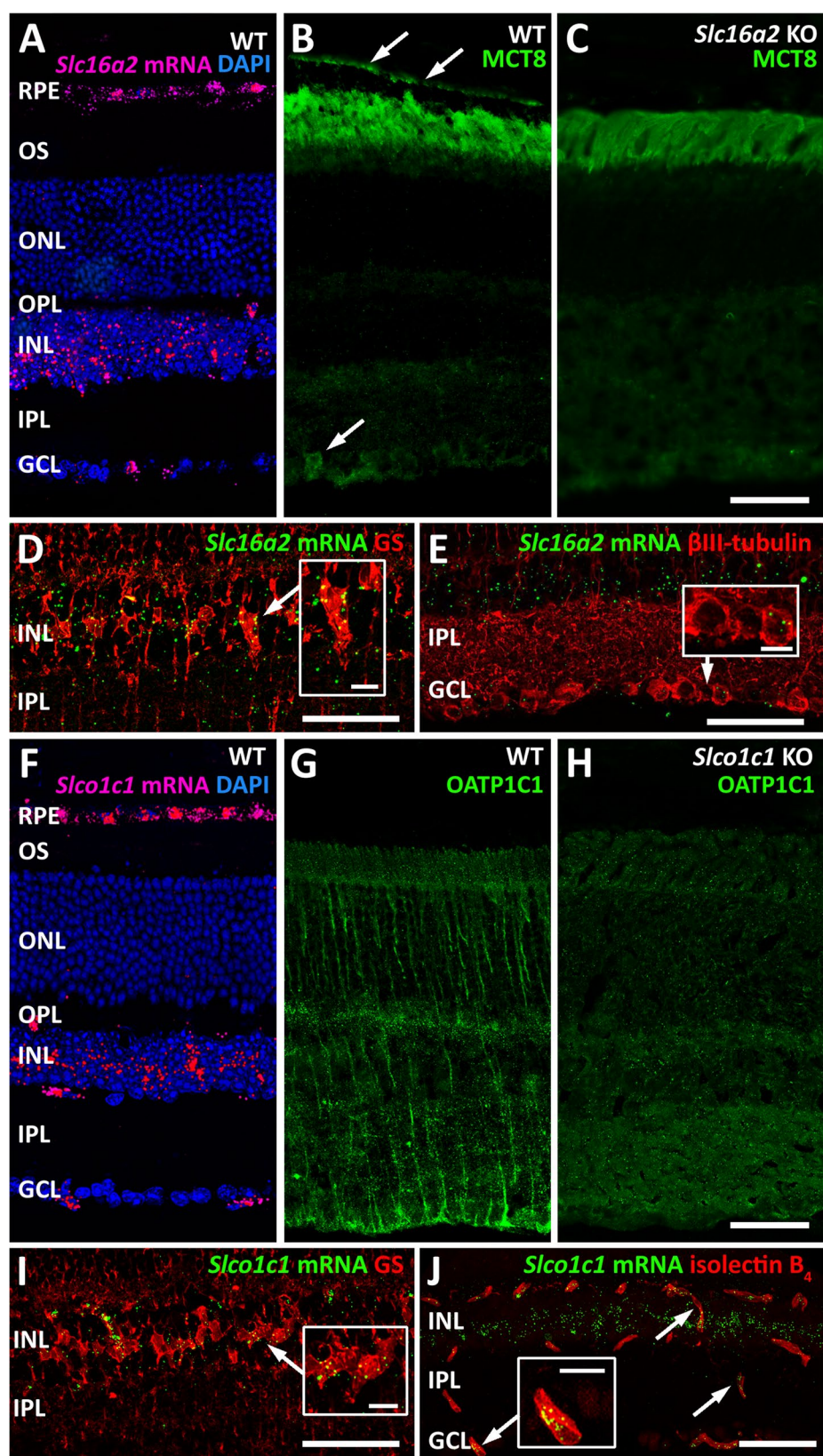


Fig. 7 (See legend on next page.)

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Fig. 7 MCT8 and OATP1C1 localization patterns. *Slc16a2* mRNA visualized with RNAscope in situ hybridization (**A**) and MCT8 immunoreactivity in WT (**B**) and in *Slc16a2* KO (**C**) retinas. The arrows in (**B**) point to specific MCT8 immunoreactivity in the RPE and in the GCL. (**D**) *Slc16a2* mRNA in glutamine synthetase (GS) immunoreactive Müller cells (a double labeled cell is shown at higher power in the inset). (**E**) *Slc16a2* mRNA in sparsely distributed β -III tubulin immunoreactive ganglion cells (one cell only β -III tubulin immunolabeled and one double labeled cell are shown at higher power in the inset). *Slco1c1* mRNA visualized with RNAscope in situ hybridization (**F**) and OATP1C1 immunoreactivity in WT (**G**) and in *Slco1c1* KO (**H**) retinas. (**I**) *Slco1c1* mRNA in GS immunoreactive Müller cells (a couple of double labeled cells is shown at higher power in the inset). (**J**) *Slco1c1* mRNA in retinal blood capillaries labeled with isolectin B₄ (arrows; a double labeled capillary is shown at higher power in the inset). Scale bars in (**C**) and in (**H**), 50 μ m (referring to panels **A-C** and **F-H**, respectively); Scale bars in D, e, I, and J, 30 μ m (10 μ m in the insets). See Fig. 1 for abbreviations of the retinal layers

DIO3 protein in rods, horizontal cells, ganglion cells and GABAergic amacrine cells.

A study using a β -gal reporter system described the presence of T3 signaling in the inner retina of mice, mainly in glycinergic, AII amacrine cells and in ganglion cells [46], which is consistent, at least in part, with our findings regarding THR α and THR β localization. A previous paper reported the presence of mRNA of the *Thra1* isoform in the ONL, INL, and GCL using RNAscope in situ hybridization in retinas of mice at postnatal day 20 [47], while scRNA-seq indicated the presence of *Thra* mRNA in rod and cone bipolar cells, Müller cells, amacrine, horizontal, and ganglion cells of mouse retinas [13]. Overall, our findings confirm and expand these observations, revealing a previously unnoted prevalence of *Thra* mRNA expression in the inner retina together with an abundant THR α protein localization in ganglion cells. The expression of *Thrb* in cone photoreceptors has been assessed in different reports. The THR β 2 isoform was detected using immunohistochemistry in developing cones of mouse retinas [48], while *Thrb*2 mRNA expression in cones was also described using reporter mice with a *lacZ* knockin [28, 29]. The THR β 1 isoform was also localized to cone photoreceptors as well as to amacrine and putative ganglion cells by *lacZ* reporter transgenes [27]. In addition, diffuse localization of *Thrb* mRNA was reported in ONL, INL, and GCL using RNAscope [47], while scRNA-seq indicated the presence of *Thrb* mRNA in cone bipolar cells of mice [13] and of primate [26] retinas. Overall, our observations are consistent with these previous data and also add some previously unreported features of *Thrb* expression patterns in the retina. For instance, both the bioinformatic analysis and the RNAscope observations indicate *Thrb* mRNA expression not only in cone but also in rod photoreceptors and in cells of the inner retina, in particular in retinal ganglion cells. Finally, the bioinformatic data indicate possible expression of both *Thra* and *Thrb* mRNAs in the RPE.

Concerning TH transporters, the distinctive traits of *Slc16a2* and *Slco1c1* expression patterns were the presence of their mRNAs and/or proteins in the RPE, in Müller cells, and in ganglion cells, together with the presence of strong RNAscope signal of *Slco1c1* in blood capillaries. Similar features were reported, at least in part, by previous studies using t-sne [13], *lacZ* reporter transgenes

[49], and immunofluorescence in mouse [50] or in rat [4] retinas.

Divergent localization patterns between mRNA and proteins of TH regulatory elements

As noted above, the data from the bioinformatic analysis, the RNAscope investigation, and the immunofluorescence study are not always convergent in a unitary picture. In particular, three different types of mismatches could be identified: (i) a mismatch between the bioinformatic predictions and the mRNA localization; (ii) a mismatch between mRNA and protein localization with presence of the mRNA but absence of the protein; (iii) a mismatch between mRNA and protein localization with absence of mRNA but presence of the protein. Figure 9A summarizes the findings reported in Tables 2, 3 and 4 with color-coding of the expression / signal intensity of each marker in different cell populations for direct comparison of the bioinformatics, RNAscope, and immunofluorescence data.

i) Mismatch between the bioinformatic predictions and the mRNA localization

Evident differences were those concerning the lack of bioinformatic evidence of *Dio2* and *Dio3* mRNA expression in ganglion cells as compared to the presence of the RNAscope signals in these cells and those at the level of retinal blood vessels, where bioinformatic data seem to indicate absence of *Slco1c1* mRNA while a strong *Slco1c1* RNAscope signal was detected. On the other hand, RNAscope in situ hybridization failed to validate bioinformatic results on the presence of *Thrb* mRNA in RPE. These mismatches may be explained considering that there may be differences in the expression patterns of cells within a tissue and isolated cells in a single cell suspension [51], or that the used techniques have different resolutions [52].

ii) Mismatch between mRNA and protein localization: presence of mRNA and absence of protein

High density of RNAscope signal but lack of immunofluorescence was observed for MCT8 in the RPE and in Müller cells, and for OATP1C1 in the RPE and in vascular endothelial cells. A mismatch between mRNA expression and protein synthesis may depend on different factors, for instance the presence of regulatory molecules, such

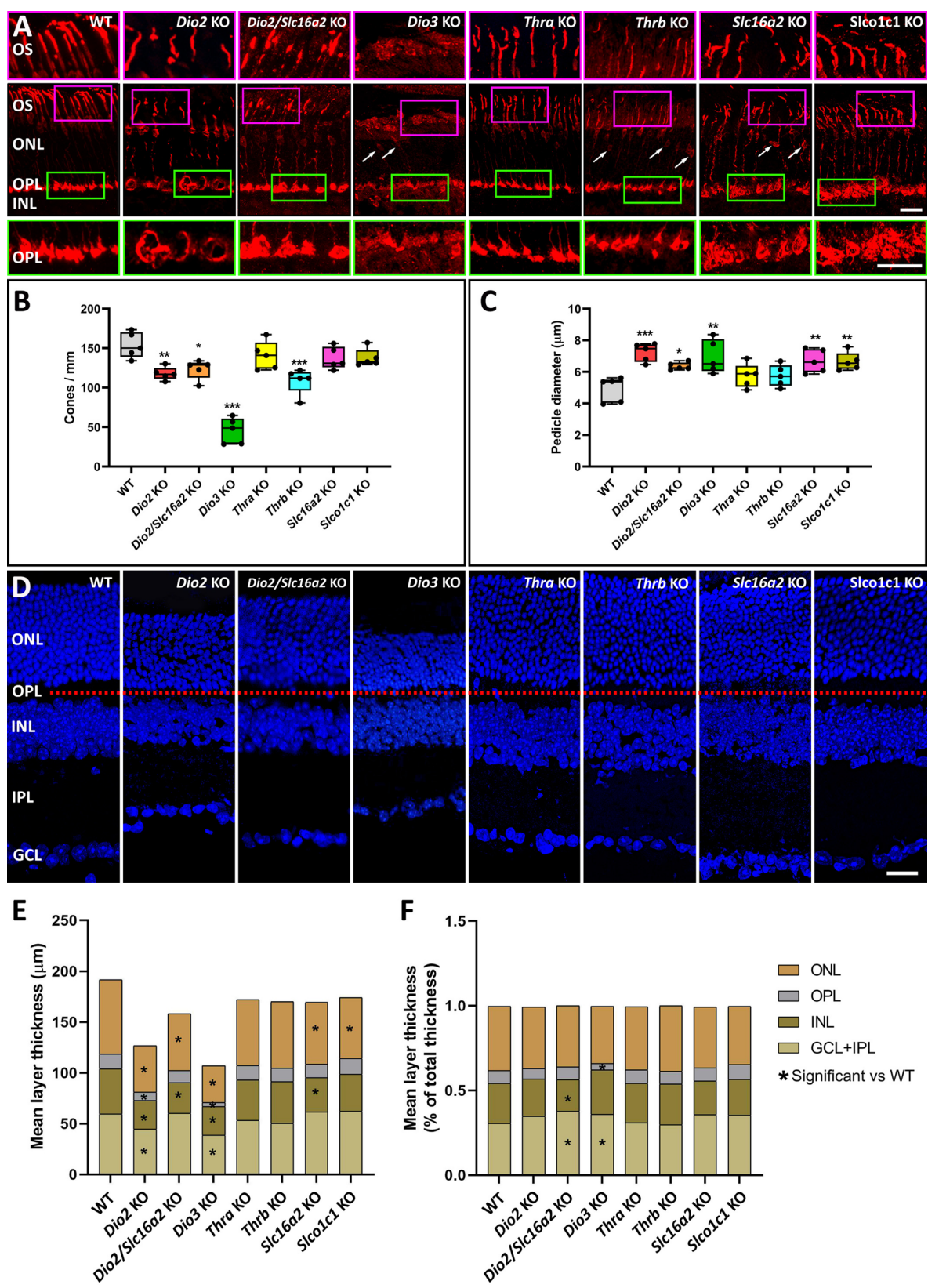


Fig. 8 (See legend on next page.)

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Fig. 8 Analyses of KO retinas. **(A)** Confocal images of the outer retina from sections of WT and KO retinas immunolabeled with an antibody directed to cone arrestin. Higher power images show cone outer segments (pink-edged panels) and cone pedicles (green-edged panels). The arrows indicate misplaced cone somata in the ONL. Scale bar, 25 μm . **(B)** Quantitative analysis of cone density. **(C)** Quantitative analysis of cone pedicle diameters. **(D)** DAPI stained sections from WT and KO retinas for comparison of retinal layer thicknesses. The images are aligned to the OPL (dashed red line). Scale bar, 20 μm . The results of the quantitative analysis of layer thickness are shown in **(E)** as mean layer thicknesses measured in μm and in **(F)** as mean layer thicknesses expressed as percentages of the total retinal thickness. Detailed graphs showing means and standard errors, together with the different levels of statistical significance, are reported in figure S7. Mean differences among groups were tested using one-way ANOVA followed by Tukey's post-hoc test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. WT. $N = 5$. See Fig. 1 for abbreviations of the retinal layers

as miRNA, that may bind to mRNA and block its translation or promote its degradation [53], or different rates of mRNA turnover [54]. On the other hand, the lack of immunofluorescent signal might also be due to limited ability of the primary antibodies to detect their specific antigens in some cell types due to differential post-translational processing, as typically observed for proteins of the Slc transporter family [55].

iii) Mismatch between mRNA and protein localization: absence of mRNA and presence of protein

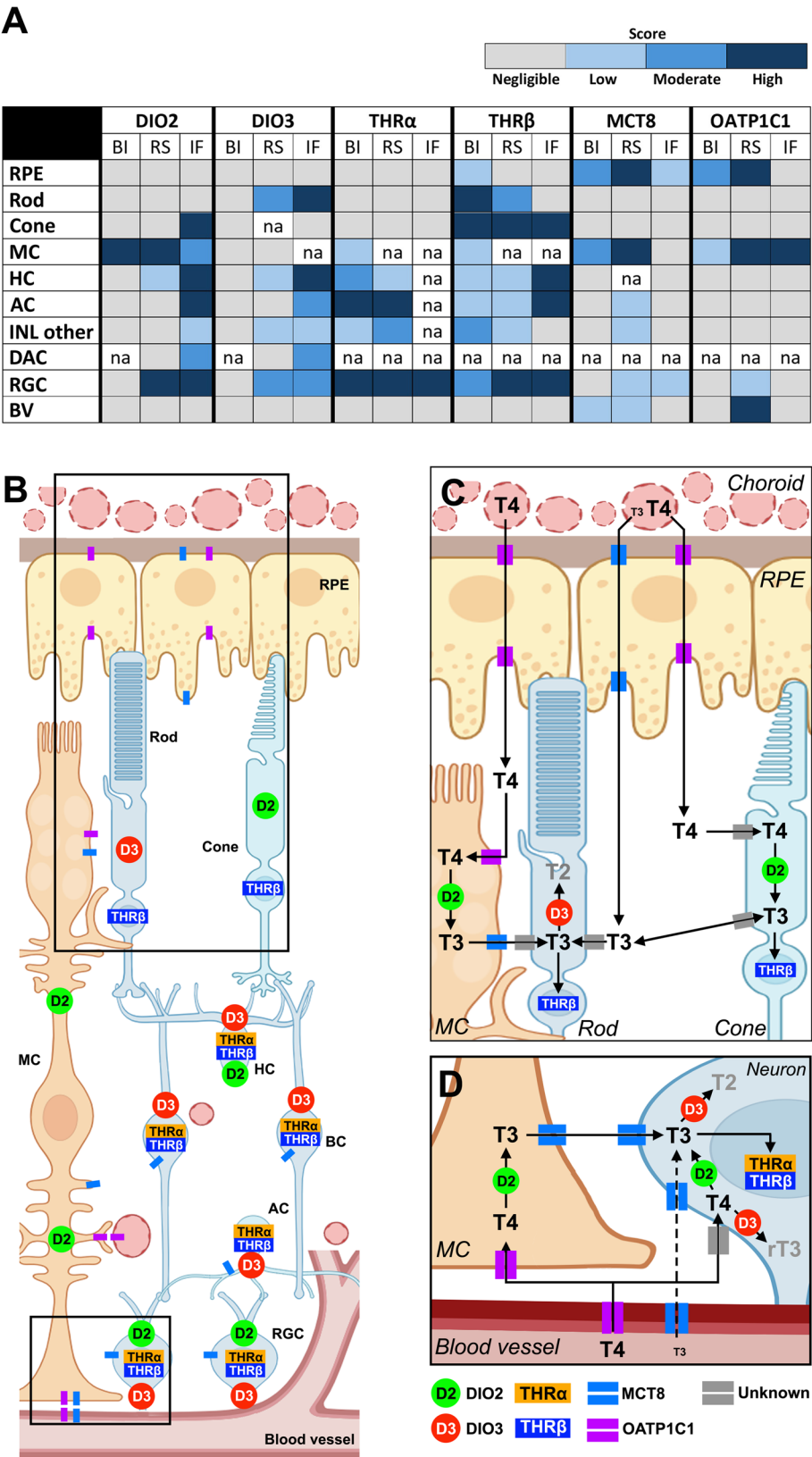
The most impressive mismatch of this kind was the presence of specific DIO2 immunostaining in cone photoreceptors and in amacrine cells, which were devoid of any RNAscope signal. Similar to our observations, an immunofluorescence study in rats reported DIO2 immunoreactivity localized to photoreceptors, the INL, and the GCL in dark adapted retinas, although specificity controls of the DIO2 antibody used in those studies were lacking [44]. Another study suggested the presence of a circadian control of DIO2 expression in cones, although direct demonstration of *Dio2* mRNA in cones was not provided [56]. The possibility exists that a protein is detected in the absence of its mRNA. Indeed, it is known that some proteins may have high stability and long half-life, so that they can persist in the cell after degradation of their mRNA [57]. However, the reason underlying the different stability of *Dio2* mRNA in cone photoreceptors as compared with other DIO2-expressing cells (i.e. Müller cells) would be difficult to explain. Another intriguing possibility is that DIO2 protein reaches cone photoreceptors and other cells through extracellular vesicles (EVs) released by Müller cells. Indeed, it has been shown that Müller cells may release EVs in vivo [58], which have been shown by in vitro studies to contain small amounts of proteins and mRNAs as well as miRNAs [59]. The EVs released by Müller cells can be internalized by both neural and non-neural retinal cells and may play important roles in communication between glial and neuronal cells both in physiological and in pathological conditions [59, 60]. If Müller cells were able to deliver DIO2 containing EVs, then these cells would act as main controllers of T3 production in virtually all types of retinal cells, and in particular in highly metabolically active cells such as the cone photoreceptors.

Functional implications

TH signaling relies on three main processes: hormone transport from circulation to target cells, hormone processing by deiodinases (activation by DIO2 or inactivation by DIO3) and hormone binding to its receptors [6]. Similar to findings in the brain [61, 62], the present data allow to propose a possible reconstruction of the functional organization of the local TH system in the retina, as reported in Fig. 9B-D.

In the outer retina, both MCT8 and OATP1C1 are expressed by RPE cells. This is not surprising, since the RPE is the major component of the outer blood-retina barrier acting as the primary checkpoint for the selective transport of molecules from choriocapillaris circulation [63]. Then, circulating T3 and T4 would reach the outer retina through MCT8 and OATP1C1, respectively, expressed by the RPE. T4 would be internalized in Müller cells through OATP1C1 to be converted into T3 by DIO2 expressed by Müller cells. In contrast to neurons of the inner retina, photoreceptors do not seem to express either MCT8 or OATP1C1, but they display a marked expression of *Thrb*, confirming a strong influence of TH in their physiology, as extensively described in previous reports [7]. Therefore, T3 released by Müller cells through MCT8 would be taken up by photoreceptors via transporters other than MCT8 or OATP1C1 [64]. Rods may use DIO3 to exert a negative regulation of T3 by transforming it into T2. On the other hand, in cones the presence of DIO2 protein, although not of *Dio2* mRNA, suggests that these photoreceptors might also receive T4 directly from RPE and exert a positive control of TH signaling by converting T4 into T3.

In the inner retina, the vasculature displays a marked expression of OATP1C1, whose affinity for T4 is higher than that for T3. In particular, OATP1C1 would facilitate both the influx of T4 into endothelial cells and the efflux of T4 from endothelial cells to the extracellular matrix, thus allowing the translocation of T4 from the vascular lumen to retinal cells. Circulating T3, although minimally represented as compared to T4, could still reach retinal cells by carrier-mediated transport through MCT8 expressed at the level of blood vessels. Among the cells of the inner retina, Müller cells are the only type displaying high levels of OATP1C1 expression, indicating that these cells are likely to be the main acceptors of T4 from the circulation. Müller cells also have a prominent



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Fig. 9 (A) Summary of the results with color-coding of the expression / signal intensity of each marker in different cell populations for direct comparison of the bioinformatics (BI), RNAscope (RS), and immunofluorescence (IF) data. (B) Graphical summary of the localization data. (C) and (D) are enlargements of the boxed areas in (B) showing reconstructions of the possible functional organization of the local TH system in the outer and in the inner retina, respectively, as deduced from the experimental findings. See the text for explanations. AC(s), amacrine cell(s); BCap, Blood capillaries; BC(s), bipolar cell(s); DACs, displaced amacrine cells (GABA immunoreactive cells in the GCL); HC(s), horizontal cell(s); MC(s), Müller cell(s); na, not available; Other INL, Other cells in the INL; RGC(s), retinal ganglion cell(s); RPE, retinal pigment epithelium. Panels (B-D) were partially created with BioRender

expression of DIO2, thus suggesting that they act as the primary retinal converters of T4 into active T3. Our observations show that Müller cells also express MCT8, which has higher affinity for the T3 than for T4 [65]. Therefore, after DIO2 mediated conversion from T4, T3 may exit Müller cells through a carrier-mediated mechanism involving MCT8 and, as MCT8 is also expressed by inner retinal neurons, T3 released by Müller cells would be taken up by neurons of the INL and GCL. These neurons have been found to express DIO3, although at low levels, and therefore they could participate in the regulation of TH function by allowing inactivation of T3 into T2. On the other hand, the presence of DIO2 protein in inner retinal neurons, although not fully confirmed by *Dio2* mRNA, raises the possibility that T4 may directly enter retinal neurons. For instance, the uptake of T4 by ganglion cells may take place via OATP1C1, although it is expressed only by sparsely distributed ganglion cells, or via low affinity transport through MCT8. T4 would then be either converted into T3 by DIO2 or into rT3 by DIO3. Therefore, the effective T3 levels available to bind THRs and activate TH signaling would depend upon the combined effect of DIO2 and DIO3 in retinal neurons. Although the present reconstruction based on localization data should be validated with in depth mechanistic analyses (for instance using cell-specific gene knock out models), still it remains a key reference for upcoming studies focusing on TH signal transduction in the retina.

Effects of genetic deletions

The observation of retinal alterations caused by the genetic deletion of specific components of the retinal TH system may provide information on the roles played by these elements, mainly during retinal development. In this regard, retinas from *Dio2* or *Dio3* KO mice displayed an overall damage of the cone structure, in line with the known functional roles played by DIO2 in photoreceptor maturation and in the maintenance of photoreceptor physiological function [56, 66]. In particular, in *Dio2* KO retinas we noted an evident increase in size of cone pedicles, indicating presynaptic or axonal swelling typical of degenerating synapses, as observed in rats after brain damage [67] and in a mouse model of Alzheimer disease [68]. Still, since the deletion of *Dio2* results both in decreased T3 tissue levels and accumulation of circulating T4 [69], the analysis of *Dio2* KO alone could not establish the differential contribution of these

two conditions. Therefore, we used the *Dio2* / *Slc16a2* double KO model, which combines the *Dio2* KO effects (low T3 tissue levels and accumulation of circulating T4) with those of *Slc16a2* KO, consisting in reduced release of T4 into the circulation from thyroid gland with unaltered tissue T3 [69–71]. This model would reproduce a low T3 state in the retina with attenuated T4 accumulation, thus allowing to estimate the T4 contribution to the phenotypical alterations. Interestingly, the effects on cone pedicle hypertrophy were reduced in retinas from *Dio2/Slc16a2* KO mice as compared to *Dio2* KO, suggesting a possible exacerbating role of T4 accumulation on cone alterations.

On the other hand, a total absence of cones was observed in *Dio3* KO retinas, in line with previous findings describing the key role of DIO3 for the survival and the maturation of cone photoreceptors through the control of T3 availability [72].

Regarding the effects of genetic deletions of the *Thrs*, it seems that a normal overall retinal development may still occur, despite the key role of *Thrb* in cone differentiation and maturation as reported by previous studies [39, 73]. These observations are consistent with only minor changes in opsin expression and normal electroretinographic responses reported in *Thrb1* KO mice [27]. A slight, but statistically significant, decrease in ONL thickness was observed in both *Slc16a2* and *Slco1c1* KO retinas, which was likely to be the result of cone suffering, as indicated by the presence of cone pedicle hypertrophy detected in these retinas. Indeed, the expression of both *Slc16a2* and *Slco1c1* in the RPE is extremely important to ensure cone differentiation during development. In this respect, MCT8-deficient mice have been found to display reduced electroretinographic responses, demonstrating an overall photoreceptor loss of function [49].

Most studies regarding the role of TH in retinal development and the effects of genetic deletions of deiodinases, THRs or TH transporters have been dedicated to the maturation of cone photoreceptors. However, THs may play an important role for the overall retinal development. Indeed, the alterations induced by genetic deletion of deiodinases observed in the outer retina were concomitant with a reduction of the whole retinal thickness, affecting all retinal layers. These findings suggest that the functional role of DIO2 and DIO3 are not limited to the maturation and survival of photoreceptors but may also affect the inner retina inducing a dysregulation

of TH signaling. Interestingly, in line with this view, a significant correlation between insufficient thyroid function markers and retinal thinning has been reported recently in women affected by type 1 diabetes [74]. Similar to the observations in cone photoreceptors, the less evident alterations observed in *Dio2/Slc16a2* KO retinas compared to those in *Dio2* KO retinas suggest possible negative effects of circulating T4 on the development of the retinal layers.

The genetic deletion of either the *Thrs* or the TH transporters did not result in notable alterations in retinal layer thickness as compared to retinas with genetic deletion of the deiodinases. The lack of overt retinal damage or effects on retinal layer thicknesses in *Thra* or *Thrb* KO mice was probably the result of compensatory mechanisms involving one receptor isoform in the absence of the other, as previously reported [75, 76]. The hypothesis of compensatory mechanisms involving different elements of the TH regulatory system in the retina would be particularly relevant for the retinal ganglion cells, as they appear to express all the elements of this system. In this line, our findings indicate no changes in ganglion cell density in any of the KO retinas analyzed in this study, thus supporting the hypothesis of a sort of cooperation involving the existing elements of the TH system in response to the lack of one of them.

These observations in KO retinas are in line with those of previous studies and indicate that TH functions in retinal development may go well beyond regulation of cone photoreceptor maturation and affect the whole retinal structure. However, from this data it is difficult to ascertain whether the observed effects reflect either direct or indirect effects of altered TH signaling on retinal cells. To establish this point, analysis involving cell-specific KO of each TH signaling component for each major retinal cell type should be realized in a series of dedicated studies. Likewise, conditional KO mice for each TH signaling component could be used to ascertain whether the effects observed might derive from retinal progenitors and/or mature retinal cells. In addition, if any of the morphological changes observed in the KO models have an impact on visual function remains an open question that will be considered in future studies using electroretinography and behavioral tests.

Conclusions

To our knowledge, the present work is the first detailed, comprehensive analysis of the expression and localization of the elements comprising the local TH regulatory system in the mouse retina. This data allow us to recapitulate some mechanisms that are likely to regulate T3 availability in the retina and provide a solid structural basis for past [14], ongoing, and future studies on the functional roles played by THs in retinal health and disease.

Abbreviations

AC	Amacrine cell
ANOVA	Analysis of variance
BV	Blood vessel
BC	Bipolar cell
Calb	Calbindin
DAC	Displaced amacrine cell
DAPI	4', 6-diamidino-2-phenylindole
DIO2	Deiodinase 2
<i>Dio2</i>	Deiodinase 2 encoding gene
DIO3	Deiodinase 3
<i>Dio3</i>	Deiodinase 3 encoding gene
EVs	Extracellular vesicles
FITC	Fluorescein isothiocyanate
GABA	γ -aminobutyric acid
GCL	Ganglion cell layer
GS	Glutamine synthetase
HC	Horizontal cell
INL	Inner nuclear layer
IPL	Inner plexiform layer
IRL	Inner retinal layer
KO	Knockout
LAT1	L-type amino acid transporter 1
LAT2	L-type amino acid transporter 2
<i>Llat2</i>	L-type amino acid transporter 2 encoding gene
MC	Müller cell
MCT10	Monocarboxylate transporter 10
MCT8	Monocarboxylate transporter 8
na	Not available
OATP1C1	Organic anion-transporting polypeptide 1C1
ONL	Outer nuclear layer
OPL	Outer plexiform layer
OS	Outer segments
PBS	Phosphate-buffered saline
PLAE	PLatform for Analysis of scEiad
RBPMs	RNA-binding protein with multiple splicing
RGC	Retinal ganglion cell
RPE	Retinal pigment epithelium
scEiaD	Single-cell Eye in a Disk
scRNA-seq	Single-cell RNA sequencing
<i>Slc16a10</i>	Solute Carrier Family 16 Member 10 (MCT10 encoding gene)
<i>Slc16a2</i>	Solute Carrier Family 16 Member 2 (MCT8 encoding gene)
<i>Slc7a5</i>	Solute Carrier Family 7 Member 5 (LAT1 encoding gene)
<i>Slco1c1</i>	Solute carrier organic anion transporter family member 1C1 (OATP1C1 encoding gene)
T2	3,5-diiodothyronine
T3	3,5,3'-triiodothyronine
T4	Thyroxine
TH	Thyroid hormone
THR	Thyroid hormone receptor
<i>Thra</i>	Thyroid hormone receptor alpha encoding gene
<i>Thrb</i>	Thyroid hormone receptor beta encoding gene
THRa	Thyroid hormone receptor alpha
THR β	Thyroid hormone receptor beta
TX	Tyrosine hydroxylase
WT	Wild type
β -gal	β -galactosidase

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12964-026-02913-0>.

Supplementary Material 1.

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of Neurological Diseases and Aging, Spanish National Research Council, Madrid, Spain; eyes from other Dio2 KO and from Dio3 KO mice were provided by Arturo Hernandez, of the Center for Molecular Medicine, Maine Health Institute for Research, Scarborough, Maine, USA.

Authors' contributions

AM, RA, and GC conceived and designed the study. AM, AC, EA, SC, and RA acquired and analyzed the data. SB, FF, FB, ML, GN, CP, MR, and RA interpreted the data. AM, RA, and GC drafted the manuscript. All Authors revised the manuscript and approved its final version.

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Data availability

The datasets analyzed during the current study (bioinformatic analysis) are available in: PLatform for Analysis of scEiad (PLAE) dataset (<https://plae.nei.nih.gov/>). All the other datasets generated, used, and analyzed during the current study are available from the corresponding authors on reasonable request.

Declarations

Ethics approval and consent to participate

All the animals used in these studies were treated in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and with the European Communities Council Directive (2010/63/UE). Animal procedures and organ collection were approved by the Italian Ministry of Health (decree number 256/2024-PR) and by the institutional animal care committee of the Technische Universität Dresden and the Landesdirektion Sachsen (TVT 03/2023), according to the 3Rs principles for ethical use of animals and ARRIVE guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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