Expression of the inactivating deiodinase, Deiodinase 3, in the pre-metamorphic tadpole retina

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Abstract

Thyroid hormone (TH) orchestrates amphibian metamorphosis. Thus, this developmental phase is often used to study TH-dependent responses in specific tissues. However, TH signaling appears early in development raising the question of the control of TH availability in specific cell types prior to metamorphosis. TH availability is under strict temporal and tissue-specific control by deiodinases. We examined the expression of the TH-inactivating enzyme, deiodinase type 3 (D3), during early retinal development. To this end we created a Xenopus laevis transgenic line expressing GFP from the Xenopus dio3 promoter region (p dio3) and followed p dio3–GFP expression in pre-metamorphic tadpoles. To validate retinal GFP expression in the transgenic line as a function of dio3 promoter activity, we used in situ hybridization to compare endogenous dio3 expression to reporter-driven GFP activity. Retinal expression of dio3 increased during pre-metamorphosis through stages NF41, 45 and 48. Both sets of results show dio3 to have cell-specific, dynamic expression in the pre-metamorphic retina. At stage NF48, dio3 expression co-localised with markers for photoreceptors, rods, Opsin-S cones and bipolar neurons. In contrast, in post-metamorphic juveniles dio3 expression was reduced and spatially confined to certain photoreceptors and amacrine cells. We compared dio3 expression at stages NF41 and NF48 with TH-dependent transcriptional responses using another transgenic reporter line: ThhbZIP-GFP and by analyzing the expression of T3-regulated genes in distinct TH availability contexts. At stage NF48, the majority of retinal cells expressing dio3 were negative for T3 signaling. Notably, most ganglion cells were virtually both dio3-free and T3-responsive. The results show that dio3 can reduce TH availability at the cellular scale. Further, a reduction in dio3 expression can trigger fine-tuned T3 action in cell-type specific maturation at the right time, as exemplified here in photoreceptor survival in the pre-metamorphic retina.
Introduction

Tissue specific availability of the two main forms of thyroid hormone, thyroxine (T4) and the most biologically active form, triiodothyronine (T3), orchestrates the complex developmental program of amphibian metamorphosis. As in other vertebrates, development of the central nervous system is highly T3-dependent [1]. This is the case for retinal development, [2, 3]. TH receptors (TR), TRa and TRb, have been detected in the retina of mice, chicken and the African clawed frog Xenopus laevis [4, 5]. We previously showed that disrupting thrb receptor during development impairs Xenopus retinal formation [4]. More specifically, inducing a mutation in Xenopus laevis thrb ligand binding domain causes defects in eye development in embryos [4]. These data suggested that TH could have tissue specific responses during early stages of eye development. However, nothing is known on the cell specific role of TH in amphibian eye development at pre-metamorphic stages and whether certain cell types would be particularly sensitive to excess T3.

Thyroid hormone availability in specific tissues is determined by reciprocal activity of activating and inactivating deiodinases. The main activating deiodinase is deiodinase 2 (D2), whereas deiodinase 3 (D3, encoded by dio3) is the principal inactivating enzyme in tissue. Deiodinases are essential regulators of TH levels in target cells, notably in the nervous system [6, 7, 8]. The central role of deiodinases in controlling TH availability is exemplified by control of retinal development in multiple vertebrate species [5, 9, 10, 11]. In particular, fine regulation of TH availability by activating and inactivating deiodinases ensures survival and maturation of cone photoreceptors in mice [12]. Deiodinase knockdown in zebrafish eye affects eye size, retinal lamination and strongly reduces the number of rods and cone cells [5]. This result is consistent with the lamination defect observed when impairing deiodinase activity with iopanoic acid in earlier pre-metamorphic Xenopus embryos [4]. When TH deiodination is impaired by iopanoic acid treatment, T3 treatment further enhances the deleterious eye phenotype, revealing a crucial role for thrb as aporeceptor and pinpointing that TH availability is tightly controlled [4] during eye development. D3 plays an essential protective role in inhibiting TH-induced proliferation in CMZ, resulting in an asymmetric growth in Xenopus laevis retina during metamorphosis [13, 11], or in protecting certain photoreceptors from excessive T3 signaling in the mouse and zebrafish retina [5, 12, 14]. These findings favor the hypothesis of a control of local TH availability mostly by the inactivating deiodinase D3.

The second question raised by this data set is the scale at which local TH availability is controlled, and more precisely, if the control occurs at the tissue level or cellular level. TH roles in proliferation and in the differentiation of cone photoreceptors are well known, but little is known about the protective role of D3 during retinal neurogenesis.

Furthermore, previous studies in Xenopus retina addressed the question of the regulation of the stem cells and progenitor cells proliferation [15] or the genesis of different cell types [16, 17, 18, 19] in early development. Other studies addressed the determination of cone and rod photoreceptors during embryonic stages NF33-41 [20, 21]. These studies raised the importance of local environment and extrinsic factors but did not address the role of TH.

As to other species, the role of TH on retinal development has been addressed during embryogenesis in rodents [10, 14], during pro-metamorphosis in fish [5] as well as during metamorphosis in Xenopus [4, 11].

As to deiodinase expression, different studies have shown the general pattern expression of dio3 during embryonic chicken brain development [3, 12] and increasing mRNA levels during zebrafish embryonic development [5, 9] and in brain between stages NF45 and NF48 in Xenopus tadpoles [8]. But, no studies have addressed the control of TH availability by D3 on retinal neurogenesis in Xenopus pre-metamorphic tadpoles.
To address the question of TH availability in specific retinal cell types, we used a number of complementary experimental approaches. First, we compared expression of dio3 using both in situ hybridization (ISH) and exploiting a pdio3-GFP construct, where GFP expression is controlled by part of the Xenopus dio3 promoter. Second, we compared dio3 ISH expression with immunohistochemistry for specific retinal cell types. Third, we determined which of these cells types responded to T3 signaling by using a well-established TH/bZIP-GFP reporter system [22, 23, 24]. Taken together, the results show that dio3 expression could limit T3-dependent responses in specific retinal cells at specific developmental stages.

Material and methods

1. Transgenic and wild type animals

X. laevis tadpoles and juveniles were obtained by in-house breeding. To obtain eggs, adult frogs were injected with human chorionic gonadotropin (Chorulon) (400 U/female and 200 U/male). Some adult frogs were bought to the CRB (Xenope Biology Resources Centre, Centre de Resource Biologie Xénopoe), France (University of Renéssis; http://xenopus.univ-rennes1.fr/). Animals were reared under a 12-hour light/12-hour dark cycle at 22–23°C. Animals were staged according to Nieuwkoop and Faber (NF) (1956). Juveniles were used when they had developed beyond NF66 and weighed between 1 and 2 g.

To determine T3 responsiveness in the retina, we used the X. laevis transgenic TH/bZIP reporter line [22, 23, 24]. The transgenic TH/bZIP reporter line contains a series of T3 sensitive response elements upstream of the GFP coding sequence providing a direct T3 readout. F1 founders were crossed together to produce F2 homozygous transgenic tadpoles.

All animal studies were carried out in accordance with the European Union regulations concerning the protection of experimental animals and approved by the Museum National d’Histoire Naturelle Animal Care and Use Committee, Paris, France. All procedures were approved by the institutional Ethics Committee (Animal Housing Agreement Number: C75-05-01-2, Committee Approval 68.031).

2. Generation of the Xenopus laevis deiodinase 3–GFP transgenic line

The X. laevis transgenic line deiodinase 3-GFP (pdio3-GFP) reporter was obtained by sperm nuclei-mediated REMI method transgenesis [25] using a dio3 promoter driven-GFP expressing construct. Injections were carried out so that the transgene was inserted into the genome prior to the first cleavage. The DNA plasmid construct used for this reporter line corresponds to a clone representing a part of the Xenopus tropicalis dio3 promoter locus and upstream regulatory elements. The DNA clone represents 1448 pb of the full Xt dio3 4915 pb promoter locus. The cDNA clone was from 454 pb of the 3’ end Xt dio3 promoter locus. The PCR product was initially obtained with the ExTaq TaKaRa kit (Ozyme, ref: TAKRR013A) from X. tropicalis tadpole tail genomic DNA extract. PCR primers were designed against the promoter dio3 locus. Forward and reverse primers were 5’ CGGGGAAGATATGGAAG and 5’ GGGCTCCCGGATGATCTGA 3’, respectively. The latest Xenopus tropicalis genome annotation, confirms the position of the clone in the promoter. This PCR product was sub-cloned into a pGlow-GFP vector (Invitrogen pGlowTOPO cloning kit ref: 45–0021) and verified by sequencing. F0 or F1 transgenic founders were crossed with wild type adult to produce F1 or F2 transgenic tadpoles that were used for the characterization experiments.
3. Tadpole treatment
The *X. laevis* transgenic (pdio3-GFP) reporter tadpoles NF48 were treated for 24 h, with $10^{-8}$ M T$_3$ (Sigma, St Quentin Fallavier, France), with $5.10^{-6}$ M IOP (TCI Europe, Zwijndrecht, Belgium) or with $10^{-8}$ M T$_3$ + $5.10^{-6}$ M IOP (diluted in 0.1% ethanol) [4]. Vehicle control group is used in 0.1% ethanol. The experiment was performed twice, with 5–8 animals per group.

4. Sample collection and preparation
Tadpoles and juveniles of both transgenic reporter lines were deeply anesthetized by submersion in 0.1% tricaine methanesulfonate anesthesia (MS-222, Sigma-Aldrich) and euthanized by decapitation. Whole heads of NF48 tadpoles and juveniles were fixed overnight at 4°C in 4% paraformaldehyde (PFA, Sigma-Aldrich, Saint Quentin Fallavier, France) in phosphate buffered saline (PBS pH 7.4). Samples were briefly washed three times (15 min.) in PBS and placed overnight in 15% sucrose PBS. Samples were embedded in Tissue-Tek and stored at -80°C. Coronal cryosections (16 micrometers) were performed on whole heads and analyzed on eyes. Sections were stored at -80°C prior to immunohistochemistry or in situ hybridization.

5. Immunohistochemistry for GFP and Opsin Blue, GFP and Rhodopsin, GFP and ChX10
Coronal whole heads cryosections were briefly rehydrated with phosphate buffer salt PBS (pH 7.4) and Tween 20 (0.1%), then post-fixed with 4% PFA for 10 min. Sections were washed in PBS-Tween 20 (0.1%) and blocked with 10% normal goat serum in PBS-Tween 20 (0.1%) (both from Sigma-Aldrich) to reduce non-specific binding. Sections were then incubated overnight at 4°C with primary antibody chicken polyclonal GFP (Abcam, Ab 13970) or rabbit polyclonal GFP (Invitrogen, ref: 11122) diluted 1:300 in PBS-Tween 20 (0.1%) containing 5% normal goat serum (NGS), according to the species in which the second primary antibody was generated. The slides were rinsed with fresh PBS-Tween 20 (0.1%) and incubated two hours at room temperature (RT) with secondary antibody (Alexa fluor 488 goat anti-rabbit, Invitrogen A-11034 Carlsbad, CA, USA) diluted 1:500 in PBS-5% normal goat serum-0.1% Tween 20. Then, sections were incubated with the second primary rabbit polyclonal antibody either Opsin Blue, a marker of Opsin S (Millipore AB 5407) diluted 1:250 or mouse monoclonal Rhodopsin (Sigma-Aldrich R 5403) diluted 1:500 or sheep polyclonal ChX10 (Exalpha XI180P) diluted 1:200 in PBS-Tween 20 (0.1%) containing 5% normal goat serum. Slides were rinsed with fresh PBS-Tween 20 (0.1%) and incubated two hours at RT with secondary antibody Alexa fluor 568 goat anti-rabbit, (Invitrogen A-11036) for Opsin-Blue or Alexa fluor 594 donkey anti-mouse, (Invitrogen A-21203) for Rhodopsin or Alexa 594 donkey anti-sheep IgG (Invitrogen A-11016) for ChX10 diluted 1:500 in PBS-Tween 20 (0.1%) with 5% NGS. Immunostained retinas were rinsed several times in PBS-Tween 20 (0.1%) and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen P-36931) and a cover slip.

6. Immunohistochemistry for GFP and GABA or GFP and Parvalbumin
Coronal cryosections were briefly rehydrated with phosphate buffer salt PBS (pH 7.4) and Triton X-100 (0.3%). Sections were blocked with 10% normal goat serum in PBS-Triton X-100 (0.3%) (both from Sigma-Aldrich) to reduce non-specific binding during 2 hours at room temperature. Sections were then incubated overnight at 4°C with primary antibody chicken polyclonal GFP (Abcam, Ab 13970) diluted 1:300 in PBS-Triton X-100 (0.3%) containing 5% normal goat serum (NGS). The slides were rinsed with fresh PBS-Tween 20 (0.1%) and incubated two hours at room temperature (RT) with secondary antibody (Alexa fluor 488 goat anti-rabbit,
anti-chicken, Invitrogen A-11039 Carlsbad, CA, USA) diluted 1:500 in PBS-5% normal goat serum-0.3% Triton X-100. Then, sections were incubated with the second primary rabbit polyclonal antibody GABA, gamma-Aminobutyric Acid (Immunostar 20094) diluted 1:1500 or mouse monoclonal Parvalbumin IgG1 (Millipore MAB1572) diluted 1:100 in PBS-Triton X-100 (0.3%) containing 5% normal goat serum. Slides were rinsed with fresh PBS-Tween 20 (0.1%) and incubated two hours at RT with secondary antibody Alexa fluor 647 goat anti-rabbit, (Invitrogen A-21245) for GABA or Alexa fluor 568 goat anti-mouse IgG1, (Invitrogen A-21124) for Parvalbumin diluted 1:500 in PBS-Triton X-100 (0.3%) with 5% NGS. Immunostained retinas were rinsed several times in PBS-Tween 20 (0.1%) and stained by DAPI (Sigma-Aldrich) for 7 min, rinsed several times in PBS-Tween 20 (0.1%), another time and mounted with Prolong Gold antifade reagent (Invitrogen P-36930) and a cover slip.

The immunostained retinas were visualized with a LEICA DM 5500 B microscope equipped with a LEICA CTR 5500 lens and for epifluorescence with a PRIOR Lumen 200 system. Fluorescent image acquisitions were carried out using a ZEISS LSM 710 system confocal microscope by channels or spectral mode acquisitions and the Zen 2011 software acquisition at the J. Monod Institute (ImagoSeine platform, Jussieu University, Paris, France).

7. In situ Hybridization (ISH)
Probes for dio3 cDNA were isolated by RT-PCR using a pool of RNA extracted from embryos and tadpoles at several stages. The PCR fragment for dio3 (296 bp, forward: TCGGTGCACAA TATGTCGGG and reverse: CTTCTGCCCCTGGAACAC) were cloned using the TOPO TA cloning kit dual promoter (Invitrogen, Carlsbad, CA, USA) and sequenced to check orientation. The dio3 mRNA probe was synthesized using T7 enzyme 25 (Roche, Basel, Switzerland) and labeled with digoxigenin (Roche, Basel, Switzerland). ISH was performed on cryosections. Slides were briefly rehydrated with PBS (pH 7.4) then post-fixed with 4% PFA during 10 min. Then slides were washed with PBS Tween 20 (0.1%). Tissues were permeated with proteinase k (5.10^{-6} g/ml, Sigma, ref. P4850) for two minutes. Slides were then post-fixed with 4% PFA during 10 min follow by wash with PBS Tween 20 (0.1%). To prevent RNA interaction with protein, slides were incubated with 100 mM Triethanolamine/0.25% anhydride acid acetic during 10 min. Slides were washed with PBS Tween 20 (0.1%) and then pre-hydrated with hybridization buffer (formamide 50%, SSC 5X, ARNt grade VI à 1 mg/ml, heparin 100.10^{-6} g/ml, Denhart’s 1X, Tween 20 (0.1%), CHAPS 10 mM 0.1%, EDTA 10 mM) during 1 hour. After denaturation dio3 probe was used at 1 ng/μl concentration and incubated on slides overnight at 60°C. Slides were washed several times with SSC buffer (saline sodium chloride citrate Sigma, ref.93017) at 65°C and then blocked with 10% NGS conjugated to alkaline phosphatase (1/2000, Roche) in 5% NGS/PBS/Tween 20 (0.1%). Slides were washed several times in maleic acid buffer. Signals were revealed using BM purple (Roche, ref.11442074001). Reactions were stopped by washes with PBS Tween 20 (0.1%). Slides were mounted with Prolong (Invitrogen, Carlsbad, CA, USA). ISH on retina sections was visualized using a LEICA DM 5500 B microscope equipped for visible light with LEICA CTR 5500. Image acquisition exploited a DFC 450 C Camera and the Leica Application suite LAS version 4.1.0 software acquisition.

8. RNA isolation and reverse transcription (RT)
RNA extraction was performed by RNA isolation from micro-scale, using RNAqueous Micro kit (Invitrogen AM 1931). For mRNA extraction from eyes, two steel balls were used for each sample in 100 microliters of lysis solution. Eyes were homogenized by using a tissue lyser at 30 Hz twice during 1 min. Ethanol 100% (50 microliters) was added and the lysate was placed on a micro-filter cartridge before centrifugation (16,400 x g, 20 sec.). The cartridge was rinsed
three times with a wash solution before RNA elution. A warm (75°C) RNA elution solution (18 microliters) was used before centrifugation (twice 16,400 x g, 30 sec. at room temperature). A DNase was used (22 min. at 37°C) and an inactivation reagent was added before centrifugation (16,400 x g, 2 min.). Concentrations of RNA were determined by using a NanoDrop (ThermoScientific, Rockford, IL). RNAs were stored in TRIS 10 mM /EDTA 0.1 mM (PH 7.4) at -80°C. Extracted total RNA (300 ng) was used for reverse transcription (RT) using a High Capacity cDNA RT kit (Applied Biosystems, Foster City, CA) with the addition of RNase inhibitor. A control for genomic DNA (RT- reaction: all reagents and RNA except reverse transcriptase) was performed for each group (Ctrl, T₃, IOP, IOP+T₃).

9. Real-time PCR quantification
The amount of each RNA transcript was estimated by relative quantitative real-time PCR (qRT-PCR) using Power SYBR green master mix and a Quant Studio Flex 6 (Applied Biosystems). 5 to 8 biological replicates were performed for each group. A 1:10 dilution of each cDNA was run in triplicate on a 384-well plate for each primer pair (Table 1) (intra assay variability) by using thermal cycling parameters: 95°C for 10 min, 95°C for 15 sec and 60°C for 1 min (40 cycles) and an additional step for dissociation curves was performed for all plates. Results were normalized with the expression of reference gene odc. DDCT method was used to estimate fold change of expression when compared to the untreated (CTL) group. PCR primer sequences are designed previously [26] for the housekeeping gene odc. Primers list is provided in Table 1.

Statistical analysis
For multiple comparison analysis, Non-parametric ANOVA was performed, followed by a Kruskall Wallis test (PRISM7). Heatmap was performed on Fold Changes (PRISM7), and correlation analysis was performed on XLSTAT.

Results
Dynamic expression of retinal dio3 in the pre-metamorphic Xenopus laevis retina
To address the role of dio3 in pre-metamorphic retina, we cloned part of the X. tropicalis dio3 promoter and generated a transgenic pdo3-GFP reporter line. GFP immunostaining was used to follow pdo3-reporter GFP expression. To validate the dio3 transgenic GFP reporter signal in the retina, we compared it to the endogenous signal obtained with ISH using antisense

| Gene | Forward primer | Reverse primer |
|------|----------------|----------------|
| eGFP | 5’ ACA GGA TGA GGA TGG TCT TTT CG 3’ | 5’ TGT CTG TTG CCA GTC AT 3’ |
| dio3 | 5’ CAC AAA AAG TGC GAC CAA ACG 3’ | 5’ GCC TTG TTG CAG TTT ACT 3’ |
| thrb | 5’ ATA GTT AAT GCG CCC GAG GGT GGA 3’ | 5’ CTT TTC TAT TCT CTC TCC AGC CTA GC 3’ |
| thibz | 5’ GCC GTC TCC GTG CTG AAG T 3’ | 5’ GGT CAC GTA CCA GGC CAA A 3’ |
| klf9 | 5’ TGT GGC AAA GGG TAT GGG AAG TCT 3’ | 5’ GGC GTP CAC CTG TAT GGA CTC T 3’ |
| odc | 5’ GCT TCT GGA GGC AAG GGA 3’ | 5’ CCA AGG TCA GCC CCC ATG TCA 3’ |

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probes against dio3 (Fig 1A–1F). Expression of dio3 (Fig 1A and 1B) and pdio3-reporter GFP
(Fig 1D, 1E, 1H and 1I) were examined by ISH on head cryo-sections in tadpoles at stages NF45 and NF48 and in the froglet at stage NF66 (Fig 1C and 1F).

At stage NF45, both the dio3 ISH signal and GFP expression were limited to a very few ganglion cells and in a subset of the photoreceptors of the outer nuclear layer, as judged by cell morphology and anatomical localization (Fig 1A and 1D). At stage NF48, both the dio3 ISH signal and GFP expression showed higher intensity and a wider distribution, being present in all retinal cell types: ganglion cells, inner nuclear layer cells (bipolar neurons, horizontal neurons and amacrine cells) and in the outer nuclear layer cells (basal part of the photoreceptors) (Fig 1B, 1E, 1H and 1I). Cell morphologies were determined according to Wong et al. [27]. At stage NF66 corresponding to a post-metamorphic juvenile stage, dio3 expression was more restricted and localized in the outer nuclear layer and in the inner nuclear layer. More specifically, both ISH and GFP immunocytochemistry showed dio3 expression in certain photoreceptor types and in Amacrine cells (Fig 1C and 1F).

D3 expression generally corresponds to lack of T3 responsiveness

To determine which retinal cells express D3 and those that respond to T3, we compared the GFP expression in F2 tadpoles for two transgenic reporter lines, respectively p dio3-GFP and TH/bZIP-GFP. Retinal GFP immuno-labeling was examined on 16 micrometers coronal whole head cryo-sections at stage NF 41–42 (Fig 2) and NF48 (Fig 1E, 1H, 1I and 1G) (see Materials and Methods).

At stage NF41–42 D3 activity, as judged by GFP expression in p dio3-GFP tadpoles, is observed in numerous photoreceptors of the outer nuclear layer (yellow arrow heads, Fig 2A, 2C, 2E and 2F and Table 2) and in some bipolar neurons (blue arrow heads, Fig 2A, 2C, 2E and 2F and Table 2), whereas no T3-response is detected in photoreceptors nor in bipolar neurons of the inner nuclear layer (INL) (Fig 2G, 2I, 2J and 2L and Table 2). Furthermore, the photoreceptors that are GFP positive in p dio3-GFP tadpoles are in the dorso-central area (Fig 2A).

The horizontal neurons (magenta arrow heads) of the outer plexiform layer that are GABA positive cells (Fig 2B and 2H) are also GFP positive in p dio3-GFP (Fig 2C and 2F and Table 2) and in TH/bZIP reporter tadpoles (Fig 2I, Table 2). Similarly, numerous amacrine cells (white arrow heads) in the inner nuclear layer, that are GABA/PARV positive cells (Fig 2B, 2D, 2E and 2K) are GFP positive in p dio3-GFP (Fig 2C, 2E and 2F and Table 2) and in TH/bZIP-GFP lines (Fig 2I and 2L and Table 2). However, they are less numerous in the TH/bZIP reporters. A ganglion cells subset (white asterisk) is Parvalbumine PARV positive (Fig 2D) in the ganglion cell layer and some of them are also GFP positive, in p dio3-GFP tadpoles (Fig 2E and 2F and Table 2). In TH/bZIP-GFP tadpoles, GFP expression is seen in a large subset of ganglion cells (Fig 2K and 2L and Table 2).

At stage NF48, widespread GFP expression was observed in p dio3 tadpoles (Fig 1, 1E, 1H and 11), a fact that was reflected by limited T3 responses (lower TH/bZIP-driven GFP expression) notably in retinal ganglion cells, noted as “*” in Fig 1 and horizontal neurons (outer plexiform layer), noted as “+” (Fig 1G, Table 2). The p dio3-GFP expression displayed a strong dorso-ventral gradient (Fig 1E), which contrasted with the higher levels of TH/bZIP-driven GFP in the ventral area, mostly for the outer plexiform layer (Fig 1G). Thus, higher D3 levels appear to restrict T3-responsiveness in the dorsal retina at this stage.

Furthermore, at this stage, no TH/bZIP-driven GFP signal was seen in any cell type in the inner nuclear layer as in bipolar neurons or amacrine cells (Fig 1G, Table 2), which corresponded to the high expression of dio3-driven GFP at the same stage (Fig 1E, 1H and 11); note the magenta arrow indicating a bipolar neuron in Fig 1E and 11 and the white arrow indicating an amacrine cell in Fig 1E and 1H).
Fig 2. Comparison of p dio3-GFP and TH/bZIP-GFP expression pattern in NF41–42 reporter tadpoles. Fig 2A–2F: p dio3-GFP transgenic reporter tadpoles, Fig 2G–2L: TH/bZIP-GFP (T3 sensor) reporter tadpoles. Fig 2A–2I: lateral plane sections. Fig 2J–2L: median plane section. Fig 2A, 2G and 2J: DAPI/GFP co-staining, Fig 2B and 2H: GABA labeling in horizontal neurons (magenta arrow heads) and amacrine cells (white arrow heads). Fig 2C and 2I: GFP/GABA co-labeling in horizontal neurons (magenta arrow heads) and amacrine cells (white arrow heads). Fig 2Dand 2K: Parvalbumin PARV labeling in amacrine cells (white arrow head) and ganglion cells (asterisks). Fig 2E and 2L:
Similarly, at stage NF48, the outer plexiform layer was GFP positive in both transgenic lines, reflecting a certain level of T<sub>3</sub> responsiveness despite discernable Dio3 activity. Indeed, some interneurons (horizontal cells, noted as “+”) respond to T<sub>3</sub> (Fig 1G, Table 2) and yet display limited Dio3 expression (Fig 1E and 1I and Table 2).

At stage NF48, most ganglion cells, (noted as “”) did not express Dio3 and therefore could respond to T<sub>3</sub> as confirmed by their positive TH/bZIP response (Fig 1G, Table 2). As expected, most photoreceptors express Dio3 (noted as “white head arrow in Fig 1E and 1I) and do not respond to T<sub>3</sub> (Fig 1G, Table 2). These results suggest that Dio3 expression limits T<sub>3</sub> responsiveness in specific cells too.

**Opsin S-expressing cones, rods and bipolar neurons express pDio3-GFP at stage NF48**

To determine more specifically in which retinal cell types pDio3-GFP expression was found we used double immunochemistry with established markers of each retinal cell type (see Materials and Methods). At stage NF48, pDio3-driven GFP expression was found to co-localize with markers of a large proportion of cone cells expressing Opsin Blue (Opsin S), noted by a magenta arrow in (Fig 3A, 3B and 3C), certain bipolar neurons (ChX10), noted by white head arrows (Fig 3D and 3H) and with all Rhodopsin positive rods, noted by white arrows (Fig 3E–3G).

Thus, as shown in Fig 3A, at stage NF48 GFP expression (green) was found in all retinal cell types in all layers, except in the ganglion cells layer where GFP expression is localized in few ganglion cells. In the outer nuclear layer, it only co-localizes with the body of the Opsin S cones (Fig 3A, in red). Co-localization of pDio3-GFP with several Opsin S cone body was observed and shown with magenta arrow in Fig 3B and 3C (zoom) (in red, with magenta arrow).

Another section revealed co-localization of GFP with Rhodopsin (in red), a specific marker of red rods (Fig 3E–3G for different magnifications). These results suggest that at stage NF48, Dio3 expression limits T<sub>3</sub> availability, thereby controlling Opsin S cones number and rods survival.

**Dio3 expression prevents induction of T<sub>3</sub>-responsive genes**

In order to determine whether Dio3 expression contributes to local control of TH availability in the developing eye, we treated NF48 pDio3-reporter tadpoles with T<sub>3</sub>, IOP or IOP+T<sub>3</sub> (see Materials and Methods for details) and analyzed T<sub>3</sub> target genes expression (Table 1). A highly significant increase (p<0.001) in Dio3 expression was seen between IOP and IOP+T<sub>3</sub>, but not between CTL and T<sub>3</sub> treated groups (Fig 4A). This difference in T<sub>3</sub> responsiveness can be interpreted in...
the light of the sensitivity of both activating and inactivating deiodinases to IOP [4] (Fig 4B). In the presence of $T_3$, despite the presence of IOP, we observed an increased expression of the canonical $T_3$-responsive genes $kfl9$ and $thibz$. Of note, IOP treatment itself, induces no significant change (as compared to controls) in $GFP$, $dio3$ and $T_3$ target gene expression (Fig 4A). This result indicates that the local $T_4$ to $T_3$ conversion by D2 is insufficient to trigger $T_3$ target gene expression in the retina as a whole. In IOP+$T_3$ treated group, $dio3$, $kfl9$, $thibz$ and $thrb$ expression increases when compared to control group, showing that the cells expressing these genes are $T_3$-
responsive. When comparing dio3 and GFP expression (all observations pooled), a significant correlation ($p = 0.007$) is seen between dio3 and GFP transcript levels ($r = 0.525$) (Fig 4C). This observation strengthens the argument that GFP can be used as an indicator of dio3 expression levels.

**Discussion**

Several studies have shed light on the role of deiodinases in determining sensory organ development in vertebrates. This is especially the case in the eye, where TH-related development is
controlled by the timing of Dio3 expression in mouse retina [12, 28] and dio3 in Zebrafish eye [5], as well as in Xenopus retina during metamorphosis [11].

In mouse retina, Dio3 is expressed in immature mouse retina. Ng et al. [12] showed that Dio3-/- mice lost 80% of cones through neonatal cell death. Their results suggest that Dio3 expression limits hormonal exposure of the cones ensuring cone survival and opsin (S and M) patterning, required for cone adaptive function during development. In zebrafish, knockdown of dio3 by morpholinos causes reduced eye size and a strong reduction in rods and all four cones types. This result suggests the importance of dio3 as a central player for zebrafish eye development. However, the roles of dio3 on the survival and the patterning of Opsin type photoreceptors have not yet been addressed in Xenopus retina development, notably during pre-metamorphic stages. What is more, in Xenopus, there were no data on TH availability nor on the role of deiodinase 3 expression in other retinal cell types.

Our experiments were designed to address these questions: how TH availability is controlled, and more precisely, if the control occurs at the tissue or cellular level and in which cells.

In the mouse retina, TH availability appears to control survival and patterning of specific retinal cells. Notably, a TH gradient is observed that may play a role in establishing the gradient of M-opsin [29]. In contrast, in Xenopus retina, there is a spatial gradient of dio3 during metamorphosis, with a higher level in the dorsal retina [11]. These authors demonstrated that the dorsal ciliary margin zone (CMZ) cells are resistant to exogenous TH at stage 50–54, but they noted that an increase in proliferation of these cells was induced with a low concentration of T3 when D3 activity was inhibited. In contrast, D3 overexpression inhibited TH-induced proliferation of the ventral CMZ cells [11]. This localized expression of dio3 in the dorsal CMZ leads to the asymmetric growth of the frog retina, but the question of the role of dio3 expression in the eyes of pre-metamorphic tadpoles was not addressed. Interestingly, in pre-metamorphic tadpoles, maximal dio3 mRNA levels are found in whole tadpoles at NF46-NF48 stages [22], crucial stages before the first pro-metamorphosis stage NF 53, where generalized competence to respond to T3 is observed [30].

Our study shows a specific and dynamic spatio-temporal pattern for dio3 transcripts and dio3 promoter activity in the retina of pre-metamorphic Xenopus tadpoles and post-metamorphic juveniles. The results suggest that the timing of retinal maturation in pre-metamorphic tadpoles is mostly controlled by local, cell-specific D3 activity following modulations between stages NF 41 and NF48. Moreover, D3 activity follows a dorso-ventral gradient at NF48. And the differential-T3 responsiveness of retinal cells in our TH sensor model strongly suggests that some photoreceptors and bipolar neurons are more specifically protected from TH-driven maturation at stages NF41 and NF48. Likewise, the same result is observed for amacrine cells at NF48. Our results suggest too that more ventral horizontal neurons and more ganglion cells needed an active TH signaling between NF41 and NF48 (Fig 5).

Therefore, we show that in the pre-metamorphic retina, TH availability is not controlled at the tissue level but at the cellular level by D3, in most of the cell types in each retinal layer. In particular, D3 is expressed in photoreceptors and bipolar neurons at NF41 and NF48 whereas at NF45, only a photoreceptors subset expressed D3. At stage NF48, D3 is expressed specifically in Opsin S cones, rods and bipolar neurons (Fig 5).

The more restricted expression of D3 at stage NF45 could allow the recruitment and the maturation of more cell types of the inner nuclear layer and more pre-existing photoreceptors types. In parallel, maternal stocks of TH decrease at this stage [22]. Then, as the tadpole thyroid gland is formed, T4 levels increase at NF46. So, we can speculate that the small subset of photoreceptors expressing D3 at NF45 may correspond to a subtype protected from TH maturation, just before TH levels increase.
At stage NF48, the higher D3 expression observed in most photoreceptors and in cells of the inner nuclear cell layer, could be related to the fact that retina need to be protected from the step increase in TH levels that will initiate pro-metamorphosis at NF54 [31]. Pro-metamorphosis is a step where TH signaling is active and T₃-dependent tissue remodeling starts [30], illustrated by the onset of green rod differentiation in retina [21]. This result is in accordance with previous studies showing a retinal photoreceptors sensitivity to TH excess during retina development in several species. In vertebrates, TH and TRs are involved in opsin expression and cone development [10, 28, 32, 34, 35]. Furthermore, TH can modulate the M-opsin/S-opsin ratio in the developing mouse retina [29]. Moreover, in trout, excess of TH during smoltification results in a loss of UVS cones [34, 36, 37, 38]. Likewise, another study shows that systemic TH may induce retinal remodelling in juvenile rainbow trout [39].

By analyzing dio3 and T₃-target genes transcriptional regulation by T₃ in the presence or absence of a deiodinase inhibitor (IOP), we show that dio3 plays a pivotal role in controlling local T₃ availability in dio3 expressing cells. No effect of exogenous T₃ is observed when
Deiodinases are not inactivated by IOP. The fact that expression of T₃-responsive genes increases when exogenous T₃ is added in the absence of IOP reflects the fact that these genes must be expressed in cells devoid of dio3, such as in the ventral zone.

A final point is that the p dio3-GFP reporter tadpoles are a useful tool for following dio3 expressing cells and for displaying the regulation of TH availability by D3 at the cellular scale. Moreover, the expression level of dio3 and the reporter gene are reasonably well correlated, despite a certain variability due to the very dynamic D3 expression at specific stages, a fact that could be compounded by the difficulty of obtaining homogeneous batches of tadpoles. However, even if dio3 is transcriptionally responsive to T₃, its range of T₃-responsiveness is far below that of the TH/bZIP. As such the TH/bZIP reporter remains the best T₃-sensor available to date in xenopus.

Conclusions

Our findings show that dio3 displays a dynamic and cell-specific expression in the pre-metamorphic retina between NF41 and NF48. At stage NF41 and NF48 most cells types express dio3, with a particularly high expression in rods and the S cones at NF48 whereas T₃ signaling is detectable in horizontal neurons and ganglion cells (Fig 5). After metamorphosis, dio3 expression was much more limited, only being found in amacrine cells and a sub-population of photoreceptors. The results show that dio3 plays a key role in determining TH availability during retinal development with a precise and cell-type specific timing. We suggest that D3 expression could be necessary to protect photoreceptors and bipolar neurons from out of phase TH signaling and hence that dio3 expression constrains effects of TH signaling in the retina prior to and during metamorphosis.

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References

1. Bernal J, Guadáño-Ferraz A, Morte B. Perspectives in the study of thyroid hormone action on brain development and function. Thyroid. 2003; 13(11):1005–12. https://doi.org/10.1089/105072503770867174 PMID: 14651784.

2. Harpavat S, Cepko CL. Thyroid hormone and retinal development: an emerging field. Thyroid. 2003; 13 (11):1013–9. https://doi.org/10.1089/105072503770867183 PMID: 14651785.

3. Ng L, Hurley JB, Dierks B, Srinivas M, Saltó C, Vennström B et al. A thyroid hormone receptor that is required for the development of green cone photoreceptors. Nat Genet. 2001; 27(1):94–8. https://doi.org/10.1038/sj.commands.1901356 PMID: 11138006.

4. Havis E, Le Mevel S, Morvan Dubois G, Shi DL, Scanlan TS, Demeneix BA et al. Unliganded thyroid hormone receptor is essential for Xenopus laevis eye development. EMBO J. 2006; 25(20):4943–51. Epub 2006/09/28. https://doi.org/10.1038/sj.emboj.7601356 PMID: 17006540; PubMed Central PMCID: PMCPMC1618110.

5. Houbrechts AM, Vergauwen L, Bagci E, Van Houcke J, Heijlen M, Kulemeka B et al. Deiodinase knock-down affects zebrafish eye development at the level of gene expression, morphology and function. Mol Cell Endocrinol. 2016; 424:81–93. Epub 2016/01/21. https://doi.org/10.1038/sj.mce.2016.01.018 PMID: 26802877.

6. Darras VM, Mol KA, van der Geyten, Kühn ER. Control of peripheral thyroid hormone levels by activating and inactivating deiodinases. Ann N Y Acad Sci. 1998; 898:93–6. PMID: 9629134.

7. Nunez J, Cell FS, Ng L, Forrest D. Multigenic control of thyroid hormone functions in the nervous system. Mol Cell Endocrinol. 2008; 287(1–2):1–12. Epub 2008/03/25. https://doi.org/10.1016/j.mce.2008.03.006 PMID: 18448240; PubMed Central PMCID: PMCPMC2466296.

8. Préau L, Le Blay K, Saint Paul E, Morvan-Dubois G, Demeneix BA. Differential thyroid hormone sensitivity of fast cycling progenitors in the neurogenic niches of tadpoles and juvenile frogs. Mol Cell Endocrinol. 2016; 420:138–51. Epub 2015/11/26. https://doi.org/10.1016/j.mce.2015.11.026 PMID: 26628940.

9. Houbrechts AM, Delarue J, Gabriëls LJ, Sourbrun J, Darras VM. Permanent Deiodinase Type 2 Deficiency Strongly Perturbs Zebrafish Development, Growth, and Fertility. Endocrinology. 2016; 157 (9):3668–81. Epub 2016/07/20. https://doi.org/10.1210/en.2016-1077 PMID: 27580812.

10. Kelley MW, Turner JK, Reh TA. Ligands of steroid/thyroid receptors induce cone photoreceptors in vertebrate retina. Development. 1995; 121(11):3777–85. PMID: 8982867.

11. Marsh-Armstrong N, Huang H, Remo BF, Liu TT, Brown DD. Asymmetric growth and development of the Xenopus laevis retina during metamorphosis is controlled by type III deiodinase. Neuron. 1999; 24 (4):871–8. PMID: 10624950.

12. Ng L, Lyubarsky A, Nikonov SS, Ma M, Srinivas M, Kefas Bet al. Type 3 deiodinase, a thyroid-hormone-inactivating enzyme, controls survival and maturation of cone photoreceptors. J Neurosci. 2010; 30 (9):3347–57. https://doi.org/10.1523/JNEUROSCI.5267-09.2010 PMID: 20203194; PubMed Central PMCID: PMCPMC2843520.

13. Brown DD. The role of deiodinases in amphibian metamorphosis. Thyroid. 2005; 15(8):815–21. https://doi.org/10.1089/thy.2005.15.815 PMID: 16131324.

14. Ma H, Ding XQ. Thyroid Hormone Signaling and Cone Photoreceptor Viability. Adv Exp Med Biol. 2016; 854:613–8. https://doi.org/10.1007/978-3-319-17121-0_81 PMID: 26427466.
15. Bilitou A. and Ohnuma S. The role of cell cycle in retina development: Cyclin-dependent kinase inhibitors co-ordinate cell cycle inhibition, cell-fate determination and differentiation in the developing retina. Developmental Dynamics 2010 Mar; 239:727–736. https://doi.org/10.1002/dvdy.22223 PMID: 20108332

16. Álvarez-Hemán G, Bejarano-Escobar R, Morona R, González A, Martín-Partido G, Francisco-Morcillo J. Islet-1 immunoreactivity in the developing retina of Xenopus laevis. Hindawi Publishing Corporation The ScientificWorld Journal Volume 2013, Article ID 740420. https://doi.org/10.1155/2013/740420

17. Bessodes N, Parain K, Bronchain O, Bellefroid EJ, Perron M. Prdm13 forms a feedback loop with Ptf1a and is required for glycinergic amacrine cell genesis in the Xenopus Retina. Neural Development (2017) 12:16. https://doi.org/10.1186/s13064-017-0093-2 PMID: 28863786

18. D’Autilia S, Decembrini S, Casarosa S, He RQ, Barsacchi G, Cremisi F et al. Cloning and developmental expression of the Xenopus homeobox gene Xvhsx1. Dev Genes Evol (2006) 216:829–834. https://doi.org/10.1007/s00427-006-0109-0 PMID: 17103185

19. Dullin JP, Locker M, Robach M, Henningfeld KA, Parain K, Afelik S et al. Ptf1a triggers GABAergic neuronal cell fates in the retina. BMC Developmental Biology BMC Developmental Biology 2007, 7:110. https://doi.org/10.1186/1471-213X-7-110 PMID: 17910758

20. Chang WS, Harris WA. Sequential genesis and determination of cone and rod photoreceptors in Xenopus. J Neurobiol. 1998 Jun; 35(3):227–44. PMID: 9622007

21. Parker RO, Mccarragher B, Crouch R, Darden AG. Photoreceptor development in premetamorphic and metamorphic Xenopus laevis. Anat Rec (Hoboken) 2010 Mar; 293(3):383–7. https://doi.org/10.1002/ar.21079 PMID: 20091886

22. Turque N, Palmer K, Le Mélèse S, Alliot C, Demeneix BA. A rapid, physiologic protocol for testing transcriptional effects of thyroid-disrupting agents in premetamorphic Xenopus tadpoles. Environ Health Perspect. 2005; 113(11):1588–93. https://doi.org/10.1289/ehp.7992 PMID: 16263516; PubMed Central PMCID: PMCPMC1310823.

23. Cabochette P, Vega-Lopez G, Bitard J, Parain K, Chemouny R, Masson C et al. YAP controls retinal stem cell DNA replication timing and genomic stability. eLIFE. 2015; 4:e08488. https://doi.org/10.7554/eLife.08488 PMID: 26393999

24. Wong LL, Rapaport DH. Defining retinal progenitor cell competence in Xenopus laevis by clonal analysis. Development. 2009; 136(10):1707–15. https://doi.org/10.1242/dev.027607 PMID: 19395642; PubMed Central PMCID: PMC2673759.

25. Costa PN, Santiago LA, Machado DS, Rocha FA, Ventura DF et al. Thyroid hormone action is required for normal cone opsin expression during mouse retinal development. Invest Ophthalmol Vis Sci. 2008; 49(5):2039–45. https://doi.org/10.1167/iovs.07-0908 PMID: 18436843.

26. Tata JR. Amphibian metamorphosis as a model for the developmental actions of thyroid hormone. Mol Cell Endocrinol. 2006; 246(1–2):10–20. Epub 2006/01/04. https://doi.org/10.1016/j.mce.2005.11.024 PMID: 16413959.

27. Leloup J., Buscaglia M., 1977. La triiodothyronine, hormone de la metamorphose des amphibiens. C. R. Acad. Sci. 84, 2261–2263.

28. Cossette SM, Drysdale TA. Early expression of thyroid hormone receptor beta and retinoid X receptor gamma in the Xenopus embryo. Differentiation. 2004; 72(5):239–49. https://doi.org/10.1111/j.1432-0436.2004.02705006.x PMID: 15270780.
35. Sjöberg M, Vennström B, Forrest D. Thyroid hormone receptors in chick retinal development: differential expression of mRNAs for alpha and N-terminal variant beta receptors. Development. 1992; 114(1):39–47. PMID: 1576965.

36. Allison WT, Dann SG, Veldhoen KM, Hawryshyn CW. Degeneration and regeneration of ultraviolet cone photoreceptors during development in rainbow trout. J Comp Neurol. 2006; 499(5):702–15. https://doi.org/10.1002/cne.21164 PMID: 17048226.

37. Browman HI, Hawryshyn CW. Thyroxine induces a precocial loss of ultraviolet photosensitivity in rainbow trout (Oncorhynchus mykiss, Teleostei). Vision Res. 1992; 32(12):2303–12. PMID: 1288007.

38. Browman HI, Hawryshyn CW. The developmental trajectory of ultraviolet photosensitivity in rainbow trout is altered by thyroxine. Vision Res. 1994; 34(11): 1397–406. PMID: 8023449.

39. Raine JC, Coffin AB, Hawryshyn CW. Systemic thyroid hormone is necessary and sufficient to induce ultraviolet-sensitive cone loss in the juvenile rainbow trout retina. J Exp Biol. 2010; 213(3):493–501. https://doi.org/10.1242/jeb.036301 PMID: 20086135.