Decreased thyroid hormone signaling accelerates the reinnervation of the optic tectum following optic nerve crush in adult zebrafish

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ABSTRACT

The regenerative capacity of the adult mammalian central nervous system (CNS) is poor and finding ways to stimulate long distance axonal regeneration in humans remains a challenge for neuroscientists. Thyroid hormones, well known for their key function in CNS development and maturation, more recently also emerged as molecules influencing regeneration. While several studies investigated their influence on peripheral nerve regeneration, in vivo studies on their role in adult CNS regeneration remain scarce. We therefore investigated the effect of lowering T3 signaling on the regeneration of the optic nerve (ON) following crush in zebrafish, a species where full recovery occurs spontaneously. Adult zebrafish were exposed to iopanoic acid (IOP), which lowered intracellular 3,5,3′-triiodothyronine (T3) availability, or to the thyroid hormone receptor β antagonist methylsulfonylnitrobenzoate (C1). Both treatments accelerated optic tectum (OT) reinnervation. At 7 days post injury (7 dpi) there was a clear increase in the biocytin labeled area in the OT following anterograde tracing as well as an increased immunostaining of Gap43, a protein expressed in outgrowing axons. This effect was attenuated by T3 supplementation to IOP-treated fish. ON crush induced very limited cell death and proliferation at the level of the retina in control, IOP- and C1-treated fish. The treatments also had no effect on the mRNA upregulation of the regeneration markers gap43, tub1a, and socs3b at the level of the retina at 4 and 7 dpi. We did, however, find a correlation between the accelerated OT reinnervation and a more rapid resolution of microglia/macrophages in the ON and the OT of IOP-treated fish. Taken together these data indicate that lowering T3 signaling accelerates OT reinnervation following ON crush in zebrafish and that this is accompanied by a more rapid resolution of the inflammatory response.

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1. Introduction

In adult mammals the regenerative inefficiency of the central nervous system (CNS) typically results in permanent loss of function and disability following trauma. In contrast, lower vertebrates such as fish and amphibians recover remarkably well from CNS injury, a property which makes them useful to investigate the mechanisms underlying successful CNS regeneration. One typical model used in the study of CNS regeneration is optic nerve (ON) injury. In humans/mammals the failure of ON regeneration is thought to be due to a combination of factors, including apoptosis of retinal ganglion cells (RGCs), reduced intrinsic ability for axonal regrowth and the inhibitory environment of the glial scar and myelin debris (Fischer and Leibinger, 2012). Studies on successful regeneration of the ON in fish have been very helpful to increase our knowledge about the process and its underlying molecular mechanisms. Indeed, several molecules, such as cFXIII, retinal transglutaminase (Sugitani et al., 2014), purpurin and retinol (Matsukawa et al., 2004), Jak/Stat (Elsaedi et al., 2014), reggie-1 and -2 (Schulte et al., 1997; Munderloh et al., 2009) were identified to promote ON regeneration in fish and modulation or combination of these factors in rodent models seems to at least partially overcome the block for mammalian ON regeneration (Fischer and Leibinger, 2012). As an example, reggie-1 fails to increase in rat RGCs following injury but experimental upregulation of this molecule induced axon extension (Koch et al., 2013). Another factor that is known to influence nervous system regeneration but has received little or no attention in the ON injury model so far is thyroid hormone (TH). It is an interesting candidate because it not only affects CNS development and maturation in all vertebrates (Bernal, 2005; Ahmed et al., 2008) but is also actively involved in nervous system remodeling during metamorphosis in fish and amphibians, including new RGC axonal outgrowth (Denver, 1998; Nakagawa et al., 2000; Raine et al., 2010). Many studies have shown that TH supplementation can promote the survival of neurons and glia, their proliferation to replace the lost cells, axon extension, remyelination and functional recovery after injury (recently reviewed by Bhumika and Darras, 2014). However, there are also several studies where exogenous TH failed to improve regeneration in either peripheral nervous system.
Different methods have been described to induce ON injury in zebrafish (Liu and Londraville, 2003; Zou et al., 2013). We used the optic nerve crush (ONC) model (Becker et al., 2000) and manipulated TH action. This was done by blocking the peripheral production of T3, the most active form of TH, or by using a TH receptor (TR) antagonist. We studied the impact of these treatments on axonal regeneration at the level of the retina, the ON and the optic tectum (OT). Our study focused on two possible sites of interaction. The first one is the retina where according to literature data in other models (Schenker et al., 2003; Fernandez et al., 2004; Desouza et al., 2005; Moore et al., 2009; Shulga et al., 2009) an interaction of TH with RGC cell survival, proliferation and changes in the expression of regeneration associated genes might occur. Another important site susceptible to interaction with TH is the ON where inflammation occurs. Injury in the CNS leads to the activation of microglia and the infiltration of blood borne macrophages (Perry and Gordon, 1988; Neumann et al., 2009). These cells elicit an inflammatory response together with astrocytes, which can impact the process of regeneration either favorably or adversely (Stoll et al., 2002). TH status has been shown to modulate the availability of specific subpopulations of macrophages, the phagocytic activity of macrophages and the rate of debris clearance around neuromuscular junctions in rodents but data seem to be contradictory (McsaC and Kiernan, 1975; Rosa et al., 1995; Perrotta et al., 2014). Besides these reports, clear evidence for an effect of TH on inflammation during nervous system regeneration remains elusive.

2. Experimental procedures

2.1. Animals

Adult (5 months old) zebrafish (Danio rerio) of the AB strain were used in all experiments. Fish were maintained at 28 °C under a 14/10 h light/dark cycle and fed ad libitum once daily with dry food (Dr. Bassleer Biofood, Telgte Germany). For all drug treatment experiments, 5 fish were placed together in small plastic tanks with 600 ml of system water with or without drug and all treatments were done at least twice. All animal experiments were approved by the Ethical Committee of the KU Leuven and performed in strict accordance with the European Council Directive (2010/63/EC).

2.2. Optic nerve crush

Optic nerve injury was induced by optic nerve crush (ONC) as described before (Becker et al., 2000). Briefly, fish were anesthetized in a solution of 0.03% of ethyl 3-aminobenzoate methanesulfonate (MS-222; Sigma, St. Louis, MO) and positioned appropriately under a dissection microscope (Zeiss Stemi 2000 Binocular stereo). The left eye was gently lifted by removing the surrounding connective tissue. The exposed ON was crushed using forceps (Dumont no. 5, F.S.T., Switzerland) for 10 s until a clear gap appeared inside the translucent nerve sheath, ensuring a complete crush. Care was taken not to damage the ophthalmic artery running close to the nerve. The eye was placed back in the socket. The right eye was sham operated by performing the same procedure. The exposed ON was crushed using forceps (Dumont no. 5, F.S.T., Switzerland) and a piece of gel foam soaked in biocytin (anterograde tracer, Sigma, St. Louis, MO) was placed on the distal stump of the nerve. The eye was placed back and the fish was then returned to water to recover.

2.3. Drug supplementation in tank water

Anti-thyroid drugs — lopanoic Acid (IOP) (TCI, Zwijndrecht, Belgium) and analog 1 of [(adamantan-1-yl) carbamoyl] methyl 4-methanesulfonyl-3-nitrobenzoate (or MLS000389544, described in Hwang et al., 2011 and henceforth referred in the text as Compound1 or C1) (Enamine, Riga, Latvia) were used as anti-thyroid drugs. IOP blocks activity of the deiodinase enzymes, thereby reducing the intracellular availability of T3 (Pascual et al., 1987; Bouzaffour et al., 2010). C1 irreversibly binds to TRα, thereby competitively inhibiting the binding of T3 to the receptor. A solution of 50 mM of IOP in absolute ethanol was always freshly prepared while a stock solution of 10 mM of C1 in DMSO was stored in aliquots at —20 °C. Drugs were added to the water to achieve final concentrations of 10 μM of IOP and 7 μM of C1. A stock solution of 100 mM of T3 (Sigma) was prepared in 0.1 N of NaOH and aliquots were stored at —20 °C. An appropriate amount was diluted in the system water to yield a final concentration of 5 or 15 nM.

Since IOP is sensitive to light, all the tanks were partially covered with a black sheet. The water was replaced daily, 1 h after feeding, by fresh water in the untreated control (UC) group and fresh drug containing water in the treated groups. A pilot study showed that OT reinnervation in fish exposed to vehicle only, i.e. 0.02% ethanol or 0.07% DMSO (final concentrations used in treatments) was not different from that in untreated fish. Therefore, untreated fish served as controls (UC) for all experiments.

2.4. Optic tectum reinnervation

Axon tracing was done as described previously (Becker et al., 2000). Briefly, fish were anesthetized and the ON was exposed as described above. In order to trace the regenerating axons, the ON was transected close to the ON head. A piece of gel soaked in biocytin (anterograde tracer, Sigma, St. Louis, MO) was placed on the distal stump of the nerve. The eye was placed back and the fish were returned to the system water for 3 h to allow the anterograde transport of the tracer. The fish were then euthanized in a solution of 0.1% MS-222, followed by transcardial perfusion, first with phosphate buffered saline (PBS, pH 7.4) and then with a mix of 2% paraformaldehyde (PFA) and 2.5% glutaraldehyde. Fifty micrometer vibratome cross sections of the brains were collected, stained using the Vectastain ABC kit (Vector laboratories) with DAB as chromogen and counterstained with neutral red (Sigma, St. Louis, MO) to permit brain nuclei identification. All sections from an experiment were stained simultaneously, taking care to avoid over staining. Based on the presence of specific nuclei, 5 rostral to caudal brain sections per fish were photomicrographed using a Zeiss Imager Z1 microscope and analyzed at 40× magnification with Axiovision software (Zeiss, Jena, Germany) employing an in-house developed automated program. The white matter of the OT was first delineated, which enabled the software to measure the whole target area (Fig. 1A). Next a threshold was set to precisely measure the biocytin labeled area within this target area. Brains of 5 uninjured fish were analyzed to provide the ratio of the biocytin immunopositive area over the total tectal target area. The average of this ratio per section was set as the 100% reference value for all experiments. The ratios obtained from the injury conditions were expressed in % relative to this reference that was used for all experiments. Reinnervation of the brain was calculated as the average from 5 sections for each animal and 5–10 fish were analyzed per condition.

2.5. RNA isolation and quantitative RT PCR

Whole retinas were dissected from the eye in ice cold PBS and snap frozen on dry ice. For each condition 3–5 replicates were sampled and for each sample 7 retinas were pooled. Total RNA was extracted using Tri reagent (Ambion) and RNA isolation kit (MN, Germany). RNA concentration and quality was assessed with Nanodrop and integrity was checked with gel electrophoresis. For cDNA synthesis, 1 μg of RNA was reverse transcribed using oligo dT primer and Superscript III (Invitrogen; Belgium). The qPCR was carried out on a StepOnePlus Real Time PCR system (Applied Biosystems), in a total volume of 20 μl, consisting of 25 ng of cDNA, 10 μl of SYBR green master mix (Applied
Biosystems), 250 nM Fwd and Rev primers each. Each sample was analyzed in duplicate and no-RT (minus reverse transcriptase) and water samples were included as negative controls. Three reference genes were selected for normalization based on geNorm (Vandesompele et al., 2002), namely eukaryotic translation initiation factor 1b (eif1b), hypoxanthine phosphoribosyltransferase 1 (hprt1) and glucose 6-phosphate dehydrogenase (g6pd). The primer sequences for the genes analyzed in the study are listed in Table 1. Primer pairs were designed using Primer3Plus software, verified for dimers and hairpin loops using Beacon designer by Premierbiosoft software and the predicted amplicon was checked for specificity using NCBI Nucleotide Blast. Primer pairs were verified for single peak melting curve on qPCR cycler and for amplicon size by gel electrophoresis.

2.6. BrdU labeling of proliferating cells

To label the mitotic cells in the injured retina, zebrafish were intraperitoneally injected with 0.2 mg/g body weight 5-bromo-2-

Table 1

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<td>NM_194388 McCurley and Callard (2010)</td>
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<td>R- GGAGCTCATTGACCTTGTTTTAGA</td>
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<td>F- GAGTCATGCCGACGCTG</td>
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7dpi 10dpi 14dpi
2.7. Immunohistochemistry (IHC)

Fish were euthanized at 1, 4, 7 and 10 days post injury (dpi) and fixed overnight in 4% PFA at 4 °C after lifting the cranium above the OT. The following day the brain with the eyes was dissected and washed in PBS. Tissue was cryoprotected by sequential immersion in 5%, 10%, and 30% sucrose until the tissue sank to the bottom and then embedded in a mixture of 1.5% agarose/30% sucrose. 14 μm thick horizontally cut cryosections were serially collected on glass slides (Thermo scientific SuperFrost® Plus, Menzel-Gläser) and stored at −80 °C for later use. For retinal whole mounts, the fixed retina was dissected and immediately processed for IHC.

Various IHC protocols were adapted for different antigen detection and these are summarized in Table 2. Standard IHC procedure involved permeabilization of the tissue with PBS/0.2% Triton X-100. A 2 h blocking step was carried out using PBS/2% normal goat serum/0.3% Triton X-100/1% DMSO. Next, the slides were incubated with primary antibody diluted in blocking solution overnight at room temperature. The following day sections were incubated with secondary antibody conjugated with Alexa Fluorophore (1:500, Invitrogen) in PBS/0.05% Tween20. Slides were counterstained with DAPI and mounted using mowiol (Sigma, St. Louis, MO). Some antibodies required an additional antigen retrieval step, involving immersion of slides in 10 mM of citrate buffer (pH 6) for 30 min at 95 °C prior to permeabilization. After cooling for 20 min, the sections were further processed for IHC as described above. Few other antibodies required a three step detection method for signal amplification. The procedure involved an additional endogenous peroxidase activity blocking step with 3% H2O2 in PBS for 10 min prior to the routine blocking step. The secondary antibody used in that case was biotinylated (1:300, Invitrogen) with incubation done for 45 min, followed by 30 min of reaction with Streptavidin-HRP (1:100). Fluorescent labeling was performed with 1:150 TSA–Cy3 (cyanine 3 system tyramide signal amplification, Perkin Elmer) for 8 min. Photomicrographs were acquired using a confocal microscope (Olympus FV1000).

2.8. Quantification of microglia/macrophages

A similar script to that described for biocytin quantification was used to measure microglia and macrophage staining with the 4C4 antibody (Becker and Becker, 2001) within a selected tissue area. Two locations were analyzed in detail: the proximal ON which is attached to the eye and through which the RGC axons first regrow, and the OT where the afferent endings of the injured RGCs first undergo degeneration and which later becomes reinnervated by new RGC axons reestablishing synaptic connections. In the former case, the whole proximal nerve, beginning from the optic nerve head till the site of crush, was evaluated for the 4C4 signal. In the latter case the whole white matter area of the OT was examined. Data analysis was carried out on 4–6 fish per group and on 3 proximal ON and 9–11 OT sections per fish.

2.9. Thyroid hormone extraction and radioimmuno assay (RIA)

Thyroxine (T4) and 3,5,3′-triiodothyronine (T3) levels were compared in the eye and the brain of control fish and fish treated with IOP for 3, 7, 14 and 21 days. For each time point 4 replicates were sampled per condition consisting of pools of eyes and brain from 10–12 fish. TH extraction from tissues was carried out as described earlier (Reyns et al., 2002). All samples were measured within a single assay and the values were corrected for the recovery efficiency. TH levels were expressed as pmol/g wet tissue.

3. Statistical analyses

Comparison between data from the untreated and treated group was done by unpaired Student’s t-test. For multiple comparison analysis was done by one-way ANOVA followed by Scheffé test. The results are presented as mean ± SEM and the criterion of significance was set at p < 0.05.

3. Results

3.1. The time course of optic tectum reinnervation

Before investigating the effect of drug treatment on OT reinnervation, we performed a pilot study to establish the time course of OT reinnervation in our model. Fish were subjected to ONC and after 5, 7, 10 and 14 dpi the severed axons were anterogradely traced with biocytin. Fig. 1B–F shows the gradually increasing biocytin signal in the OT of one brain hemisphere. Since all RGC axons in zebrafish normally cross over to establish connections with the contralateral OT, the absence of biocytin labeled fibers in the ipsilateral tectal hemisphere indicated that there was no misguidance of regrowing axons. The RGC axons arrived at the contralateral OT by 5 dpi and at 7 dpi the majority of the biocytin labeled axons were localized in the outer layers of the ventral half of the OT. At 10 and 14 dpi the biocytin signal was present all along the outer layers of the contralateral OT, i.e. the stratum opticum and stratum fibrosum et griseum superficiale, similar to what has previously been described (Huberman et al., 2010). The relative area covered by the biocytin labeled RGC axons in the OT target area of control fish, not subjected to ONC, was calculated to be 0.34 ± 0.03, while that observed in crushed fish at 7 dpi was 0.17 ± 0.02. Since the OT reinnervation at 7 dpi was about half that in the uninjured condition, we selected this time point for subsequent biocytin tracing experiments in drug treated fish. In all following experiments, the tectal innervation in the uninjured fish was set at 100% and all values analyzed in the experimentally manipulated fish were expressed in percentage relative to this level.

3.2. Effect of IOP treatment on the reinnervation of optic tectum

To study the effect of low T3 activity on the regeneration of severed ON, IOP was added to the water immediately after the ONC to block deiodinase activity. An untreated control (UC) group was maintained alongside. At 7 dpi, biocytin labeling was carried out and the fish were

<table>
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<th>Antibody name</th>
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<td>Gift from Dr. Pamela Raymonds, University of Michigan</td>
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HR—heat retrieval, A—amplification, IF—direct immunofluorescence.
sacrificed to study OT reinnervation. Fig. 2A shows that the fish treated with IOP exhibited an accelerated tectal reinnervation compared to the untreated fish. Quantification using the automated software showed significantly more signal in the OT of IOP-treated fish (Fig. 2B). We did not detect misguided regenerating axons in any of the IOP-treated fish, indicating that the drug had no adverse effect on axon pathfinding. The experiment was repeated on a different batch of fish with similar results.

To confirm the presence of regrowing axons, we performed an immunostaining for the growth associated protein 43 (Gap43), a protein expressed in outgrowing axons. Fig. 2C shows that more Gap43-positive axons were present at 7 dpi in the IOP-treated fish. Together these results indicate that IOP exposure facilitated the reinnervation of the OT.

3.3. Effect of IOP treatment on cell death and proliferation in the retina

Since THs are known to protect against cell death and to promote neuronal proliferation, we checked for apoptosis and proliferation in the sham and crushed retina of UC and IOP-treated fish. Immunodetection for apoptotic cells in retinal whole mounts using an anti-cleaved caspase 3a antibody at 1 dpi showed that there were only a few dying cells in the injured RGC layer and IOP treatment did not affect this number (data not shown). This also illustrated the absence of toxic effects of the drug treatment.

Cell proliferation was studied using BrdU and PCNA at 3 dpi. We noticed the presence of only a limited number of mitotic cells in the inner nuclear layer of the crushed retina of the UC group. This was similar in the crushed retina of IOP-treated fish, suggesting that IOP had no effect on proliferation either (data not shown). Together these results reveal that the observed effect of TH blockage on axonal regeneration of RGCs is not due to activities on retinal cell survival or proliferation.

3.4. Effect of IOP on the mRNA expression of regeneration associated genes and thyroid responsive genes in the retina

THs exert their effect mainly by regulating gene transcription. We therefore investigated the mRNA expression levels of two well-known regeneration associated genes, gap43 (growth associated protein 43) and tuba1 (tubulin alpha 1), in the retina at 1, 4 and 7 dpi (Fig. 2D). We indeed found upregulation of both mRNAs in the retina following ONC compared to the sham operated side, but this upregulation was not different following IOP treatment. We also checked a third gene, namely socs3b (suppressor of cytokine signaling proteins 3b), known to be upregulated after ON injury in zebrafish (Veldman et al., 2007). We observed an upregulation of its mRNA in the retina from the crushed side of UC fish but again this pattern was similar in IOP-treated fish.

We further evaluated two TH-regulated genes that have previously been shown to be involved in regeneration, klf9 (kruppel like factor 9), which is known to suppress axonal regeneration in mammals (Moore et al., 2009; Dugas et al., 2012) and bdnf (brain derived neurotrophic factor), reported to be involved in CNS regeneration in both mammals and fish (Pernet and Di Polo, 2006; Shulga et al., 2009; Chen et al., 2013). However, mRNA levels of both genes in the retina were not affected by the crush, tested at 4 and 7 dpi (klf9) and at 1 and 4 dpi (bdnf) respectively. Furthermore, the mRNA expression of these genes was unaffected by the IOP treatment. Together these results indicate that the observed effect of TH blockage on axonal regeneration of RGCs is not accompanied by changes in retinal mRNA expression of a number of genes typically associated with axon regeneration.

3.5. Effect of IOP on T₃ levels in eye and brain

IOP is commonly used to decrease intracellular levels of T₃. However, since we found no significant difference in the expression level of klf9...
Fig. 3. A. IOP treatment lowers T₃ levels in the eye and brain. Data represent mean ± SEM of T₃ concentrations (pmol/g tissue) in UC fish and fish treated with IOP for 3, 7, 14 and 21 days. (n = 4 tissue pools per group). *p < 0.05, **p < 0.005 and ***p < 0.0001 by unpaired t-test compared to UC. B. IOP treatment started 2 weeks prior to ONC also facilitates OT reinnervation. Pictures show reinnervation of the OT by RGC axons at 7 dpi in UC and IOPp fish. Brown staining indicated with arrow represents biocytin labeled RGC axons. C. Quantification of the area covered by RGC axons in the OT of UC and IOPp fish. Data represent mean ± SEM (n = 7–10 animals/group). ***p < 0.0001 by unpaired t-test. For all panels: UC—untreated control, IOPp—iopanoic acid pretreated for 2 weeks, X—optic nerve crushed, OT—optic tectum. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

Fig. 4. T₃ supplementation attenuates OT reinnervation. A. Reinnervation of the OT by RGC axons at 7 dpi in different groups. Brown staining indicated with arrow represents biocytin labeled RGC axons. B. Quantification of the area covered by RGC axons in the OT of the different groups. Data represent mean ± SEM (n = 4–5 animals/group). Data were analyzed by ANOVA followed by Scheffé test. Means with a different letter (a–b) are significantly different (p at least < 0.05). C. Immunofluorescent detection of Gap43 protein (red) in RGC axons (arrow) arriving in the OT. Cell nuclei are stained with DAPI (blue). Pictures are representative images for n = 4–5 animals/group. Scale bar = 20 μm. For all panels: UC—untreated control, IOPp—iopanoic acid pretreated for 2 weeks, IOPp + 5nM T₃ (or +15 nM T₃)—iopanoic acid pretreated for 2 weeks before crush and co-treated with 5 nM T₃ (or 15 nM T₃) starting 3 days before crush, S—sham operated, X—optic nerve crushed, OT—optic tectum.
and bdnf, we checked the efficiency of our IOP treatment in reducing T3 levels in eye and brain. We measured T3 in tissue extracts from untreated fish and fish treated with IOP for 3, 7, 14 and 21 days. Fig. 3A shows that the T3 level in the eye and the brain gradually decreased following IOP exposure to reach minimal levels from 7 days of treatment onwards. Measurements also showed that T4 levels in the eye and brain increased slightly but not significantly on the first 3 days of treatment but then decreased significantly to reach minimal levels after 7 to 14 days of treatment (data not shown).

3.6. Effect of IOP pretreatment on the reinnervation of the optic tectum

To investigate the regenerative properties in a situation where both T4 and T3 levels are minimal from the start of injury, fish were pre-exposed to IOP (IOPp) for 2 weeks prior to ONC. After ONC, drug treatment was continued as done before. Biocytin tracing performed at 7 dpi revealed that the OT reinnervation was still significantly higher in the IOPp group compared to the UC group (Fig. 3B–C), suggesting that OT reinnervation is accelerated also when T3 levels are already low at the moment of the crush.

3.7. Effect of exogenous T3 on the reinnervation of OT

Next we investigated whether the accelerated reinnervation evidenced in the case of IOP- or IOPp-treated fish could be attenuated by exogenous T3. Therefore IOP pretreatment for 2 weeks was coupled with T3 supplementation (5 or 15 nM) starting 3 days prior to ONC. Co-administration of IOP and T3 was continued following ONC. These groups were compared to IOPp treatment alone as well as to an UC group. Biocytin tracing and IHC for Gap43 were performed at 7 dpi while this effect diminished after combination with T3 supplementation. The increased reinnervation and its attenuation were confirmed by Gap43 immunostaining (Fig. 4C). Together the data clearly show that lowering the level of T3 post injury facilitates reinnervation of the OT.

3.8. Effect of a TRβ1 antagonist on OT reinnervation

Data from a microarray study on whole eye samples from ONC zebrafish had shown that thrβ1 mRNA was downregulated at 4 and 21 dpi (McCurley and Callard, 2010). There are also reports showing that drug induced hypothyroidism can decrease while TH supplementation upregulates thrβ1 mRNA expression (Santillo et al., 2012). We therefore hypothesized that the IOP-induced hypothyroidism in our experiment might lower TRβ1-mediated T3 signaling eventually resulting in better regeneration of the ON. Injured fish were treated with C1, a TRβ1 antagonist. Biocytin tracing at 7 dpi again revealed an accelerated OT reinnervation, similar to our earlier observations in IOP-treated fish (Fig. 5A and B). Gap43 immunostaining confirmed the biocytin results (Fig. 5C). These observations favored the hypothesis that antagonizing TRβ1 activity accelerates the reinnervation of OT.

We also analyzed the mRNA levels of the regeneration associated genes gap43, tub1a, and socs3b as well as the TH-responsive genes bdnf and klf9 in the retina of C1-treated fish. The results agreed with the qPCR data reported above for IOP-treated fish. Blocking TRβ1 did not affect the transcription of these genes in the retina from the crushed side.

3.9. Effect of low T3 levels on inflammation

To further investigate possible underlying mechanisms for the observed effects of low T3 levels on ON regeneration, we analyzed the effect of IOP on the infiltration of microglia/macrophages by IHC at 4, 7 and 10 dpi, using the 4C4 antibody which detects both microglia and macrophages. We observed resting or surveying microglia with ramified fibrous processes in the retina, ON and corresponding OT of the sham operated side (Fig. 6A–C), and in a pure control group which

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**Fig. 5.** Blocking TRβ1 receptor accelerates OT reinnervation. A. Brown staining represents biocytin labeled RGC axons. B. Quantification of the area covered by RGC axons in the OT of UC and C1 fish. Data represent mean ± SEM (n = 6 animals/group). ***p < 0.0001 by unpaired t-test. C. Immunofluorescent detection of Gap43 protein (red) in RGC axons (arrow) arriving in the OT. Cell nuclei are stained with DAPI (blue). Pictures are representative images for n = 4 animals/group. Scale bar = 20 μm. For all panels: UC—untreated control, C1—compound 1 treated, S—sham operated, X—optic nerve crushed, OT—optic tectum.
underwent no surgery or treatment (data not shown). After ONC however, reactive microglia and/or infiltrating macrophages with amoeboid morphology were obvious all along the injured ON and in the corresponding OT (Fig. 6A–C) while only few were present in the retina.

Next, the abundance and distribution of microglia/macrophages in the proximal ON was analyzed in more detail. At 4 dpi the total 4C4 immunopositive signal was similar in IOPp-treated and UC fish (Fig. 6D). However, the activated microglia/macrophages were clearly more concentrated close to the site of crush in the IOPp group (Fig. 6E). Their distribution became more dispersed at later time points and at 10 dpi the total 4C4 signal was significantly lower in the IOPp group compared to the UC group where it remained elevated (Fig. 6D–G).

Finally, analysis of the presence of microglia/macrophages in the OT revealed that at 4 dpi the amount of 4C4-positive cells was not different between the two experimental groups (Fig. 6H–I). However, at 7 and 10 dpi, a significantly more rapid decline in activated microglia/macrophages was evident in the IOPp group compared to the UC group (Fig. 6J–K), as confirmed by quantification (Fig. 6H).

4. Discussion

THs have been known to promote several aspects of nerve regeneration following injury but a number of studies also found potentially adverse effects. Here we report data showing that lowering the T3 level via IOP treatment or blocking its activity by a TR antagonist accelerates OT reinnervation following ONC in zebrafish. No obvious impact of the treatment was observed at the level of the retina. The accelerated OT reinnervation seemed however correlated with changes in the inflammatory response in the IOPp-treated fish at the level of the ON as well as the OT.

4.1. Lowering T3 signaling: effects at the level of the retina

THs have repeatedly been found to increase cell survival following nervous system injury, an action that in rodents is at least partially mediated via BDNF (Shulga et al., 2009; Shulga and Rivera, 2013). THs were also reported to promote neuronal proliferation in the adult rodent CNS (Desouza et al., 2005; Lemkine et al., 2005; Lopez-Juarez et al., 2012). We here show that lowering T3 did not augment the cell death of damaged RGCs and had no influence on mitosis in the zebrafish retina. The lack of TH-related changes is most probably linked to the specific conditions of the ONC model, in which most RGCs typically survive the injury and functional recovery occurs without any accountable contribution from proliferation, as has also been reported before (Zou et al., 2013). This is different in some other models used in zebrafish to induce RGC regeneration, such as injection of the toxic drug ouabain into the eye resulting in massive cell death in the inner retina (Fimbel et al., 2007) or ON transection where 25% of the RGCs die (Zou et al., 2013). Our data do not exclude that lowering T3 signaling in these more drastic conditions could have a greater impact on RGC survival and regeneration.

Fig. 6. IOP treatment accelerates the resolution of microglia/macrophages in the ON and OT. A. Overview of the presence of inactive and reactive microglia/macrophages (arrow heads) after ONC in the injured ON and OT as detected with the 4C4 antibody. B–C. Magnification of the inactive ramified form (red) and the reactive amoeboid form of microglia/macrophages (red, arrowhead) in ON and OT of the sham operated and ONC side, respectively. Nuclei are stained with DAPI (blue). Scale bar = 5 μm. D and H. Quantification of the area covered by the microglia/macrophages in the proximal ON and OT in UC and IOPp fish at 4, 7 and 10 dpi, respectively. Data represent mean ± SEM (n = 3–6 animals/group). **p < 0.005 and ***p < 0.0001 by unpaired t-test, compared to UC at the same time point. E–G. and I–K. Immunofluorescent detection of activated microglia/macrophages in the proximal ON and the OT at 4, 7 and 10 dpi. The site of crush is marked by a yellow dotted line. At 4 dpi microglia/macrophages are more densely packed in the proximal ON in the IOPp group compared to the UC group (indicated by arrowhead in panel E). At 7 and 10 dpi less microglia/macrophages are present in the OT of the IOPp group compared to the respective UC group (indicated by arrowheads in panels I and K). Scale bar = 100 μm. For all panels: UC—untreated control, IOPp—iopanoic acid pretreated for 2 weeks, S—sham operated optic nerve, X—optic nerve crushed, ON—optic nerve, OT—optic tectum.
injury models might have a negative impact on retinal cell survival and/or proliferation.

ONC in zebrafish significantly increased mRNA expression of three regeneration-associated genes, gap43, tuba1 and socs3b. Both Gap43 and Tuba1 help in neurite formation and axon outgrowth during regeneration. Interestingly, the increase in GAP43 and TUBA1 expression after injury in mammals is less compared to non-mammals (Skene, 1989; Leon et al., 2000). Lowering T₃ signaling did not reduce or augment the expression levels of these genes in our model. Contrary to GAP43 and TUBA1, SOCS3 is inhibitory towards axon growth in mammals (Miao et al., 2006; Smith et al., 2009) and its mRNA and protein levels are upregulated in rodents following nervous system injury (Lukas et al., 2009). In zebrafish the mRNA of the paralogous genes socs3a and socs3b are also upregulated following ON injury (Veldman et al., 2007) but only Socs3a, and not Socs3b, was found to have an inhibitory effect on regeneration in vitro (Elsaeidi et al., 2014). However, in vivo the Jak/STAT pathway in zebrafish efficiently overcomes the inhibition by Socs3a (Elsaeidi et al., 2014). Our data showed that ONC induced an upregulation in the socs3b mRNA expression levels, which was again not affected by lowering T₃.

Taken together these data indicate that reducing T₃ signaling has no effect on the expression of a number of well-known regeneration-associated markers in the retina. As we performed qPCR on extracts of whole retina, we cannot exclude that subtle changes in expression occurred in the RGCs. However, as our data showed a very prominent increase in expression in all crushed retinas irrespective of the drug treatment and upregulation of these regeneration markers is thought to occur mainly within the RGCs, our approach should normally have detected the impact of TH modulation if present. Of course, the present results do not exclude that lowering T₃ signaling interferes with retinal expression of other genes that can influence RGC axon regeneration in a positive or negative way.

In order to identify possible sites of interaction, we tried to check the expression pattern of TRs in zebrafish retina by IHC using several commercially available antibodies that were supposed to (cross-)react with zebrafish TRα and/or TRβ1. We detected positive signals for both receptor types in the retina, including the RGC layer, but due to inconsistencies in the cell-specific staining pattern between different antibodies the interpretation of these results remains uncertain. It is however known from the literature that RGCs from different vertebrates express TRα (e.g. in chicken Sjöberg et al., 1992, and in flounder Mader and Cameron, 2006) so this may also be true for zebrafish. Therefore we conducted a pilot study to check the effect of blocking both TRα and TRβ1 on RGC regeneration using the antagonist amiodarone (van Beeren et al., 2012). Due to its severe adverse effects on fish health further experimentation was however not possible. So, instead we blocked TRβ1-mediated signaling using C1 since this receptor was shown earlier to be downregulated in zebrafish eye following ONC (McCurley and Callard, 2010). As discussed further down, OT reinnervation was increased similar to what we observed in IOP-treated fish, suggesting that TRβ1 may be involved in the process. Taking into account that, as for IOP treatment, C1 did not change retinal expression of the tested regeneration markers, the involvement of TRβ1 does however not necessarily occur at the level of the eye.

4.2. Lowering T₃ signaling: effects at the level of the optic tectum

TH supplementation has been shown to increase axon elongation in several rodent studies (McIsaac and Kiernan, 1975; Yu and Srinivasan, 1981; Voria et al., 2006; Bessede et al., 2010) but there are also several studies where no beneficial effects were found in either PNS (Stelmack and Kiernan, 1977; Scherman et al., 2004) or CNS (Tator et al., 1983; Hoovler et al., 1985). Our data show that the use of IOP to reduce the T₃ level during the regeneration phase triggered the injured RGC axons to reach their target sooner. This was shown by labeling with the anterograde tracer biocytin and confirmed by immunostaining for Gap43, a cytoplasmic protein associated with actively growing axons (Strittmatter et al., 1994; Hocquemiller et al., 2010). Conversely, exogenous T₃ administered to fish pre-treated with IOP attenuated this effect, showing that the acceleration in reinnervation is indeed specifically related to reduced T₃ signaling. We were not able to convincingly show TR expression in the OT of zebrafish but literature data report on expression of both TRα and TRβ1 in the OT of some other species (e.g. in Xenopus laevis Denver et al., 2009, in European sea bass Isorna et al., 2008). Therefore the OT is one of the alternative/additional sites where decreased T₃ signaling via TRβ1 might favor reinnervation as observed following treatment with the TRβ1 antagonist.

Since changing T₃ signaling might locally influence RGC axon elongation in the ON, we made several efforts to quantify the rate of axon elongation. The frequent overlapping of the sham operated ON over the regrowing ON obstructed the quantification using Gap43 staining while trying to study regrowing axons in isolated ON was not possible because of its small size. We also tried to use ath5:gfp Tg fish in which RGCs express GFP protein during development. However, RGCs from adult fish, with or without ONC, do not (re-)express the fluorescent protein, rendering this model inappropriate for our goal.

4.3. Lowering T₃ signaling: effects on inflammation in ON and OT

Trauma and degenerative disease in the vertebrate nervous system activates resident microglia and attracts blood-borne macrophages to the site of damage (Kyritsis et al., 2014). In tilapia, ON lesion was reported to induce the proliferation of resident microglia in the ON and in the OT close to the degenerating RGC axons (Dowding et al., 1991). The present results indicate an increased occurrence of activated microglia and macrophages following ONC in adult zebrafish. At 4 dpi the presence of these cells was marked around the site of injury as well as further in the distal ON and in the RGC afferent layers in the OT. When comparing UC- and IOPp-treated fish, the total amount of 4C4-immunopositive inflammatory cells observed in the proximal ON and the OT at 4 dpi was similar but their distribution pattern seemed to differ. It has been shown in mammals that leukocytes express TRs and THs can affect their distribution and response (Ohashi and Itoh, 1994; De Vito et al., 2011). TRs were also detected in the leukocytes of rainbow trout (Quesada-Garcia et al., 2014) but so far we could not confirm their presence in zebrafish microglia/macrophages.

Our data also clearly revealed that the length of the inflammatory response was different, showing a faster resolution of microglia/macrophages in IOPp-treated fish in the proximal ON at 10 dpi and in the OT at 7 and 10 dpi. It has been found that hypothyroidism can increase the phagocytic activity of macrophages (Rosa et al., 1995). Since microglia and macrophages are important in debris clearance, a potentially increased phagocytic activity of these inflammatory cells in IOPp-treated fish, where T₃ signaling is indeed lower, could result in an accelerated clearance of inhibitory molecules. Furthermore, the fact that the inflammatory response following CNS injury is typically rapid and short in lower vertebrates is thought to prevent the accumulation of toxic metabolites and favor regeneration compared to mammals (Kyritsis et al., 2014). As our findings are only associative, it is however difficult to conclude at this point whether the more rapid resolution of inflammation is a cause or rather a consequence of accelerated reinnervation. Future experiments aimed at interfering with the immune response are needed to resolve this question.

In summary, our results suggest that lowering T₃ signaling is beneficial for regeneration following ONC in zebrafish. In contrast to some other ON or retinal injury models, ONC hardly induces cell death or proliferation in the retina and the upregulation of a number of well-known regeneration markers in the RGCs retina is not affected by reduced T₃ signaling. Interestingly, the accelerated OT reinnervation correlates with a more rapid resolution of the inflammatory response both at the injury site and in the OT. The exact mechanism by which low T₃ signaling favors OT reinnervation remains at this point unclear and future
studies are needed to find out whether or not the accelerated/shortened inflammatory response is a causative factor in this process.

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