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Changes in thyroid hormone reception precede SWS1 opsin downregulation in trout retina

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SUMMARY

Rainbow trout undergo natural cone degeneration and thus are interesting models for examining mechanisms of neural degeneration. They have ultraviolet-sensitive (UVS) cones that are lost over most of the retina during development; only a small functional population remains in the dorsal retina. How this spatial distribution of UVS cones is maintained is unclear. Thyroxine (T4) induces UVS cone loss, and local thyroid hormone regulation was hypothesized to control UVS cone distribution. Thyroid hormone receptor alpha (TRα), thyroid hormone receptor beta (TRβ) and Type 2 deiodinase (D2) regulate thyroid hormone exposure to target cells. Regional retinal expression of these genes was investigated during exogenous T4 treatment and natural smoltification of rainbow trout. Each retina from dark-adapted parr, T4-treated parr and natural smolts was divided into four quadrants, and total RNA was isolated. Quantitative real-time RT-PCR analysis demonstrated that all retinal quadrants had increased accumulation of *TRβ* transcripts 2 days post-T4 treatment, corresponding to initiation of *SWS1* opsin downregulation. Smolts exhibited decreased accumulation of *TRα* and *TRβ* transcripts in all quadrants, but this effect was most pronounced in the dorso-temporal (DT) retinal quadrant where UVS cones persist. By contrast, in 2 day T4-treated parr, the DT quadrant showed increased expression of *TRα* and *TRβ*. Furthermore, *D2* transcripts decreased in the DT quadrant of T4-treated parr but increased in the DT quadrant of smolts. These results suggest that T4 upregulates *TRβ* expression to initiate *SWS1* opsin downregulation, while *TRα* and *TRβ* downregulation occurs to prevent natural loss of UVS cones from the DT retina.

Key words: thyroid hormone, retina, degeneration, opsin, photoreceptor, development.

INTRODUCTION

Salmonid fishes undergo well-characterized developmental changes in their retinas during their life cycle. One of these is a selective degeneration of cone photoreceptors. The naturally occurring degeneration of cones makes the salmonid retina a useful model for study of the cellular processes underlying neuronal cell degeneration. The predictability of these cell losses offers a unique opportunity to further our understanding of the basis of major diseases such as Alzheimer's, retinitus pigmentosa and macular degeneration.

The salmonid retina contains a cone type that is sensitive to ultraviolet light, ultraviolet-sensitive cones (UVS), which express the *SWS1* opsin gene and can detect ultraviolet and polarized light (Parkyn and Hawryshyn, 2000). This ability of salmonid UVS cones to detect polarized light and wavelengths in the UV spectrum may benefit these fish in homing and migration and feeding (Hawryshyn, 2000). Over the course of development, salmonid fishes undergo the process of smoltification, in which dramatic changes in morphology, physiology and behaviour take place, enabling the developing fish to migrate from their hatching grounds in shallow freshwater rivers and streams to a relatively open marine environment where they spend the majority of their adult lives (Groot and Margolis, 1991). Smoltification is most dramatic in anadromous species, but rainbow trout (*Oncorynchus mykiss*), which are a land-locked freshwater species, demonstrate some of these same changes, and, in particular, comparable changes occur in their visual system (Veldhoen et al., 2006). During smoltification, both anadromous and land-locked species undergo downregulation of *SWS1* opsin expression and depopulation of UVS cones in the retina through apoptosis; this corresponds to a decrease in UV sensitivity (Browman and Hawryshyn, 1992; Browman and Hawryshyn, 1994; Kunz et al., 1994; Deutschlander et al., 2001; Allison et al., 2003; Allison et al., 2006; Hawryshyn et al., 2003; Kunz, 2006; Veldhoen et al., 2006).

Natural smoltification is accompanied by the elevation of several hormones, and exogenous treatment with one of these elevated hormones, thyroxine (T4; a thyroid hormone), can induce some of the changes that occur during natural smoltification, such as body silvering and a change in condition factor (Hoar, 1988). It has since been demonstrated that not only does serum T4 increase prior to UVS cone loss, but exogenous T4 treatment of rainbow trout parr induces precocial loss of UVS cones from the retina and loss of ability of these fish to detect light in the UV spectrum (Browman and Hawryshyn, 1992; Browman and Hawryshyn, 1994; Deutschlander et al., 2001; Hawryshyn et al., 2003; Allison et al., 2003; Allison et al., 2006). It was also found that cessation of T4 treatment resulted in regeneration of UVS cones in the salmonid retina (Browman and Hawryshyn, 1994; Allison et al., 2006). Interestingly, although the T4-induced loss of UVS cones from the central retina of salmonids is complete, a small population of UVS cones is retained in the dorsal temporal quadrant of the retina after natural smoltification; the ability to detect light in the UV spectrum remains intact in this quadrant (Deutschlander et al., 2001; Allison et al., 2003; Hawryshyn et al., 2003; Kunz, 2006).

T4 and triiodothyronine (T3) are the two main forms of thyroid hormone (TH) circulating in the blood of teleost fish. T4 is the predominant form of TH released from the thyroid tissue into the blood and is considered to be a prohormone for the more biologically active form of thyroid hormone, T3. T3 has the highest affinity for

its nuclear thyroid hormone receptor (TR) and acts as a ligand-regulated transcription factor to alter the transcription of target genes. Two main isoforms of TR have been identified in rainbow trout, *TRα* and *TRβ*, although other forms generated from alternate splicing have been identified in several other fish species; namely two *TRα* and two *TRβ* (Yamano et al., 1994; Yamano and Inui, 1995; Essner et al., 1997; Liu et al., 2000; Marchand et al., 2001; Nowell et al., 2001; Jones et al., 2002; Kawakami et al., 2003; Raine et al., 2004; Nelson and Habibi, 2006; Nelson and Habibi, 2008; Tang et al., 2008). *TRα* and *TRβ* appear to be differentially expressed in most tissues, both spatially and temporally, and are thought to mediate distinct responses of TH (Nunez et al., 2008).

Deiodination enzymes in peripheral tissues such as the gills, liver and kidneys provide the majority of T3 in the circulation and are important regulators of TH concentrations in the blood, enabling either increased supply of T3 or clearance of both T4 and T3 (Eales and Brown, 1993; Leatherland, 1994; Köhrle, 1999; Bianco et al., 2002; Brown, 2005; St Germain et al., 2005; Nunez et al., 2008). Furthermore, local tissue deiodination in target tissues is considered an important mechanism for supplying T3 to the tissues of the nervous system, and T3 levels for a target cell can be increased or decreased locally by deiodination (Nunez et al., 2008). Three deiodination enzymes have been identified in fish, D1, D2 and D3; however, only the D2 and D3 genes have been characterized in salmonids (Orozco et al., 1997; Orozco et al., 2000; Sambroni et al., 2001; Bres et al., 2006). D2 is primarily involved in generating T3 from T4, while D3 predominantly converts T4 and T3 into inactive metabolites (Sambroni et al., 2001; Bres et al., 2006). The actions of TRs and deiodination enzymes likely provide target cells with the ability to regulate their exposure to TH and its potential effects on gene expression.

TH appears to play a major role in vertebrate growth and development, although the precise nature of its role in teleost fish is not clear. In mammals, TH appears to play a key role in vertebrate colour vision (Ng et al., 2001). *TRα* and *TRβ* have both been detected in the retina of mice and chicks, African clawed frog (*Xenopus laevis*) and winter flounder (*Pseudopleuronectes americanus*) and have been shown to be involved in opsin expression and cone development (Sjoberg et al., 1992; Cossette and Drysdale, 2004; Mader and Cameron, 2006; Nunez. et al., 2008). *TRβ*, in particular, has been found to play a dominant role in cone development in these same species, and *TRβ* deletions in mice have resulted in colour blindness (Sjoberg et al., 1992; Cossette and Drysdale, 2004; Mader and Cameron, 2006; Nunez et al., 2008). Furthermore, it has been demonstrated that deiodinase activity is present in retina and that this activity is altered with T4 treatment (Orozco et al., 2000; Plate et al., 2002).

Although T4 treatment has been shown to consistently induce UVS cone loss and *SWS1* opsin gene downregulation in rainbow trout (Browman and Hawryshyn, 1992; Browman and Hawryshyn, 1994; Deutschlander et al., 2001; Hawryshyn et al., 2003; Allison et al., 2003; Allison et al., 2006; Veldhoen et al., 2006), there has been no evidence that TH acts directly on the retina to induce these events. It is quite possible that T4 is not directly involved in initiating UVS cone loss but it could instead upregulate other factors that are directly responsible for initiating UVS cone loss. Recently, Veldhoen et al. demonstrated that exogenous T4 treatment induces the onset of *SWS1* opsin gene downregulation as early as 2 days after the initiation of treatment (Veldhoen et al., 2006). This suggests that if T4 directly induces downregulation of *SWS1* opsin gene expression, there should be corresponding changes in the regulation of TH in the retina at this time. Furthermore, the persistence of a small, functional population of UVS cones in the DT retinal quadrant of natural smolts suggests that if TH is directly involved in UVS cone loss, there will be a corresponding change in TH regulation in this quadrant that correlates with the presence of UVS cones. Thus, experiments were conducted to determine whether T4 regulation is involved in regional UVS cone loss and SWS1 opsin downregulation and to determine whether regional differences in D2 and TRα and TRβ across the retina correlate with distributional loss of the UVS cones during natural smoltification. This study provides evidence that supports the hypothesis that regional differences in D2 and TRα and TRβ across the retina result in the small population of UVS cones present after natural smoltification.

MATERIALS AND METHODS

Existing total RNA from a previous study (Veldhoen et al., 2006) was utilized for this investigation. Total RNA was originally obtained from retinas of wild stock rainbow trout (*Oncorhynchus mykiss* Walbaum) obtained from the Vancouver Island Trout Hatchery (Duncan, BC, Canada). The trout were maintained indoors at the University of Victoria at an average water temperature of 15°C. The retina from the left eye was divided into quadrants [dorsal-temporal (DT), dorso-nasal (DN), ventro-nasal (VN) and ventro-temporal (VT)] using the location of the optic nerve head as a morphological marker (Fig. 1). The total RNA was then stored at –80°C. Fish care and treatment were performed in accordance with Canadian Council for Animal Care regulations and the University of Victoria Animal Care Committee. The fluorescent lighting [Stanpro FU32T8/65K/8 6500K; see Veldhoen et al. (Veldhoen et al., 2006) for spectral irradiance measurements] provided a 12 h:12 h light:dark cycle, and fish were fed a maintenance ration of Trout AB feed (Moore-Clark, Vancouver, BC, Canada). Prior to utilization in the current study, the rainbow trout parr were maintained in the facility for 6 weeks and the smolt for 16 months.

For the purposes of this study, the terms ‘parr’ and ‘smolt’, representing pre- and post-migratory stages, respectively, in anadromous salmonid species were used to represent the comparable developmental stages in land-locked rainbow trout. Although rainbow trout are not anadromous, they do demonstrate similar changes in their visual system during development as anadromous salmonid populations (e.g. Steelhead) (Veldhoen et al., 2006).

Rainbow trout parr and smolts were examined for changes in *TR* and *D2* gene expression between retinal quadrants (Fig. 1). Parr exhibit a full complement of UVS cones expressing SWS1 opsin in their retina, while smolt express very low levels of SWS1 opsin and retain only a small population of UVS cones in the DT retina. Rainbow trout parr were divided into three groups. Two groups of parr were used to examine the changes in TH regulation across the retina at the onset of T4-induced *SWS1* downregulation. The remaining group of parr was allowed to develop naturally to examine the changes in TH regulation across the retina during natural *SWS1* downregulation and UVS cone loss.

For the T4 treatment experiment, rainbow trout parr were placed into one of two tanks to be used as a control group (vehicle only) or a T4-treated group. L-thyroxine sodium salt pentahydrate (T4; Sigma, St Louis, MO, USA) was dissolved in 0.1 mol l^{-1} NaOH and added to the water of the treatment group to achieve a final concentration of 300 μg l^{-1}. Water and T4 treatment were replaced daily. The control group was treated in an identical manner but only received the 0.1 mol l^{-1} NaOH vehicle. Fish sampled at 2 days post-treatment were utilized for the current study, as it was shown previously (Veldhoen et al., 2006) that downregulation of *SWS1* gene expression was initiated at this time. It was expected that if

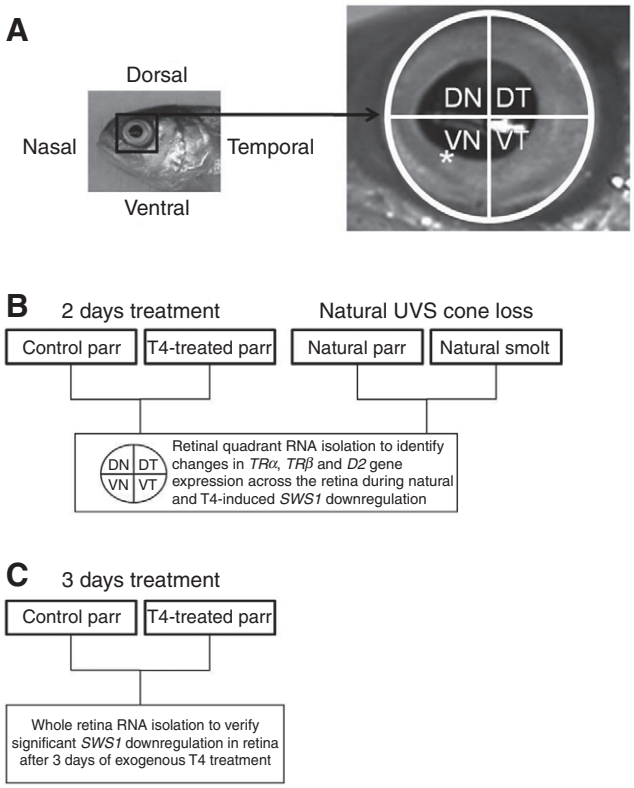

Fig. 1. An overview of the experimental design used in the current study. (A) Schematic diagram of the orientation, anatomical naming and division of the retinal quadrants dissected from the isolated retina of rainbow trout. The grid overlays the head and eye of a T4-treated parr. (B) Flowchart showing the treatments used to compare the regulation of thyroid hormone across the retina in T4-treated parr and smolt. (C) A flowchart of the treatments used to demonstrate the significant downregulation of *SWS1* expression in the retina of rainbow trout parr 3 days after the initiation of treatment with exogenous T4. DN, dorso-nasal retinal quadrant; DT, dorso-temporal retinal quadrant; VN, ventro-nasal retinal quadrant; VT, ventro-temporal retinal quadrant. * denotes the location of the optic nerve head.

TH was involved in the direct regulation of *SWS1* gene expression, there should be noticeable corresponding changes in the expression of *TR* and *D2* genes at this time.

Natural smolt and untreated parr were used for the second component of the study. Untreated parr were sampled to provide the natural control fish with a full component of UVS cones in the retina and baseline expression levels of the genes to be examined. Once the parr reached the smolt stage with natural downregulation of SWS1 gene expression and UVS cone loss throughout most of the central retina, these fish were sampled to examine the potential changes in *TR* and *D2* gene expression that were hypothesized to preserve the UVS cones remaining in the DT quadrant at this stage of development.

For both components of this study, fish were dark adapted prior to euthanasia with 150 mg l^{-1} tricaine methanesulfonate (MS-222; Crescent Research Chemical, Phoenix, AZ, USA). The neural retina was then dissected free of the retinal pigment epithelium of the left eye under deep red illumination and placed immediately in RNAlater (Ambion, Austin, TX, USA). Each isolated retina was divided into four quadrants using the optic nerve head as a landmark: dorso-nasal (DN), dorso-temporal (DT), ventro-nasal (VN) and ventro-temporal (VT) quadrants (Fig. 1). Total RNA was isolated from each quadrant of each fish using TriZol reagent (Sigma). Quantification of RNA from the original total RNA isolations was undertaken using RiboGreen (Invitrogen, CA, USA), following the manufacturer's protocol, and using a MX3000P real-time quantitative PCR thermocycler (Stratagene, La Jolla, CA, USA). 500 ng total RNA for each of six samples per quadrant was then reverse-transcribed to cDNA using a Superscript III First Strand Synthesis kit (Invitrogen Canada, Burlington, ON, Canada).

Previously utilized *O. mykiss* gene-specific primers for *SWS1*, ribosomal protein *L8* (*L8*) (Veldhoen et al., 2006) and *TR*α and *TR*β (Raine et al., 2004) were used in the current study (Table 1). *L8* was used as a housekeeping gene as it does not vary significantly during TH treatment and development in rainbow trout and anurans (Shi and Liang, 1994; Callery and Elinson, 2000; Crump et al., 2002; Veldhoen et al., 2006). An additional primer set was designed against *O. mykiss D2* (GenBank Accession number: AF207900) and generated a 207 bp product with a 100% sequence similarity against the cDNA of this gene of interest (Table 1). Unfortunately, although the deiodinase 3 gene, which codes for a TH-regulating enzyme involved in TH clearance, was recently characterized (Bres et al., 2006), the sequence data were not available in GenBank. In the current study, the authors were unable to successfully clone or amplify the gene based on the published sequence and primer information supplied. Thus, this gene was not included in the study.

Primer specificity was determined by amplifying target gene products using cDNA generated from 500 µg total RNA isolated from rainbow trout parr retina and served to confirm the presence of each gene of interest in the retina. PCR products were separated on 1.5% agarose gels and visualized using GelRed (Biotium, Hayward, CA, USA) staining with an Alpha Imager (Alpha Innotech, San Leandro, CA, USA) to ensure a single DNA band of appropriate size, which was then excised under UV light and the DNA extracted and purified using a MinElute Gel Extraction Kit (Qiagen, Missisauga, ON, Canada). The purified PCR products were cloned into PCR2.1-TOPO using a TOPO TA Cloning Kit (Invitrogen). Plasmid DNA was purified using an Eppendorf Miniprep kit (Eppendorf Canada, Mississauga, ON, Canada) and sequenced (Centre de Recherche du Centre hospitalier de

Table 1. Information on the gene-specific primers used for qRT-PCR analysis of retinal quadrants from parr, 2 day T4-treated parr and natural smolt

Gene	Forward primer	Reverse primer	Amplicon size (bp)	Primer concentration (µmol l^{-1})	MgCl concentration (mmol l^{-1})	Linear R^2 value/reaction efficiency (%)
SWS1	GGCTTTCTACCTACAGACTGC	CCTGCTATTGAACCCATGC	258	200	3	0.99/95.0
TRα	GCACAACATTCCCCACTTCT	AGTTCGTTGGGACACTCCAC	117	300	5	0.99/99.4
TRβ	TCACCTGTGAAGGATGCAAG	GACAGCGATGCACTTCTTGA	152	300	5	0.99/97.0
D2	ATTTTGTATGCCGATGCACA	TACGGCGCTAACCTCTGTTT	207	300	5	0.98/92.4
L8	GGTGTGGCTATGAATCCTGT	ACGACGAGCAGCAATAAGAC	113	200	3	0.99/100.6

l'Université Laval, Quebec, Canada). Positive identification of cloned PCR products confirmed the specificity of each primer pair.

Quantitative RT-PCR (qRT-PCR) analysis of individual total RNA samples from each retinal quadrant and fish were performed for each of the genes of interest: *SWS1*, *TRα*, *TRβ*, *D2* and the housekeeping gene, *L8*. Primer optimization reactions were run to determine the optimal concentration of primers to use in the qRT-PCR reactions; the resulting optimal equimolar amounts of primer varied between 100 and 300 μmol l^{-1} (Table 1). Standard curves were run for each primer pair using serial dilutions of rainbow trout parr retinal cDNA. The magnesium chloride concentration in the standard curve reactions was altered between 3 and 5 mmol l^{-1}, as needed, to generate repeatable R^2 values greater than 0.98 and reaction efficiencies between 90 and 100% (Table 1). Standard curves were run in triplicate and unknowns were run in duplicate. Master mixes were used whenever possible to reduce the possibility of pipetting error. Each 10 μl reaction contained Supermix-UDG (Invitrogen), ROX, 2 μl of 20-fold diluted retinal cDNA. The thermocycling program was 50°C for 2 min (although this extra step was not necessary for the samples used in the current study, it was performed to ensure consistent results with other studies using the same protocol), 95°C for 2 min followed by 40 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C. A dissociation curve was generated at the conclusion of each qRT-PCR run to ensure that all products generated during the thermocycling program, produced only one distinct DNA peak that dissociated at a consistent temperature. DNA amplification was performed using a MX3000P real-time quantitative PCR thermocycler (Stratagene), and the resultant amplification curves and data analysis performed by the accompanying software using the efficiency-corrected comparative quantitation method (Pfaffl, 2001) A relative method of qRT-PCR quantification was chosen to be the most relevant to analyze the data, as changes relative to the control parr were the target of the investigation and absolute copy numbers of RNA were not necessary for this purpose.

SWS1 opsin gene expression was examined in these same samples as an internal control, to verify that the same trends were present in this study as were found previously in Veldhoen et al. (Veldhoen et al., 2006).

It was deemed necessary to demonstrate that 2 days post-T4 treatment was the initiation of *SWS1* downregulation as hypothesized previously (Veldhoen et al., 2006), to ensure that any changes in TH regulation taking place 2 days post-T4 treatment immediately preceded *SWS1* downregulation and thus indirectly linked with the initiation of this process. Six fish were exposed to T4 or vehicle as above and were sampled after 3 days post-treatment. The whole retina was dissected free of the left eye and processed for RT-PCR and qRT-PCR as above. *O. mykiss* rod opsin, *RH1*, was used as a positive control transcript in the RT-PCR screening for *SWS1* opsin expression. RNA levels for *RH1* have been shown to remain constant over the experimental period and provide evidence for the presence of the photoreceptor layer in the extracted retina (Veldhoen et al., 2006).

Statistical analysis was performed on all data using SigmaPlot 11 software experiment. Two-way repeated-measures ANOVA was used to compare each gene of interest with the fish number (subject), retinal quadrant (Factor A) and treatment (Factor B) for all qRT-PCR data (Table 2). In an attempt to statistically compare the changes of the genes of interest in retinal quadrants between the T4-treated parr and natural smolts, a two-way analysis of variance was utilized. Retinal quadrants were designated as Factor A, and Factor B was either the T4-treated parr or the natural smolt (Table 2). The two-way ANOVA was considered most appropriate for this comparison as the two separate experiments utilized different control parr and were both expressed as fold change relative to the control parr. The Holm-Sidak method was employed to detect

Table 2. Statistical table showing selected values from the two-way repeated-measures ANOVA used to analyze the qRT-PCR values

Treatments compared	Statistical test	Source of variation	*SWS1*			*TRα*			*TRβ*			*D2*		
			d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f	*F*	*P*
2 day control and T4-treated parr	2-way repeated-measures ANOVA	Fish number (subject)	4	1.211	0.447	4	2.944	0.154	4	6.124	0.095	4	0.737	0.570
		Retinal quadrant (factor A)	3	0.989	0.428	3	0.912	0.462	3	0.878	0.479	3	4.903	0.027
		Treatment (factor B)	1	5.046	0.088	1	1.420	0.292	1	36.097	0.003	1	8.269	0.064
		Retinal quadrant × treatment	3	0.464	0.713	3	1.313	0.324	3	1.238	0.342	3	12.341	0.002
Natural parr and smolt	2-way repeated measures ANOVA	Fish number (subject)	4	1.257	0.49	4	1.110	0.467	4	0.572	0.696	4	0.672	0.656
		Retinal quadrant (factor A)	3	72.62	<0.001	3	0.665	0.589	3	0.235	0.870	3	9.865	0.001
		Treatment (factor B)	1	610.302	<0.001	1	33.383	0.004	1	46.654	0.002	1	3.189	0.147
		Retinal quadrant × treatment	3	42.105	<0.001	3	0.641	0.605	3	0.361	0.783	3	9.943	0.002
T4-treated parr and natural smolt	2-way ANOVA	Retinal quadrant (factor A)	3	15.405	<0.001	3	1.153	0.343	3	0.230	0.875	3	9.589	<0.001
		Treatment (factor B)	1	209.156	<0.001	1	67.817	<0.001	1	233.509	<0.001	1	8.548	0.006
		Retinal quadrant × treatment	3	11.223	<0.001	3	3.630	0.023	3	3.111	0.040	3	30.148	<0.001

differences between the treatment groups. A probability level of 0.05 was used in all cases to indicate significance. The parr treated with T4 or vehicle for 3 days were compared using Student's *t*-test. All data were expressed relative to control retina and as means ± standard error.

RESULTS

Quantitative RT-PCR analysis demonstrated that accumulation of *SWS1* opsin mRNA exhibited the same pattern as was found previously in Veldhoen et al. (Veldhoen et al., 2006) (Fig. 2). After 2 days of exogenous T4 treatment, initiation of *SWS1* downregulation was evident in all quadrants relative to the control parr (Fig. 2). Similarly, expression of *SWS1* opsin mRNA in the smolt retina exhibited a significant decrease in mRNA expression in all quadrants relative to the parr retina, as found previously (Fig. 3).

All four retinal quadrants of rainbow trout parr 2 days post-T4 treatment exhibited significant increases in *TRβ* expression relative to control parr (Fig. 2). Although the *TRα* gene appeared to exhibit a similar trend in mRNA expression, there were no significant changes in mRNA expression corresponding to this gene (Fig. 2). The DT quadrant showed the most pronounced apparent increase in *TRα* expression 2 days post-T4 treatment (Fig. 2). The pattern of mRNA expression for D2 was less clear; significant increases in mRNA expression were observed in the VT quadrant, while significant decreases were seen in both the VN and DT quadrants (Fig. 2).

In natural smolts, a significant decrease in all quadrants was found for expression of *TRα* and *TRβ* gene transcripts relative to natural parr, but the DT quadrant exhibited the most marked decreases in mRNA expression corresponding to these two genes (Fig. 3). Expression of mRNA from the *D2* gene did not present a clear trend across the retina, and only the VN and DT quadrant exhibited an increase in mRNA expression from the *D2* gene, with only the VN quadrant showing significance (Fig. 3). Comparison between the changes exhibited in retinal quadrants of the 2 day T4-treated parr and natural smolt revealed that, for each gene of interest, all retinal quadrants were significantly different between these two groups, except for D2 mRNA expression in the DN quadrant (Figs 2 and 3).

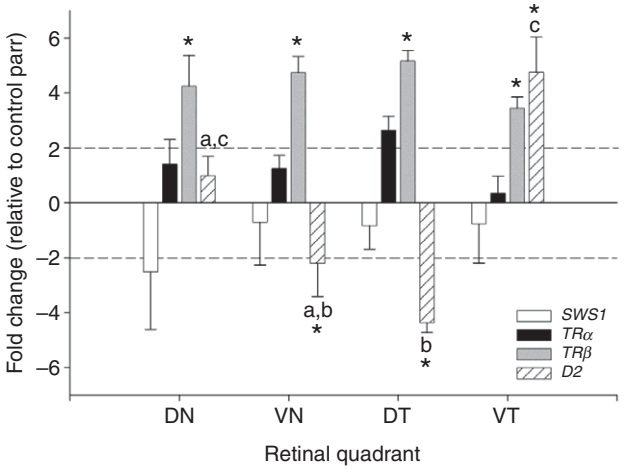

Fig. 2. Fold changes of *SWS1* opsin, *THRα*, *THRβ* and *D2* gene expression in retinal quadrants 2 days post-T4 treatment of rainbow trout parr relative to those of rainbow trout parr exposed only to treatment vehicle. DN, dorso-nasal retinal quadrant; DT, dorso-temporal retinal quadrant; VN, ventro-nasal retinal quadrant; VT, ventro-temporal retinal quadrant; *SWS1*, *SWS1* opsin gene; *TRα*, thyroid hormone receptor alpha; *TRβ*, thyroid hormone receptor beta; *D2*, deiodination type 2 enzyme. * denotes a significant difference (P<0.05) from the control fish; shared lowercase letters indicate that there is not a significant difference between quadrants for the gene of interest. Means ± s.e.m. (N=6).

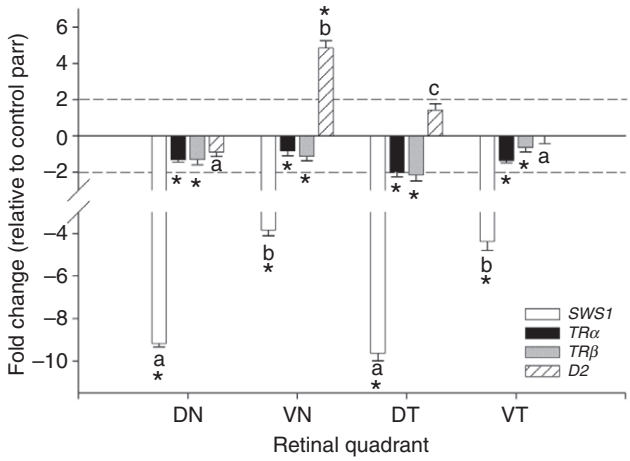

Fig. 3. Fold changes of *SWS1* opsin, *THRα*, *THRβ* and *D2* gene expression in retinal quadrants from rainbow trout smolts relative to those of rainbow trout parr. DN, dorso-nasal retinal quadrant; DT, dorso-temporal retinal quadrant; VN, ventro-nasal retinal quadrant; VT, ventro-temporal retinal quadrant; SWS1, *SWS1* opsin gene; TRα, thyroid hormone receptor alpha; TRβ, thyroid hormone receptor beta; D2, deiodinase type 2 enzyme. * denotes a significant difference (P<0.05) from the control fish; shared lowercase letters indicate that there is not a significant difference between quadrants for the gene of interest. Means ± s.e.m. (N=6).

The parr treated with T4 for 3 days lacked a visible DNA band corresponding to the *SWS1* opsin RT-PCR product from the retina, while the control parr showed a significantly brighter band at this time (Fig. 4A). Furthermore, *SWS1* mRNA expression was significantly lower after 3 days of treatment in the retina of T4-treated parr than the control (Fig. 4B). Additionally, significant increases in expression of *TRα* and *TRβ* transcripts and a significantly lower expression of *D2* mRNA were present at this time (Fig. 4B).

DISCUSSION

The results of this study indicate that T4 acts directly on the retina to induce *SWS1* opsin gene downregulation. This would lead to subsequent UVS cone loss. In addition, evidence for regional regulation of TH across the retina is indicated by the differential quadrant expression of the *TRα*, *TRβ* and *D2* genes in parr, smolt and T4-treated parr. This expression pattern supports the hypothesis that T4 acts directly on the retina and that regional regulation of *TR* receptor expression enables the preservation of UVS cones in the DT quadrant after natural smoltification has taken place.

Quantitative RT-PCR analysis of retinal quadrants from control and T4-treated parr demonstrated that *TRβ* gene expression was significantly upregulated in all four quadrants of the T4-treated parr after 2 days of T4 treatment, relative to the control parr. This upregulation of *TRβ* gene expression corresponds to data from a previous study in which UVS cones were lost 4 weeks after a measured *SWS1* opsin gene downregulation (Allison et al., 2003; Veldhoen et al., 2006). This upregulation of *TRβ* gene expression in the retina 2 days after the initiation of T4 treatment and preceding the onset of full downregulation of *SWS1* opsin gene expression indicates that TH acts directly on the retina, it is directly involved

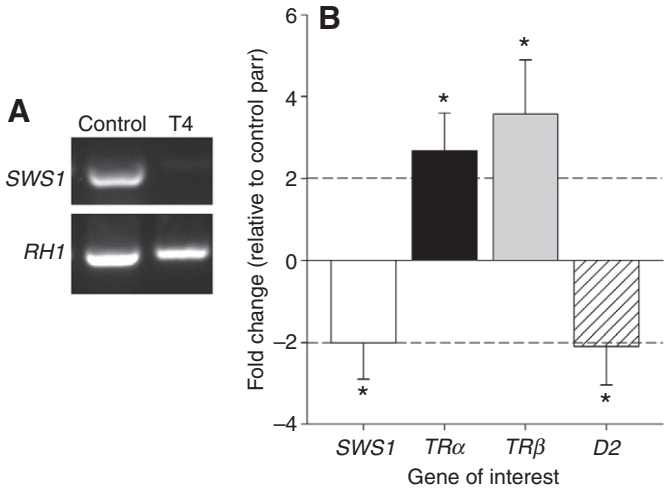

Fig. 4. *SWS1* opsin expression 3 days post-T4 treatment in the retina of rainbow trout parr. (A) Gel electrophoresis of the *SWS1* RT-PCR product from control and T4-treated parr after 3 days of treatment. *RH1* was used as a control gene to demonstrate the presence of the photoreceptor layer in the retinal samples. (B) qRT-PCR analysis of *SWS1*, *TRα*, *TRβ* and *D2* in control and T4-treated parr after 3 days of treatment. *SWS1*, *SWS1* opsin gene; *TRα*, thyroid hormone receptor alpha; *TRβ*, thyroid hormone receptor beta; *D2*, deiodinase type 2 enzyme. * denotes a significant difference (P<0.05) from the control fish. Means ± s.e.m. (N=6).

in *SWS1* opsin downregulation and it is responsible for the subsequent depopulation of UVS cones across the retina. Furthermore, the analysis of SWS1 expression after 3 days of T4 treatment demonstrates that, by this time, the whole retina exhibits a significant decrease in SWS1 mRNA accumulation. Furthermore, at this same time, significant increases in both *TRα* and *TRβ* transcripts are evident, and *D2* transcripts are significantly lower than the control fish across the entire retina. Thus, the selection of the 2 day post-T4 treatment time-point provides evidence that not only is T4 initiating changes directly at the retina but also the significant upregulation of *TRβ* (Fig. 2) takes place prior to significant changes in *SWS1* downregulation (Fig. 4B). Thus, these results provide strong evidence that TH plays a direct role in T4-induced *SWS1* downregulation.

Upregulation of *TRβ* gene expression in response to thyroid hormone treatment in fish is not unique to this study. This phenomenon has been found previously in zebrafish (*Danio rerio*) treated with TH and during Atlantic halibut (*Hippoglossus hippoglossus*) metamorphosis, where significant elevations in plasma TH levels coincide with upregulation of TR gene expression (Liu et al., 2000; Galay-Burgos et al., 2007). However, in these studies both *TRβ* and an alternate splice variant of *TRα*, *TRαA*, were upregulated. *TRαA* has also been identified in Japanese flounder (*Paralichthys olivaceus*), conger eel (*Conger myriaster*) and Atlantic salmon (*Salmo salar*), suggesting that it is likely also present in *O. mykiss*, although it has not yet been identified (Yamano et al., 1994; Marchand et al., 2001; Jones et al., 2002; Kwakami et al., 2003). Similarly, in the current study, although *TRα* gene expression did exhibit a trend towards upregulation in all retinal quadrants of T4-treated parr, there was only a marked elevation of *TRα* gene expression found in the DT quadrant. If there are, in fact, two splice variants of *TRα*, and only the *TRαA* variant tends to upregulate with T4 treatment, the *TRα* gene measured in this study may represent a net change in expression of both variants. In addition, the 3 day treatment of parr with T4 demonstrated that *TRα* is significantly upregulated by this time, although at 2 days post-T4 treatment, the upregulation of this gene is not significant. This suggests that *TRα* gene expression may have a slower response to T4 treatment than *TRβ*, or that *TRα* responds to changes in *TRβ*, *D2* or *SWS1* gene expression, or some other unknown secondary factor.

Using a mammalian model, Ng et al. found that mice lacking an alternate splice variant of *TRβ* (*TRβ2*) had an increase in S-cones (corresponding to UVS cones in teleost fish) and increased their distribution across the retina, a loss of M-cones (corresponding to MWS cones in teleosts) and disruption of the gradient of retinal cone distribution (Ng et al., 2001). The results from Ng et al. (Ng et al., 2001) indicate that *TR*s, especially the *TRβ2* splice variant of *TRβ*, may play a role in cone repression and provide some support for the proposed TRβ induced repression of *SWS1* opsin in T4-treated rainbow trout parr.

It was expected that *D2* gene expression would show a predictable pattern of expression complementing that of *TR* gene expression. However, this was not the case, and the response of *D2* gene expression was not clear. Significant upregulation of expression was observed in the VT quadrant, while significant downregulation was seen in both the VN and DT quadrants. The D2 enzyme predominantly generates T3 from T4 (Sambroni et al., 2001), and both the enzyme activity and *D2* gene expression were expected to decrease in T4-treated parr. This would slow the generation of T3 from the excessive amounts of T4 that the fish were being exposed to. However, based upon the *D2* gene expression levels found in the retina of T4-treated parr, there was no obvious pattern or correlation with receptor expression levels. It is important to note, however, that the neural retina is made up of multiple cell types and that the gene expression levels obtained in this study are the net expression level of each gene of interest in all of the cells present in the particular retinal quadrant. Although the deiodinase 3 (D3) enzyme, which predominantly converts T4 and T3 to inactive metabolites, was not examined in this study, the results obtained by Plate et al. (Plate et al., 2002) suggest that D3 plays a more prominent role in retinal TH regulation. It is likely that *D3* gene expression would be upregulated to clear excess thyroid hormone from the retina.

The expression of both *TRα* and *TRβ* genes exhibited similar expression profiles in the retinal quadrants of natural rainbow trout smolts. Both genes showed a trend towards downregulation in the retina, with significant downregulation found in only the DT quadrant. However, *D2* gene expression again did not exhibit a recognizable pattern of expression across the retina. There did seem to be a trend towards upregulation of *D2* gene expression in the DT quadrant, and the only significant change in expression was found in the VN quadrant of smolt, where the *D2* gene exhibited a >4-fold increase in expression relative to parr. It was interesting that the VN quadrant of both the T4-treated parr and the natural smolt retina exhibited opposite significant differences in *D2* gene expression. While this observation may not be important for this present study, future investigation should be carefully considered.

When the gene expression results were compared between the retinal quadrants of 2 day T4-treated parr and natural smolts, a pattern began to appear. Upregulation of both *TRα* and *TRβ* gene expression and downregulation of *D2* gene expression occurred in the DT quadrant 2 days post-T4 treatment, while natural smolts had the opposite trend in this quadrant, where UVS cones are retained (Allison et al., 2006). Although the statistical test used in this study suggests that all quadrants in the natural smolt exhibit significantly lower amounts of both *TRα* and *TRβ* mRNA expression relative to

the natural parr, the differences observed in the DT quadrant are most evident. This indicates that a gradient of thyroid hormone regulation does exist across the retina and that the changes in TR regulation in the DT quadrant may play a role in preserving the UVS cones still present in this quadrant. In addition, the changes in *TRα*, *TRβ* and *D2* gene expression in the DT quadrant were significantly different between the T4-treated parr and the natural smolt. This further demonstrates that these genes play a role in both preventing and initiating UVS cone loss. Recently, Applebury et al. found that murine cone photoreceptor distribution is likely patterned by TRβ2-regulated dorsal repression of S-opsin (Applebury et al., 2007). Activation of S-opsin in the ventral hemisphere, however, was not dependent on TRβ2. These results provide further support of those found in Ng et al. (Ng et al., 2001) and the present study. T4 may act directly on the retina through TRβ to initiate UVS cone loss, and regional TH regulation may preserve UVS cones in the DT quadrant during natural smoltification (Fig. 5). Clearly, this is not the only possible explanation for these results and more work is required to fully test this hypothesis; future studies will be done to address this issue. In particular, cellular localization of the TRs is essential to understanding how T4 initiates *SWS1* opsin downregulation and UVS cone loss. Once the cell or cone type that expresses these receptors has been identified, the specific target genes will also need to be determined before the mechanism of T4 involvement can be determined. It is well known that TRs mediate their changes in gene transcription by binding to specific sites on the promoters of target genes called thyroid response elements (TREs) (Hulbert, 2000). In order to show unquestionably that T4 targets a particular cell and gene, the isolation and sequencing of the promoters of potential target genes, and subsequent mapping of the location of the TREs on the segment of DNA, is required. Identifying potential TREs and demonstrating their ability to bind TRs and alter target gene transcription is ultimately necessary to show that TH is acting directly on the target gene. Thus, in order to provide unequivocal evidence for the ability of TH to alter transcription of *TRα*, *TRβ* and *SWS1* genes as hypothesized in this study, the presence of TREs on the promoters of these genes is necessary.

If natural parr exhibit upregulation of TRβ during natural *SWS1* opsin downregulation and UVS cone loss as hypothesized, this would likely only be seen during the elevation of plasma TH levels and earlier stages of UVS cone loss. A time course would be necessary to examine this phenomenon, but that was not the objective of this study. The objective of this study was to determine if T4 directly initiates *SWS1* downregulation and if topographical changes in TH regulation across the retina could explain the presence of UVS cones that persist in natural smolts; the results of this study strongly support our working hypothesis.

LIST OF ABBREVIATIONS

cDNA	complimentary deoxyribonucleic acid
D1	type 1 deiodinase
D2	type 2 deiodinase
D3	type 3 deiodinase
DN	dorso-nasal
DT	dorso-temporal
MWS	middle wavelength sensitive
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RH1	rod opsin
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SWS1	short-wave sensitive 1
T3	triiodothyronine
T4	thyroxine
TH	thyroid hormone
TR	thyroid hormone receptor
TRα	thyroid hormone receptor alpha
TRβ	thyroid hormone receptor beta
UVS	ultraviolet sensitive
VN	ventro-nasal
VT	ventro-temporal

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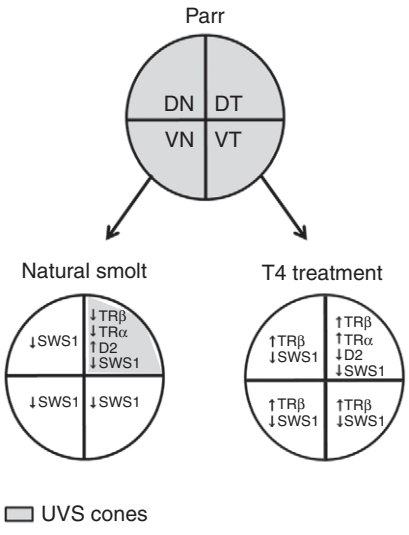

Fig. 5. Proposed model for the topographical changes in retinal regulation of thyroid hormone in response to T4 treatment and natural smoltification. DT, dorso-temporal retinal quadrant; VN, ventro-nasal retinal quadrant; VT, ventro-temporal retinal quadrant; *SWS1*, *SWS1* opsin; *TRα*, thyroid hormone receptor alpha; *TRβ*, thyroid hormone receptor beta; *D2*, type 2 deiodinase.

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