The Journal of Experimental Biology 203, 1925–1936 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JEB2743

VERTEBRATE ANCIENT (VA) OPSIN AND EXTRARETINAL PHOTORECEPTION IN THE ATLANTIC SALMON (*SALMO SALAR*)

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Accepted 30 March; published on WWW 23 May 2000

Summary

A member of a new photopigment family first isolated from teleost fish, vertebrate ancient (VA) opsin, has recently been shown to form a functional photopigment and to be expressed within a subset of horizontal and amacrine cells of the inner retina. These sites of expression (and structural features) of VA opsin suggest that this photopigment might mediate non-image-forming light-detection tasks. We attempted to gain support for this hypothesis by examining the expression of VA opsin within the central nervous system (CNS) (pineal and deep brain) of the Atlantic salmon *Salmo salar*. In addition, we examined the sites of rod-opsin, cone-opsin and α-transducin expression within the salmon CNS to provide a more complete description of the extraretinal photoreceptors of a teleost vertebrate. We show that multiple populations of cells within the salmon CNS appear to contain photoreceptors: VA opsin was strongly expressed in the pineal organ and in bilateral columns of subependymal cells in the epithalamus; anti-cone-opsin antibodies labelled cells within the pineal and numerous cells in the anterior hypothalamus (suprachiasmatic nucleus, nucleus preopticus magnocellularis, nucleus preopticus parvocellularis); anti-rod-opsin antibodies labelled cells within the pineal but no other areas within the central brain; and anti-α-transducin antibodies labelled cells within the pineal and the ventral telencephalon. Collectively, our results suggest that VA opsin is a photopigment specialised for irradiance detection tasks within the eye, pineal and central brain, and that the salmon has multiple and varied populations of photoreceptors within the CNS. We review the significance of these findings within the broad context of vertebrate extraretinal photoreception.

Key words: extraretinal photoreceptor, teleost fish, immunocytochemistry, *in situ* hybridisation, opsin, pineal, VA opsin, vertebrate photoreception, Atlantic salmon, *Salmo salar*.

Introduction

Vertebrate photopigments are remarkably conserved, consisting of an opsin protein coupled to a chromophore derived from an 11-*cis* form of vitamin A retinaldehyde (Wald, 1968). Photoisomerisation of 11-*cis* retinal to the all-*trans* state induces conformational changes in the opsin which, in turn, activate a G-protein (transducin) phototransduction cascade (Rodieck, 1998). Opsin-based photopigments are expressed in a number of different regions of the vertebrate central nervous system (CNS) and are classified as: (i) intracranial pineal organs (epiphysis cerebri), which are photoreceptive in all non-mammalian vertebrates; (ii) parapineal organs, which are unique to the Agnatha and teleost fish; (iii) extracranial 'third eyes', termed frontal organs in frogs and parietal eyes in lizards; (iv) deep brain photoreceptors, found in all non-mammalian vertebrates; and (v) lateral eyes (Foster and Soni, 1998). Traditionally, these diverse photoreceptor organs have been associated with two broad photosensory tasks. The lateral eyes, employing rod and cone photoreceptors, mediate image detection (vision), whilst the extraretinal photoreceptors are thought to use environmental irradiance cues for tasks that include the regulation (entrainment) of circadian rhythms, behavioural orientation, the regulation of body pigmentation and coloration, and the regulation of pupil size (Shand and Foster, 1999). In recent years, this separation of photosensory tasks into 'ocular vision' and 'extra-ocular irradiance detection' has become blurred. For example, mice in which both rod and cone photoreceptors have been genetically ablated are still capable of regulating both circadian rhythms of behaviour and pineal melatonin synthesis in response to light. Removal of the eyes blocks these responses, suggesting that the mammalian retina contains unrecognised, non-visual photoreceptors that mediate irradiance detection tasks (Freedman et al., 1999; Lucas et al., 1999).

The hypothesis that the inner retina of mammals, and

perhaps of all vertebrates, contains irradiance detectors is supported by the discovery of a new photopigment family in teleost fish, the vertebrate ancient (VA) opsins (Soni and Foster, 1997; Soni et al., 1998). Preliminary studies showed that VA opsin is not expressed in retinal rods and cones but in a subset of horizontal and amacrine cells of the teleost retina (Soni et al., 1998). Furthermore, VA opsin differs from rod and cone opsins in ways that suggest a non-image-forming function (Soni and Foster, 1997). Collectively, the sites of expression and structural features of VA opsin suggest that this photopigment might mediate non-visual light-detection tasks. This hypothesis would be strengthened if VA opsin were shown to be expressed in those extraretinal areas of the CNS (the pineal and deep brain) known to regulate physiological responses to gross changes in environmental light levels (Shand and Foster, 1999).

The aims of the present study were to place VA opsin within the framework of retinal and extraretinal photoreception and to provide a more complete description of the extraretinal photoreceptors of a teleost. To this end, we have addressed the following related questions. (i) Is VA opsin expressed in extraretinal photoreceptors? (ii) Do teleosts possess multiple extraretinal photopigments? (iii) Can sites of opsin expression be correlated with areas of the brain known to regulate physiological responses to light?

Materials and methods

Animals

Pre-smolt Atlantic salmon (*Salmo salar*) were collected from rivers in Asturias (Northern Spain) and then maintained in a 400 l aquarium and exposed to a 12 h:12 h light:dark photoperiod generated using fluorescent lights.

VA opsin cRNA in situ *hybridisation histochemistry*

Fish were selected at random time points during the light:dark cycle (08.00–12:00 h), deeply anaesthetised in tricaine methanesulphonate (MS-222, Sigma) and perfused through the heart with 4 % buffered paraformaldehyde at room temperature (20 °C). The eyes (lenses removed) and brains were excised, and the tissue was fixed for a further 12–24 h at 4 °C. Following a series of washes in 0.1 mol l^{-1} phosphate-buffered saline (PBS) (pH 7.2), the tissue was immersed in 0.1 mol l^{-1} PBS containing 30 % sucrose, mounted in Tissue Tek cryoprotection medium (Sakura Finetechnical Co, Japan) and frozen in iso-pentane cooled on dry ice. Consecutive 10 µm sections were cut using a Leica 1900M cryostat and collected onto slides coated with 3-aminopropyltriethoxysilane (TESPA, Merk). Slides were maintained at 35 °C for 1.5–2 h preceding storage in an airtight box at −80 °C.

Digoxigenin (DIG)-labelled riboprobes (sense and antisense) were transcribed using a DIG RNA labelling kit (Boehringer Mannheim/Roche). The cDNA template used was a full-length VA opsin clone in Bluescript SK+ (Stratagene), previously sequenced (Soni and Foster, 1997) to confirm insert identity and orientation. Following the manufacturer's instructions, T3 or T7 RNA polymerase was used to produce antisense or sense probes respectively. The probes were reduced to approximately 200 nucleotides in length by partial alkaline hydrolysis (0.4 mol l^{-1} $NaHCO_3$ and 0.6 mol l^{-1} Na_2CO_3 incubated at 60 °C). Unincorporated nucleotides were removed by passing the probes through ProbeQuant G50 micro columns (Amersham Pharmacia Biotech). Further purification was achieved by ethanol precipitation with 4 mol l^{-1} LiCl, prior to dilution in hybridisation buffer and storage at −80 °C.

Slides were brought to room temperature and re-fixed with 4 % paraformaldehyde in PBS for 20 min. Tissue was permeabilised using a 200 µg ml^{-1} solution of Proteinase K for a further 20 min, prior to incubation with 0.2 mol l^{-1} HCl and subsequent acetylation in acidified triethanolamine (10 min). Prehybridisation was undertaken at 65 °C in hybridisation buffer (50 % formamide, 5× standard saline citrate, SSC, 50 µg ml^{-1} yeast RNA, 50 µg ml^{-1} heparin, 0.1 % Tween-20) using sections covered with a clean coverslip. The coverslips were carefully removed, and the pre-hybridisation solution was replaced with 100 ng of probe in 200 µl of fresh hybridisation buffer. Slides were again covered with a coverslip and hybridised at 65 °C for 16–20 h. The coverslips were removed, and the slides were incubated in 25 % formamide, 2× SSC solution at 65 °C. A solution of 20 µg ml^{-1} RNAase A was applied to the slides at 37 °C for 30 min prior to the application of labelling kit blocking solution. A monoclonal anti-DIG antibody conjugated with alkaline phosphatase (Boehringer Mannheim/Roche) was applied for 1 h at room temperature. Colour developing of immunopositive cells was achieved by washing the sections with maleic acid buffer (100 mmol l^{-1} maleic acid, 150 mmol l^{-1} NaCl, pH 7.5 at 20 °C) incorporating 1 % Tween 20 and applying a drop of BM Purple AP substrate (Boehringer Mannheim/Roche). Slides were subsequently incubated in a light-tight damp container. Developing time varied between 18 and 30 h before the colour reaction was terminated by immersion in water.

All results were viewed and photographed using a Zeiss Axiopan photomicroscope, and anatomical figures were prepared by scanning photomicrographs into Photoshop (Adobe). Anatomical boundaries were defined using the brain atlas of the Atlantic salmon (*Salmo salar*) (Peter et al., 1991) and the definitions provided by Davis and Northcutt (1983).

VA opsin mRNA reverse transcriptase polymerase chain reaction

Pre-smolt Atlantic salmon (as above) were selected between 08:00 and 12:00 h and deeply anaesthetised as described previously. Pineal glands and retinae were dissected using dedicated and autoclaved instruments to prevent cross-contamination, and immediately frozen at −80 °C. Poly(A+) mRNA was extracted from 15 pineal glands and two retinae using a QuickPrep micro mRNA kit (Pharmacia). cDNA was synthesised using the 3′ RACE system (Gibco BRL) for use in polymerase chain reactions (PCRs) using components and cycling conditions similar to those described by Bellingham et al. (1997). Gene-specific primers were designed from the

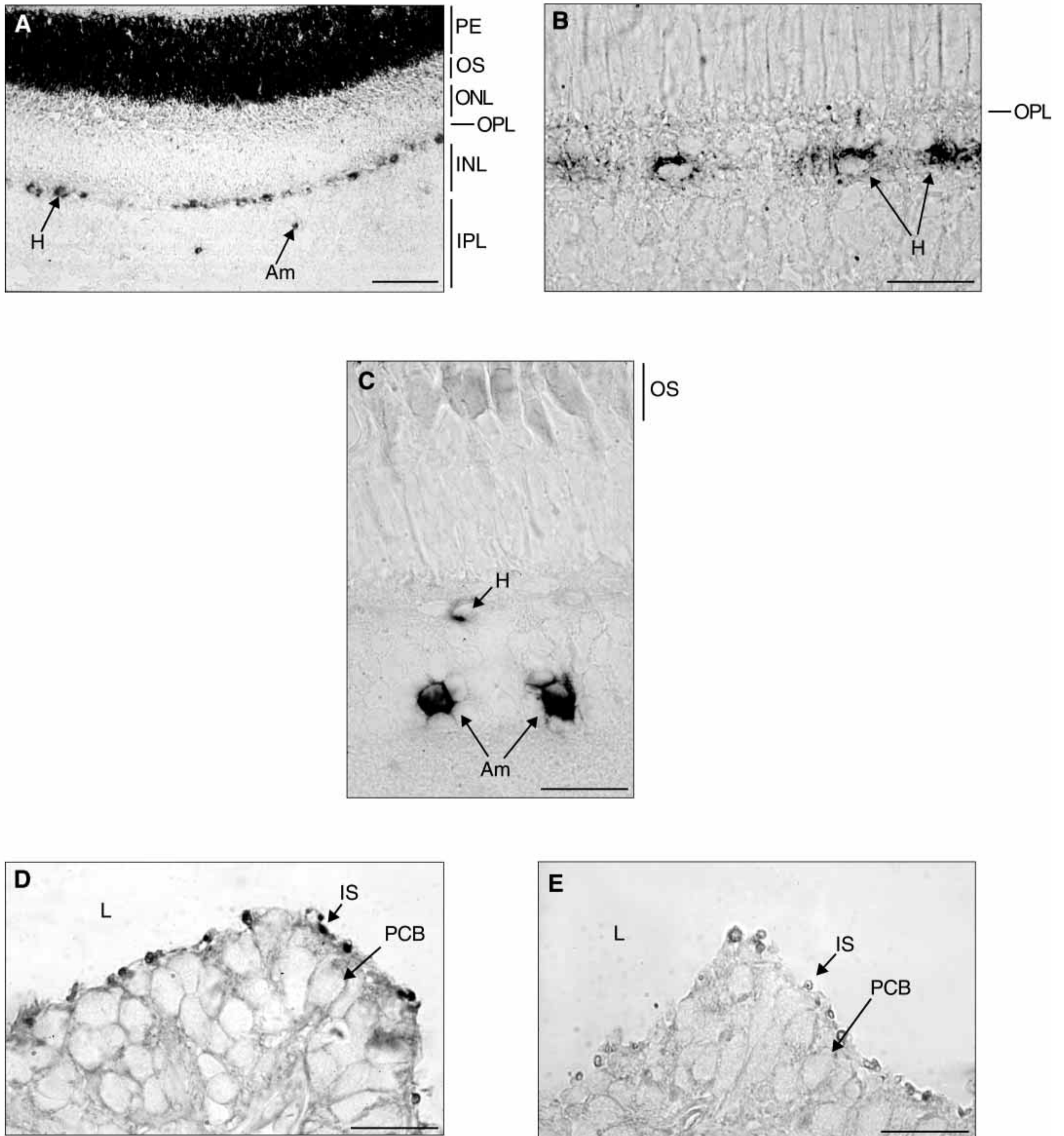

Fig. 1. *In situ* hybridisation using digoxigenin-labelled cRNA probes transcribed from vertebrate ancient (VA) opsin clones and used on cryostat-cut retina and pineal sections from the Atlantic salmon. (A) The distribution of VA-opsin-expressing horizontal (H) and amacrine (Am) cells across the retina; (B,C) higher magnification of VA opsin expression in horizontal (B) and amacrine (C) cells; (D) antisense probe labelling of pineal inner/outer segments; (E) sense probe showing no labelling of a pineal section. This negative result illustrates the specificity of the procedure. Am, amacrine cell; H, horizontal cell; INL, inner nuclear layer; IPL, inner plexiform layer; IS, pinealocyte inner/outer segments; L, pineal lumen; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, photoreceptor outer segments; PCB, pinealocyte cell body; PE, pigment epithelium. Scale bars: A, 100 μm; B–E, 25 μm.

sequence of salmon VA opsin on the basis that the predicted product spans three introns (P. Moutsaki, J. Bellingham and R. G. Foster, unpublished data), so providing an appropriate control against genomic template contamination.

Primer sequences were as follows: VASALF, 5′-TATAAC-GAGGCATCAGAGATC-3′ (corresponding to bases 37–57 of GenBank AF001499), and VASALR, 5′-AGGTAAGCGACG-ATCATCACC-3′ (complementary to bases 759–779 of GenBank AF001499). Primers were used at an annealing temperature of 62 °C and in 1.5 $mmol\,l^{-1}$ $MgCl_2$. The resultant PCR products were visualised by agarose gel electrophoresis and ethidium bromide staining. The 742 base pair (bp) PCR products were excised, gel-purified using a QIAquick gel extraction kit (Qiagen), and cloned into pGEM-T-Easy (Promega). Ligations were used to transform DH5α subcloning efficiency competent cells (Gibco BRL). Recombinant clones of interest were sequenced on an ABI PRISM 377 DNA sequencer (Perkin Elmer) using the ABI PRISM BigDye terminator cycle sequencing kit (Perkin Elmer) and M13 primers to confirm sequence identity. Both strands of all clones were sequenced.

Opsin and transducin immunocytochemistry

Salmon were anaesthetised with MS-222 and fixed by intracardiac perfusion with Bouin's fixative. Brains were removed and placed in the same fixative for 48 h prior to dehydration, paraffin-embedding and sectioning. Transverse and sagittal sections (8 μm thick) were collected on gelatin-coated slides and hydrated for 30 min in 0.1 $mol\,l^{-1}$ PBS containing 0.2 % Triton X-100 (PBS-T).

Endogenous peroxidase activity was blocked by incubation for 10 min in 0.1 $mol\,l^{-1}$ PBS (pH 7.4) containing 1 % methanol and 0.3 % H_2O_2, followed by three washes in PBS-T. Possible background staining was blocked by incubating the section for 20–30 min in blocking serum (diluted 1:30 in PBS-T). Sections were then transferred into one of several primary antisera (72 h in a humid chamber at 4 °C). The primary antisera used in this study have been used previously to describe ocular and extraocular photoreceptors in other groups of vertebrates (Foster et al., 1993, 1994; Garcia-Fernandez and Foster, 1994; Garcia-Fernandez et al., 1997; Grace et al., 1996) and were as follows. (i) CERN 874 antiserum was raised in rabbits against purified cone opsin. This antibody is specific for vertebrate cone opsins and will not cross-react with rod opsins at the dilutions used in this study (1:4000 in PBS-T). (ii) α-Transducin (AS), a polyclonal antibody, was raised against a decapeptide corresponding to the C terminus of the α-subunit of retinal G-protein (α-transducin). This decapeptide sequence is identical in both rod and cone α-transducins (Simonds et al., 1989). AS was used at a dilution of 1:200. (iii) CERN 858 antiserum was raised in rabbits against purified lipid-free bovine rhodopsin (Margry et al., 1983; Schalken and DeGrip, 1986). This antiserum is monospecific for rod opsin (Janssen, 1991; Schalken, 1987) and was used at a dilution of 1:2000 in PBS-T. Following fixation, several isolated pineal glands and retinae were embedded in Araldite resin (Durcupan, Fluka). Blocks were sectioned (1.5 μm) with glass knives on a Reicher ultracut ultramicrotome. After removal of the resin with sodium ethoxide, immunocytochemical protocols were identical to those used for paraffin sections. Immunocytochemical results were visualised and analysed as described for the *in situ* hybridisation (see above).

Results

VA opsin in situ *hybridisation and reverse transcriptase polymerase chain reaction (RT-PCR)*

Expression of VA opsin was restricted to a number of different cell types in the retina and brain of Atlantic salmon. Our failure to identify any labelling using 'sense' DIG-labelled cRNA probes (see Fig. 1E) confirmed the specificity of these findings in all tissue types and locations. In the retina, cells expressing VA opsin were restricted to two subsets of neurons of the inner retina. One cell type had a location and morphology typical of horizontal cells, and the other neurons resembled amacrine cells. These cells were more concentrated towards the periphery of the retina and were never found in the central portion of the retina (Fig. 1A–C). VA opsin expression was never observed in the retinal rods or cones of the outer retina. VA opsin was also expressed in the pineal within the inner/outer segments of pinealocytes located in the more caudal regions of the pineal (Fig. 1D). RT-PCR using gene-specific primers designed against salmon VA opsin amplified the predicted 742 bp fragment from both retinal and pineal cDNA (Fig. 2). The nucleotide sequence of this product confirmed the presence of VA opsin within both these tissues. In the diencephalon, VA opsin was expressed at high levels within bilateral columns of cells in the epithalamus. These cells were subependymal. We have yet to demonstrate whether these cells have a sensory-like dendrite projecting into the ventricular lumen and could therefore be called cerebrospinal fluid (CSF)-contacting neurons (Fig. 3A–F). The most rostral cells were localised in the subhabenular region, forming a

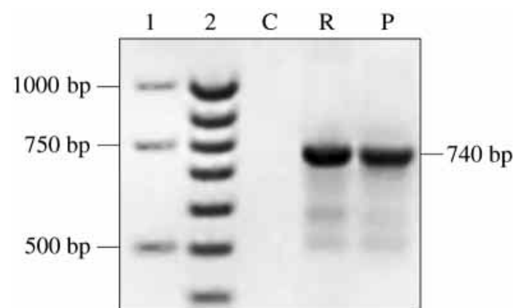

Fig. 2. Reverse transcriptase polymerase chain reaction (RT-PCR) isolation of vertebrate ancient (VA) opsin from the salmon retina (R) and pineal (P). Gene-specific primers amplified a 742 base pair fragment that was sequenced and identified as VA opsin. Lane C contains a non-DNA negative control. Lanes 1 and 2 are DNA size ladders. Numerical values indicate the number of base pairs (bp).

compact groups of neurons interspersed with non-VA-opsin-expressing cells (Fig. 3A,B). From the subhabenula, the column of VA-opsin-expressing cells extends caudally and ventrally, dropping below the fasciculus retroflexus and

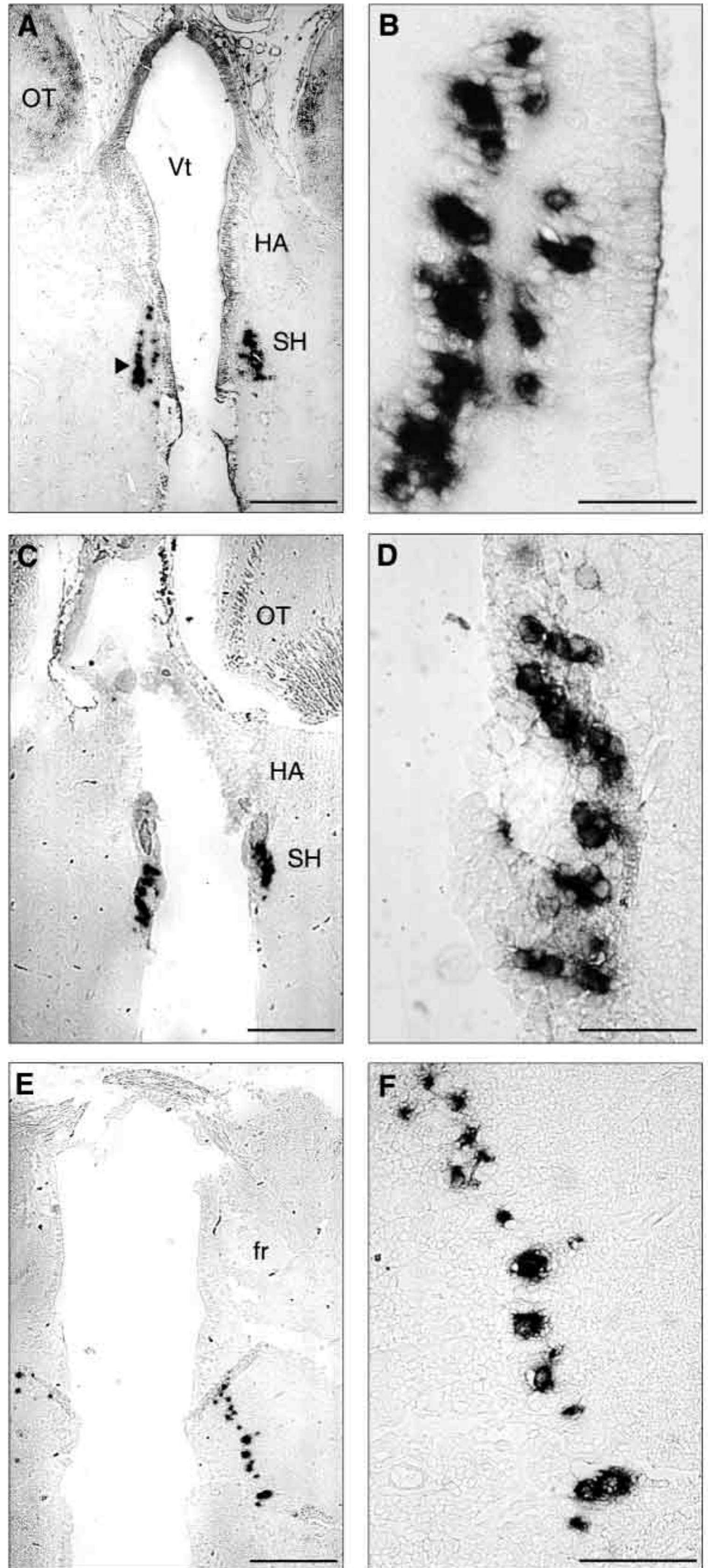

Fig. 3. *In situ* hybridisation using antisense digoxigenin-labelled cRNA probes transcribed from vertebrate ancient (VA) opsin clones on a rostral-to-caudal (A–E) series of frontal sections through the salmon diencephalon. (A) The most rostral section of the series, showing bilateral groups of cells expressing VA opsin within the subhabenula (arrowhead); (B) higher magnification of a group of VA cells in the subhabenula; (C) a more caudal section showing the distribution of VA-opsin-expressing cells within the subhabenula; (D) higher magnification of part of C showing one bilateral cluster of VA-expressing cells; (E) view of the more scattered distribution of VA-opsin-expressing cells in the caudal subhabenula; (F) higher magnification of part of E showing one bilateral cluster of VA-expressing cells. fr, fasciculus retroflexus; HA, habenula; OT, optic tectum; SH, subhabenular area; Vt, ventricle. Scale bars: A,C,E, 200 μm; B,D,F, 50 μm.

terminating at the level of the posterior commissure. As the column of VA neurons extends caudally, the density of cells becomes reduced (Fig. 3E,F; see also Figs 6, 7). Results were similar for all the animals tested irrespective of the time at which they were selected (08:00–12:00 h).

α-Transducin-like immunoreactivity

In the retina, as anticipated, both rod and cone photoreceptors were labelled by the α-transducin antiserum. In the pineal, most pinealocytes were also immunopositive. In the brain, α-transducin-like immunoreactivity was observed in cells and fibres of the ventral telencephalon, specifically the lateral and commissural parts of the area ventralis telencephali, with occasional fibres scattered rostral and dorsal to the anterior commissure (Fig. 4A,B; see also Figs 6, 7). No other areas of the brain were labelled by these antibodies.

Cone opsin immunoreactivity (CERN 874)

In the retina, CERN 874 labelled cone cell outer segments, producing a distinctive retinal mosaic (Fig. 4C). In the pineal,

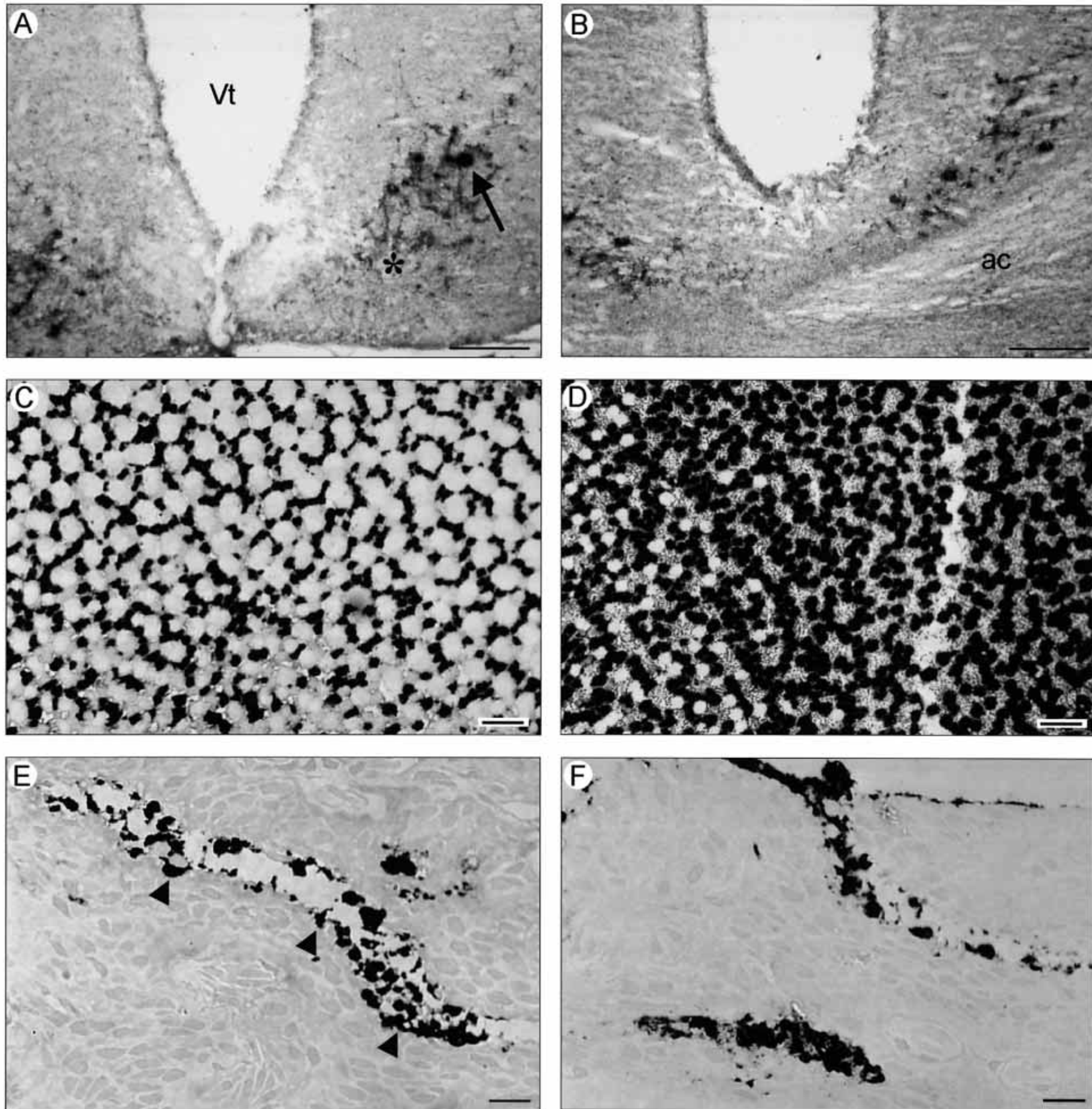

Fig. 4. Immunoreactive cells in the central nervous system of the Atlantic salmon. (A,B) Frontal sections through the telencephalon showing cells (arrow) and fibres (*) immunolabelled with α-transducin antibodies within the lateral and commissural parts of the ventral telencephalon. (C,D) Semi-thin sections through the retina showing the retinal 'mosaic' of photoreceptors labelled with (C) anti-cone-opsin antiserum (CERN 874) and (D) anti-rod-opsin antiserum (CERN 858). (E,F) Semi-thin sections through the pineal showing pinealocyte outer segments (arrowheads) labelled with (E) anti-cone-opsin antiserum (CERN 874) and (F) anti-rod-opsin antiserum (CERN 858). ac, anterior commissure; Vt, ventricle. Scale bars: A,B, 100 μm; C–F, 25 μm.

numerous cell bodies and outer/inner segments of pinealocytes were labelled (Fig. 4E). In the central brain, various regions of the anterior hypothalamus showed strong labelling. Serial reconstruction of this area of the brain identified bilateral clusters of immunoreactive cells that extended through the suprachiasmatic nucleus (SCN, also referred to as the nucleus anterioris periventricularis), the dorsal preoptic recess, the nucleus preopticus parvocellularis and the nucleus preopticus magnocellularis (NPOm) (Figs 5A–D, 6, 7). The density of immunoreactive neurons in the caudal part of the NPOm was low, numbering only 3–4 per 8 μm frontal section. Labelled neurons appeared to lack an intraventricular processes. Bilateral groups of immunoreactive fibres originated from the preoptic area and ran caudally and ventrally before passing

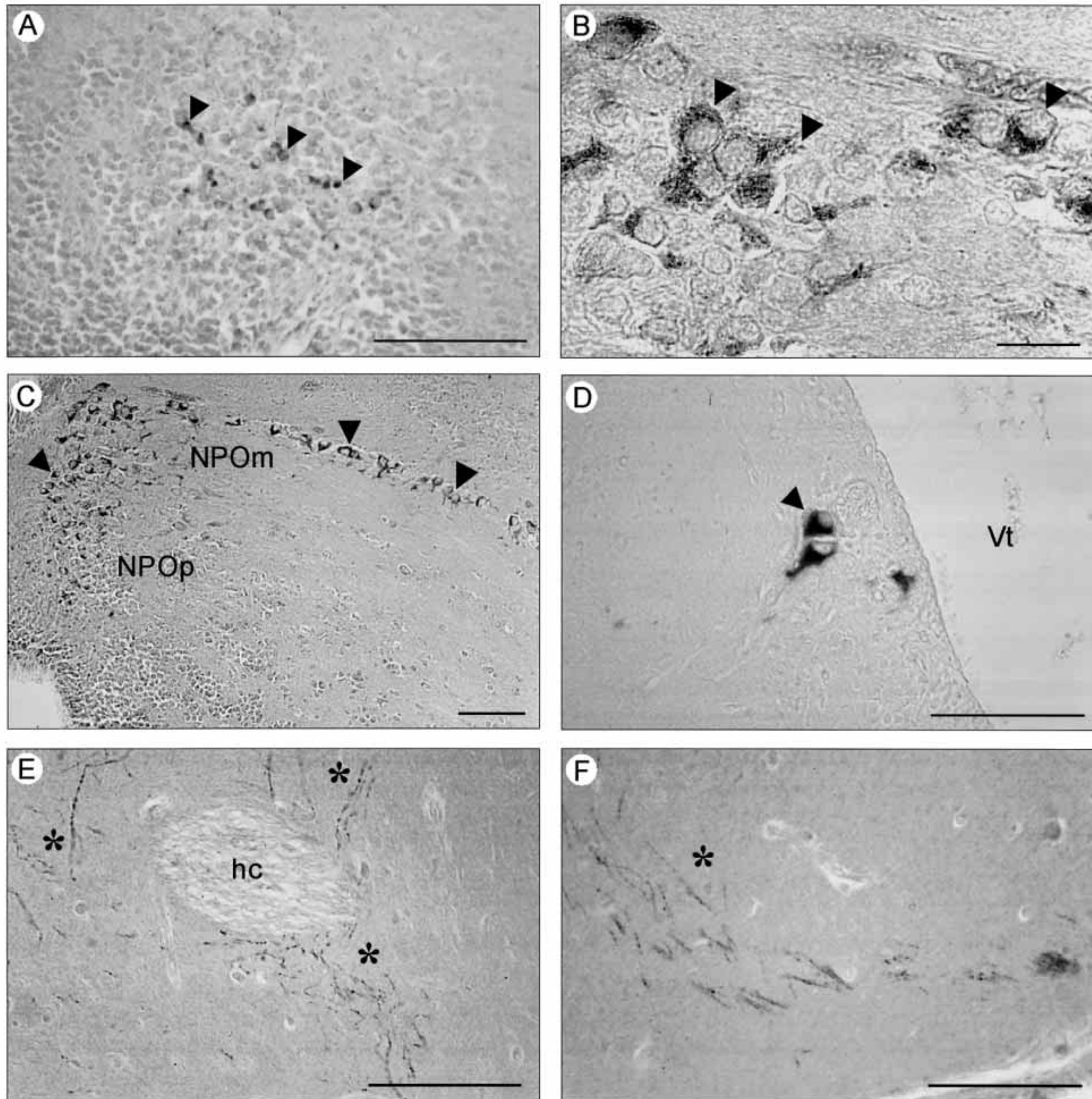

Fig. 5. Immunopositive cells and fibres in the salmon diencephalon labelled with anti-cone-opsin antiserum (CERN 874). (A) Sagittal section through the diencephalon showing a small group of immunopositive cells (arrowheads) in the area of the suprachiasmatic nucleus; (B) transverse section showing cone-opsin-immunopositive cells (arrowheads) in the NPOm area of the hypothalamus; (C) parasagittal section through the diencephalon showing the distribution of cone-opsin-immunopositive cells (arrowheads) extending from the NPOp to the caudal portion of the NPOm (see Figs 6, 7); (D) transverse section through the caudal part of the NPOm showing the low density of cone-opsin-immunopositive cells (arrowhead); (E) sagittal section through the diencephalon showing cone-opsin-immunopositive fibres (*) passing around the horizontal commissure; (F) sagittal sections through the diencephalon showing cone-opsin-immunopositive fibres (*) passing into the basal hypothalamus. hc, horizontal commissure; NPOm, nucleus preopticus magnocellularis; NPOp, nucleus preopticus parvocellularis; Vt, ventricle. Scale bars: A,D–F, 100 μm; B, 50 μm; C, 200 μm.

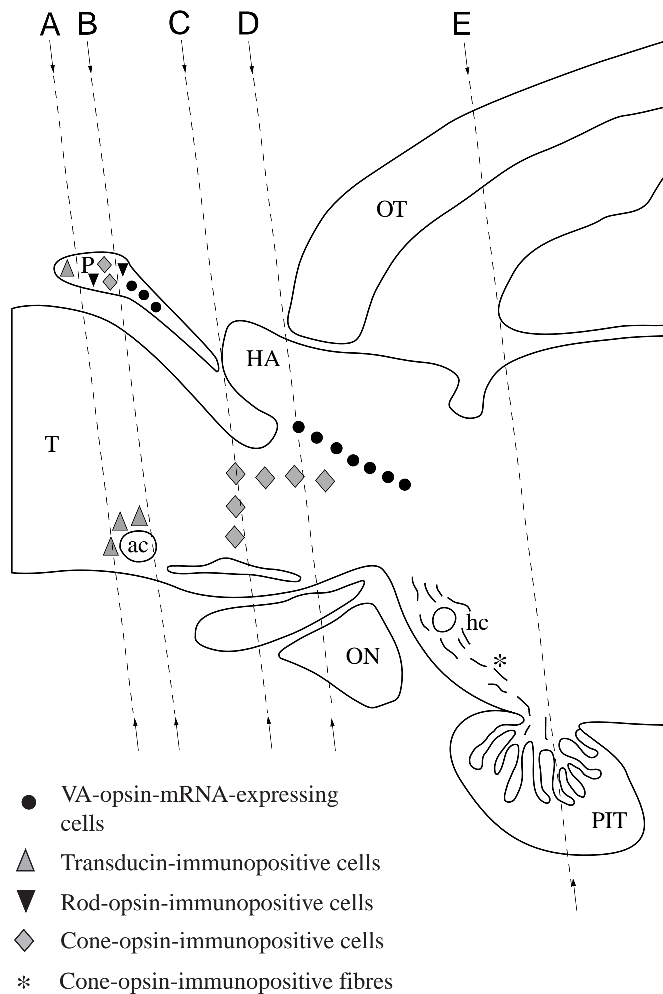

Fig. 6. Summary diagram representing a sagittal view of the salmon brain showing the distribution of cells and fibres immunolabelled by opsin and α-transducin antibodies and expressing vertebrate ancient (VA) opsin. A–E refer to the plane of frontal sections represented in Fig. 7. Anterior is to the left. ac, anterior commissure; HA, habenula; hc, horizontal commissure; ON, optic nerve; OT, optic tectum; P, pineal organ; PIT, pituitary; T, telencephalon.

down into the basal hypothalamus. The fibres dispersed around the horizontal commissure before regrouping into small fascicles traversing the nucleus lateralis tuberis to the neurohypophysis (Figs 5E,F, 6, 7).

Rod opsin immunoreactivity (CERN 858)

In the retina, rod outer segments were strongly labelled and formed a mosaic complementary to that of CERN 874 (Fig. 4D). In the pineal, outer/inner segments of the pinealocytes were labelled in a manner similar to the pattern observed with the cone opsin antibodies (Fig. 4F).

Discussion

The results presented here have confirmed our initial observations that VA opsin is expressed in a subset of horizontal and amacrine cells of the salmon retina (Soni et al., 1998). In addition, we have shown that VA opsin is expressed within the pineal and subhabenular regions of the brain. One of the primary aims of the current study was to determine whether VA opsin is expressed outside the retina. We reasoned that the inner retinal location (Soni et al., 1998) and the structural features of VA opsin (Soni and Foster, 1997) suggested a specialised non-visual role for this photopigment. This hypothesis is strongly supported by the findings presented in this paper. We have shown that VA opsin is a photopigment uniquely expressed within the inner retina, pineal and epithalamus of a teleost fish and has a pattern of expression apparently specialised for irradiance detection tasks.

The sites of strongest VA opsin expression in the salmon CNS were bilateral columns of subependymal cells in the epithalamus. These cells extend caudally and ventrally from the subhabenular region to the posterior commissure. We have yet to establish whether these cells are CSF-contacting neurons and, hence, similar to the putative encephalic photoreceptors described in birds (Silver et al., 1988) and reptiles (Foster et al., 1993) or neurosecretory cells of the NPOm and similar to the putative encephalic photoreceptors of amphibians (Foster et al., 1994; Provencio et al., 1998; Yoshikawa et al., 1994). Expression of VA opsin within epithalamic cells provides the first evidence that this area of the teleost brain contains photoreceptors. However, antibodies to opsin and α-transducin have previously been shown to label cells in an area homologous to the subhabenula in the river and silver lamprey (Garcia-Fernandez et al., 1997).

The salmon pineal was also shown to express VA opsin using both *in situ* hybridisation and RT-PCR methodologies. The salmonid pineal has been shown to be directly light-sensitive (Ekström and Meissl, 1997) and to regulate melatonin synthesis directly in response to light (Begay et al., 1998), so VA opsin might contribute to these or as yet unidentified photic responses. We were unable to find any evidence for expression of VA opsin within the parapineal of the salmon. Little is known of this region of the teleost brain (Vollrath, 1981). A few photoreceptor-like structures have been reported in this structure (Rudeberg, 1969; Ekström et al., 1983), and a novel fish opsin, parapineal opsin, has recently been isolated from the parapineal of catfish (Blackshaw and Snyder, 1997). Like VA opsin, the role of parapineal opsin is currently unknown, but it may include irradiance detection tasks.

In parallel with VA studies *in situ*, we determined the distribution of rod-, cone- and α-transducin-like proteins within the salmon retina and brain using immunocytochemistry. The selective distribution of rod and cone opsins within the retinal mosaic of the salmon has been well documented (Wagner, 1990), and our duplication of these results confirms the specificity of the rod and cone antibodies used in the present study. In the retina, α-transducin antibodies labelled rod and cone photoreceptors in a similar pattern to previous reports (e.g. van Veen et al., 1986). Despite our findings of expression of VA opsin in a subset of horizontal and amacrine cell, no cells within the inner retina were labelled

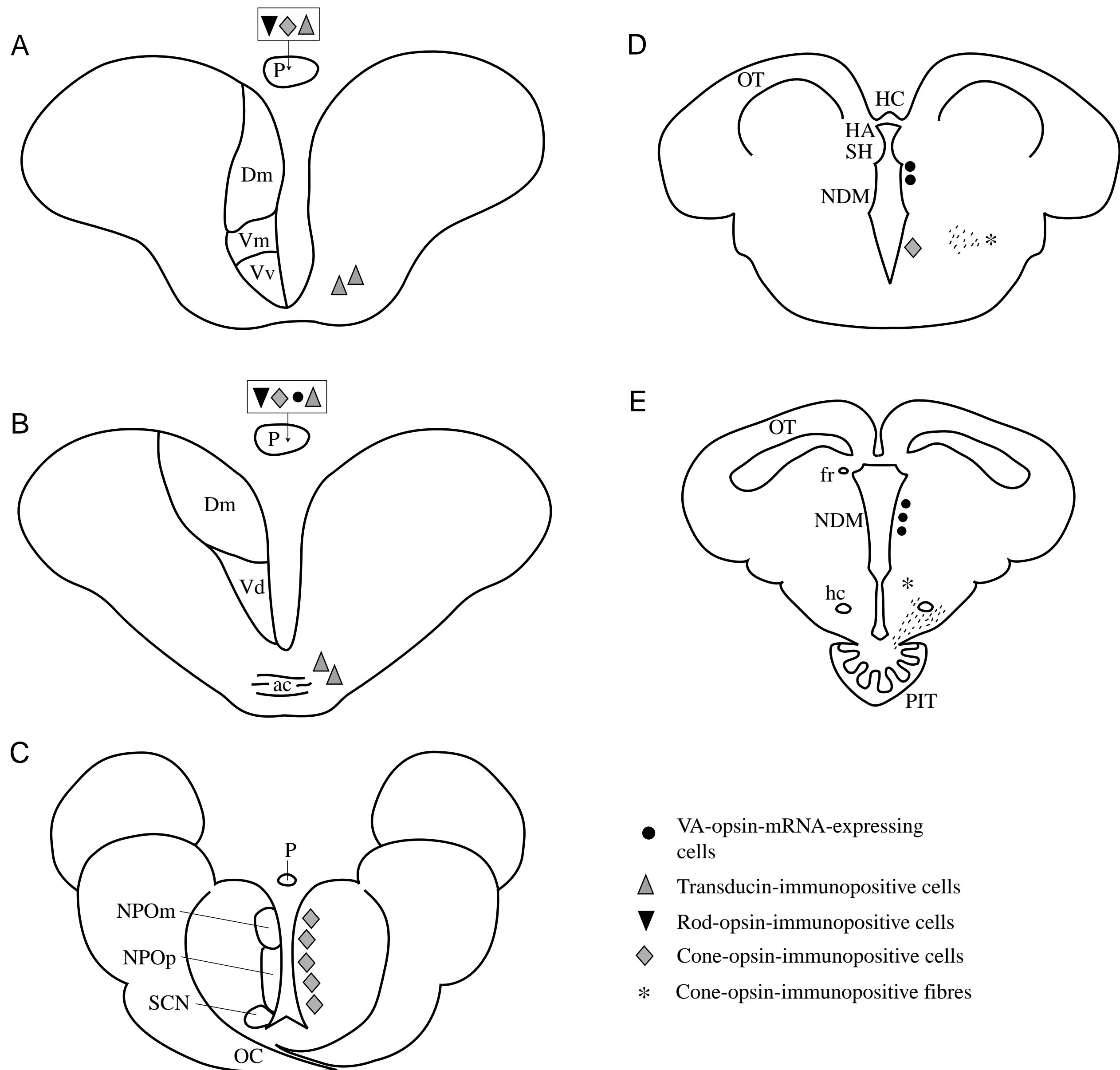

Fig. 7. Diagram showing a rostral-to-caudal series of transverse sections of the salmon brain showing the distribution of cells and fibres immunolabelled by opsin and α-transducin antibodies and expressing vertebrate ancient (VA) opsin. A–E refer to the planes of section indicated in Fig. 6. ac, anterior commissure; Dm, dorsomedial telencephalic area; fr, fasciculus retroflexus; HA, habenula; HC, habenular commissure; hc, horizontal commissure; NDM, nucleus dorsomedialis; NPOm, nucleus preopticus magnocellularis; NPOp, nucleus preopticus parvocellularis; OC, optic chiasm; OT, optic tectum; P, pineal organ; PIT, pituitary; SH, subhabenular area; SCN, suprachiasmatic nucleus; Vd, area ventralis telencephali pars dorsalis; Vm, ventromedial telencephalic area; Vv, area ventralis telencephali pars ventralis.

with α-transducin antibodies. The pineal, like the outer retina, was labelled by the rod, cone and α-transducin antibodies. By comparing the opsin labelling of consecutive (1.5 μm) semi-thin sections, we demonstrated that rod-like and cone-like opsins were not co-expressed in the same pinealocytes. Thus, the salmon pineal contains at least two types of pinealocyte, rod-like and cone-like. We have recently shown that the rod-like opsin expressed in the pineal of Atlantic salmon (ERrod-like opsin) shares only 74 % amino acid identity with the rod opsin expressed in the retina (Philp et al., 2000). Similar observations have also been made in zebrafish (Mano et al., 1999). We have yet to establish whether VA opsin is co-expressed with these opsins or expressed in another subclass of pinealocyte. Many, but not all, pinealocytes were labelled by α-transducin antibodies, suggesting that some pinealocytes may possess a G-protein α-subunit different from that detected in the rod and cone photoreceptors. Note that the rod, cone and α-transducin antibodies failed to label the parapineal.

In the central brain, numerous cell bodies in the anterior hypothalamus (suprachiasmatic nucleus, SCN; nucleus

preopticus magnocellularis, NPOm; nucleus preopticus parvocellularis, NPOp) were labelled with CERN 874 (anti-cone-opsin) antibodies. In contrast, no central brain structures were labelled with CERN 858 (anti-rod-opsin) antibodies. In previous studies (Garcia-Fernandez et al., 1997), we have used the same antibodies to undertake a detailed analysis of the adult lamprey CNS. In the lamprey, opsin labelling was similarly identified in the anterior hypothalamus, specifically within an area defined as T5 that is homologous to the NPOm of teleosts (Garcia-Fernandez et al., 1997). A putative novel photopigment called melanopsin has been localised within the SCN and ventral part of the NPOm of *Xenopus laevis* (Provencio et al., 1998). Furthermore, the photopigment P-opsin, originally isolated from the chicken pineal (Okano et al., 1994; Max et al., 1995), has been identified in the anterior nucleus preopticus of the toad (Yoshikawa et al., 1998).

In the salmon brain, α-transducin-like immunoreactivity was observed in cells and fibres of the ventral telencephalon (lateral and commissural parts of the area ventralis telencephali) but failed to co-localise to any areas of the central brain expressing VA or cone-like opsins. Perhaps this is not surprising because, as discussed previously, VA opsin may utilise a novel subtype G-protein α-subunit (Soni and Foster, 1997). These findings suggest that there may be yet more opsin-based photopigments within the salmonid brain that are not detected using either VA opsin *in situ* hybridisation or retinal rod- or cone-opsin-specific antibodies. The ventral telencephalon has been strongly implicated in containing photoreceptors in other vertebrate classes. For example, a homologous area has been shown to contain opsin-immunoreactive CSF-contacting neurons in both lizards (Foster et al., 1993, 1994) and birds (Silver et al., 1988; Wada et al., 1998). On the basis of this comparative analysis, we suspect that the ventral telencephalon is photoreceptive in a range of different vertebrate groups.

The salmon CNS appears to contain multiple photopigments expressed in several distinct sites (subhabenula/epithalamic, preoptic, ventral telencephalon, pineal, parapineal). While each of these photoreceptor populations might act independently, regulating distinct photic responses, it is also possible that they interact to regulate a range of different responses in concert. The habenula could be well placed to act as the site for this integration. In the salmonids, the habenula receives inputs from the retina that terminate in the lateral habenula and the subhabenula (Ebbesson et al., 1988). The habenula also receives afferents from the pineal complex (Ekström et al., 1987), the ventral telencephalon (Yanez et al., 1996) and the telencephalic/diencephalic boundary (entopeduncular nucleus and preoptic nucleus) (Yanez and Anadon, 1996). Since all these regions are thought to contain photoreceptors (see above), it is possible that the teleost habenula might act as a major site for the integration of retinal and extraretinal light information. The habenula would be further suited to this task because of its extensive connections to other regions of the teleost brain, including regions of the hypothalamus involved in the regulation of sexual behaviour and reproduction and the motor systems of the basal mesencephalon and rostral medulla (Demski, 1983; Peter and Fryer, 1983; Sloan and Demski, 1987; Yanez et al., 1996).

In the present study, we have presented evidence that the salmon CNS contains at least three different types of photopigment (rod-like, cone-like and VA opsin), and possibly yet another photopigment in the ventral telencephalon (see above). Currently, no single explanation can account for this multiplicity of photopigments, and none of the possibilities listed below is mutually exclusive. (i) It is possible that multiple photopigments, which differ in their spectral responses, might be used to gather information about spectral changes within the environment. For example, at twilight, the overall spectral quality of light changes in a precise manner. By monitoring this spectral change, the phase of twilight can be accurately determined (Roenneberg and Foster, 1997). (ii) Multiple photopigments, again with differing spectral responses, could also be used to provide a measure of irradiance over a broad spectral range (Roenneberg and Foster, 1997). (iii) Different photopigments might be associated with different phototransduction and effector pathways specialised for either neural or endocrine responses. (iv) Finally, encephalic photoreceptors might be specialised for the detection of slow gradual changes in environmental light rather than for the detection of transient light stimuli demanded by the visual system (Ekström and Meissl, 1997; Shand and Foster, 1999). Support for this argument comes from some of the unique structural features associated with VA opsin (Soni and Foster, 1997). Brain lesion, electrophysiological recording and transgenic ablation studies are needed to help resolve these alternatives.

Evidence for ‘deep encephalic photoreceptors’ was first demonstrated in fish by Karl von Frisch (1911). He showed that light-induced colour changes in the skin of the European minnow (*Phoxinus laevis*) could still occur in the absence of the eyes and pineal complex, and that lesions within the basal brain would block this response to light, and he concluded that there must be photoreceptive elements within the diencephalon of fish (von Frisch, 1911). Almost 90 years later, we have evidence for not one, but multiple, populations of photoreceptors within the diencephalon of fish. The future challenge is to place these different photoreceptors into a physiological and behavioural context.

This publication is the result of research sponsored by the Biotechnology and Biological Sciences Research Council (BBSRC), UK, EU BioMed2 and DGE PB97 1285 (Spain). The authors are grateful for the help and assistance provided by Tania Joyce, Ian Morris (Imperial College) and Dr J. Partridge (University of Bristol). We thank Dr W. J. deGrip and Dr P. H. Spiegel for the generous gift of anti-opsin and anti-α-transducin antibodies.

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