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Identification of extraretinal photoreceptors in the teleost *Phoxinus phoxinus*

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Summary. The existence of cells capable of detecting changes of the photoperiod within the deep brain, the so-called deep brain photoreceptors, was proposed in the early years of the twentieth century.

By using immunocytochemistry with antisera against phototransductory proteins on paraffin and vibratome sections, we have localized several positive areas in the brain of the teleost *Phoxinus phoxinus*. These areas were restricted to two encephalic regions: the epithalamus and the hypothalamus. Immunopositive (rod-opsin- and α-transducin-like) pinealocytes and parapinealocytes, as well as some sparse neurons in the habenula, were seen in the epithalamus. The immunoreaction of the hypothalamus was represented by α-transducin-like positive (magnocellular and parvicellular) neurons of the Nucleus Preopticus, as well as by α-transducin- and arrestin-like positive fibers corresponding to the hypothalamic-hypophyseal tract and a few fibers running towards the basal telencephalon.

These findings corroborate the data published on other teleost fish and fully support the hypothesis of the presence of photosensitive cells in the encephalon of lower vertebrates. The labelling with antisera against different components of the phototransductory cascade also strengthens the idea that such cells employ a biochemical mechanism similar to that in the retinal visual photoreceptor cells, rods and cones. Although the function is still unclear, the detection of the photoperiod seems to be the most likely role for these extraretinal photoreceptors.

Key words: Non-visual photoreceptors, Opsin, Pineal complex, Teleost, Hypothalamus

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Introduction

All the reported visual pigments of vertebrates possess two main components, a hydrophilic protein, the opsin, and a vitamin A-derived prostetic group, the chromophore. Light provokes a change at the chromophore that continues with an enzymatic cascade known as phototransduction. Different opsins as well as other proteins involved in this process have been found at different areas of the central nervous system (CNS) of vertebrates: retina, pineal complex and the so-called deep brain photoreceptors (Vigh et al., 2002). As is known, only the retinal cones and rods are devoted to visual perception. Other photoreceptors seem to have developed different tasks, e.g. to synchronise the biological rhythms and to mediate the regulation of non-rhythmic physiological processes in response to light.

The existence of the deep brain photoreceptor cells was firstly proposed by Karl von Frisch (1911), as an indirect evidence of his experiments about changes of skin pigmentation that occurred in absence of other photoreceptors in *Phoxinus phoxinus*. Scharrer (1928, 1964) proposed that such cells were located at the hypothalamic Nucleus Preopticus Magnocellularis (NPOM); however, some later attempts to localise them by immunohistochemistry and to study their ultrastructure were a complete failure. Other authors observed that the action spectrum of the photoperiodic response in the quail brain implied the presence of an opsin with an 11-cis retinoid (Foster and Follet, 1985; Foster et al., 1985). Another study demonstrated cone-opsin-like immunoreaction in neurons of the septum lateralis of the lizard Anolis carolinensis, but no positive cells were seen at the hypothalamus (Foster et al., 1993). Later on García-Fernández and Foster (1994) identified some cells with intraventricular projections at the medial and posterior hypothalamic areas of the *Petromyzon marinus* ammocoete. Rod and cone-opsin, as well as α-transducin and arrestin (S-antigen) immunopositive neurons were finally found in several hypothalamic and non-hypothalamic prosencephalic areas of three species of lampreys (García-Fernández et al., 1997).

As a brief summary, several photoreceptor cell populations have been reported in the encephalon of

diverse groups of nonmammalian vertebrates to date: lampreys and teleosts (García-Fernández et al., 1997; Philip et al., 2000a), amphibians (Yoshikawa et al., 1994, 1998; Álvarez-Viejo et al., 2003), reptiles (Foster et al., 1993) and avians (Silver et al., 1988).

In the recent years some new opsins have been identified: pinopsin, at the pineal complex of the chick (Okano et al., 1994; Max et al., 1995); parapinopsin, in the parapineal of some teleosts (Blackshaw and Snyder, 1997); exo-rhodopsin, in the pineal gland of zebrafish (Mano et al., 1999); the so-called vertebrate ancient (VA) opsin, which was identified at the pineal, the subhabenular area and the inner retina of the salmon (Soni and Foster, 1997; Philp et al., 2000a,b); finally, melanopsin, which was firstly described in melanophores, the brain and inner retinal layers of amphibians (Provencio et al.,1998) and recently in teleosts (Drivenes et al., 2003). Moreover, mammals have shown to express melanopsin in a subpopulation of retinal ganglion cells which project to the suprachiasmatic nucleus, the central biological clock (Hattar et al., 2002; Provencio et al., 2002).

Although it needs further demonstration, current data suggest that both retinal and non-retinal photoreceptors employ the same mechanism of photoreception based on an opsin coupled with a vitamin A-derived prostetic group, as well as a similar phototransductory cascade.

Given the data presented above, we decided to study the extraretinal photoreceptive areas in *Phoxinus phoxinus*, the first species in which non-visual photoreception was proposed, specially the hypothalamus, which has been only superficially studied to date. For this purpose, we employed diverse antibodies against components of the phototransduction cascade in an attempt to demonstrate that such areas express proteins similar to those of the retinal photoreceptor cells, or other better known extraretinal photosensitive organs such as the pineal and the parapineal, which also were subjects of our investigation.

Materials and methods

In the present study a total of 15 adult European minnows, *Phoxinus phoxinus* L. (Cyprinidae, Teleostei), which were collected from rivers in Asturias (Northern Spain), were analysed. The animals were deeply anesthetized with tricaine methanesulphonate (MS-222, Sigma) and killed by decapitation. Brains were removed and placed in Bouin's fixative for 48 hours. After fixation, 10 brains were dehydrated in a graded series of ethanol and embedded in paraffin. 10 μm-thick paraffin sections were obtained in a microtome (Reitchert-Jung, Vienna, Austria). The remaining 5 brains were directly embbeded in gelatin after fixation and 30 μm-thick slices were obtained with a Series 1000 vibratome (Energy Beam Sciences Inc., Agawam, Massachusetts, USA). Paraffin sections were collected on gelatin-coated slides, deparaffined in xylene, hydrated in ethanol and placed in phosphate-buffered saline solution (PBS) containing 0.4% Triton-100 (PBS-T) for 30 min. Vibratome cut tissue was processed as free floating sections and washed in PBS-T for 30 min. Endogenus peroxidase was blocked in both types of sections by 0,3% hydrogen peroxide in PBS (20-30 min.), they were followed by 3 washes in PBS-T. Possible background staining was blocked by incubating the sections for 30 min. in normal goat serum. Sections were then transferred into primary antisera (for 72 h in a humid chamber at 4 °C) directed against several phototransduction proteins (Hicks and Molday, 1986; Schalken and DeGrip, 1986; Cantera et al., 1990):

Rho4D2, the only monoclonal antibody used in this study, was raised against purified rod outer segments of rat and is monospecific for rod photoreceptors in mammalian species; the dilution used was 1:1000. This antiserum was provided by Dr. D. Hicks (INSERM, Strasburg, France).

CERN-9412 was raised against bovine rod α-transducin, and was used at a dilution of 1:4000. CERN-886, raised against purified lipid-free bovine rhodopsin, was used at a dilution of 1:10000. The CERN antisera are rabbit polyclonal and were provided by Dr. W. J. DeGrip (NCMLS, University of Nijmegen, The Netherlands)

Anti-S-antigen (SA): rabbit polyclonal antiserum raised against human arrestin (S-antigen) used here at a dilution of 1:1000. This antibody was provided by Dr. R. Foster (Imperial College of Science and Technology, London, UK).

Localisation of the antibody-antigen complex was accomplished by using the Vectastain Elite Kit (Vector Labs) and incubated in a solution of 0.025% diaminobenzidine containing 0.03% hydrogen peroxide in TRIS buffer. Sections were dehydrated in ethanol, cleared in xylene and mounted in a synthetic medium (Entellan; Merck, Darmstadt, Germany). Negative controls were performed by replacing the primary antiserum with normal nonimmune rabbit serum.

Results

As described in detail below, immunolabelled neurons were found in several regions belonging to two encephalic areas: the epithalamus and the hypothalamus. No labelling was found at any of the sections used as negative controls. All the figures shown in this study correspond to cross sections of the brain.

Epithalamus

Pinealocytes and some cellular somata and fibers in the habenula and posterior commisure were found to be immunopositive for several of the antibodies used. The pineal gland showed rod-opsin- (Fig. 1) as well as transducin α-subunit-like (Fig. 2) immunoreactivity (CERN-886 and CERN-9412 antibodies, respectively) at

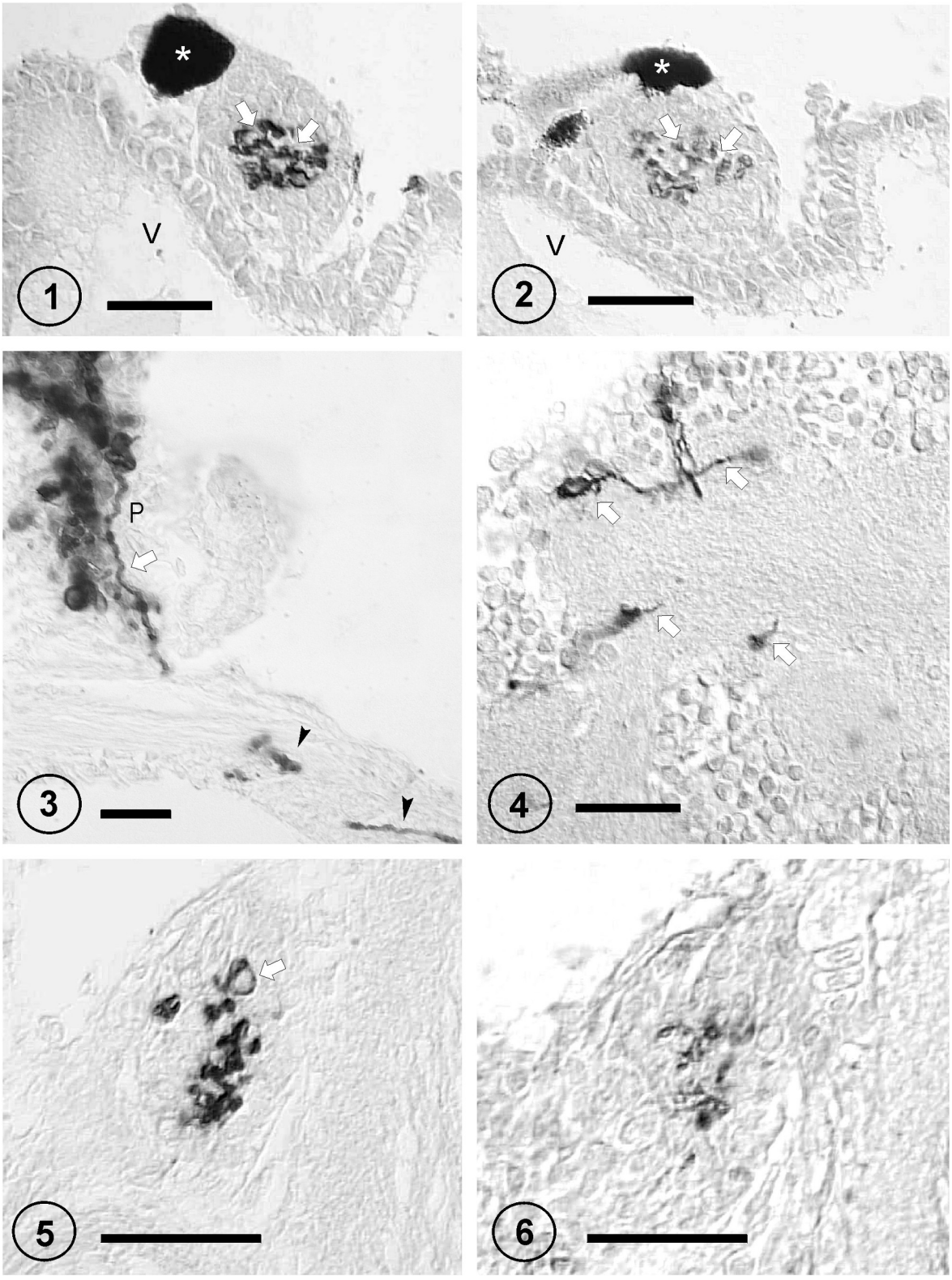


Fig. 1. Immunolabeling with the CERN-886 (anti rod-opsin) antibody in a cross section of the pineal gland. Immuno-positive pinealocyte outer segments (arrows) can be observed in the pineal core. (Asterisk, attached pigment cells; V, 3rd ventricle). x 650. Calibration bar: 25 μm.

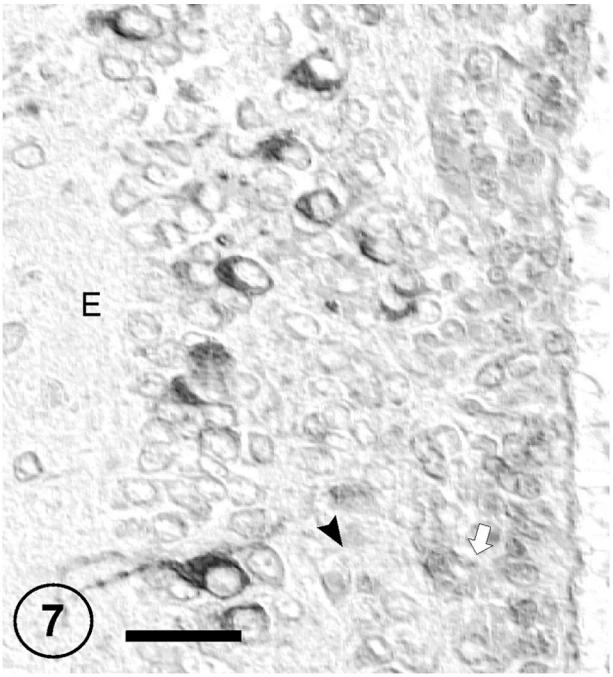
Fig. 2. CERN-9412 (α-transducin-like-) immunopositive pinealocyte outer segments (arrows) in a cross section of the pineal. This section was obtained caudally to the previous one, from which is separated by just 10 μm. (Asterisk, attached pigment cells; V, 3rd ventricle). x 650. Calibration bar: 25 μm.

Fig. 3. The Rho4D2 (anti rod-opsin) antibody labelled intensely the pinealocytes in their entirety. The arrow points to a stained pinealocyte process. Some immunoreactive elements can also be observed in the interhabenular commisure (arrowheads). x 450. Calibration bar: 25 μm.

Fig. 4. Some rod-opsin-like (CERN-886) positive somata and axonal processes of neurons can be seen in the habenula (arrows). x 550. Calibration bar: 25 μm.

Fig. 5. Rod-opsin-like (CERN-886) immunoreaction in a section of the parapineal. The arrow points to a cell in which the labelling seems to be perinuclear. x 650. Calibration bar: 25 μm.

Fig. 6. α-transducin-like immunoreaction in the parapineal in a section 10μm far from the preceding one. x 650. Calibration bar: 25 μm.



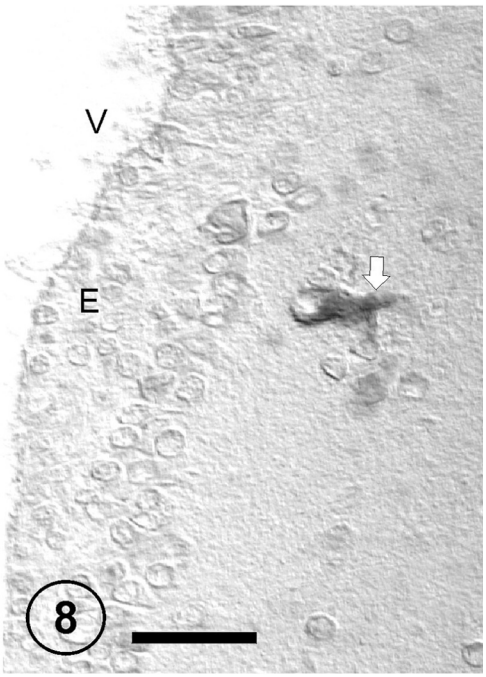
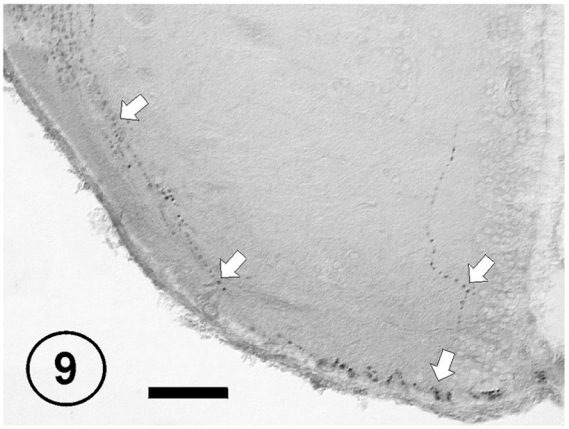


Fig. 7. Numerous magnocellular and parvicellular neurons of the NPO were labelled with the CERN-9412 antibody (against α–transducin). Parvicellular neurons are rounded and magnocellular neurons are piriform and show an axonal process (arrow). An arrowhead points to a magnocellular neuron which is not labelled. (E, ependimal layer) x 650. Calibration bar: 25 μm.

Fig 8. The caudal area of the NPO shows few immunopositive cells. The arrow points to an α-transducin-like neuron. x 650. (E, ependimal layer, V, 3 rd ventricle). Calibration bar: 25 μm.



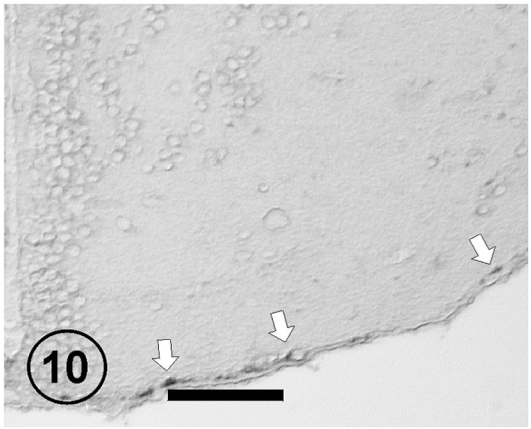
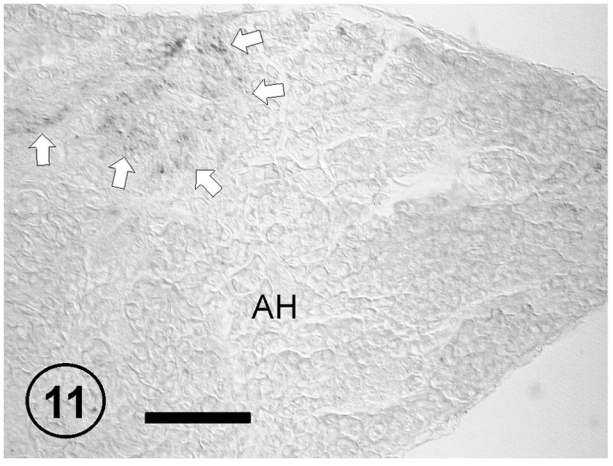


Fig. 9. Some α-transducin-like positive fibers (arrows) join together at the medial part of the hypothalamus and run towards the hypophysis. x 100. Calibration bar: 0.1 mm.

Fig. 10. A more caudal section shows some other arrestin-like positive fibers (arrows) at the basal area of the hypothalamus. x 150. Calibration bar: 0.1 mm.



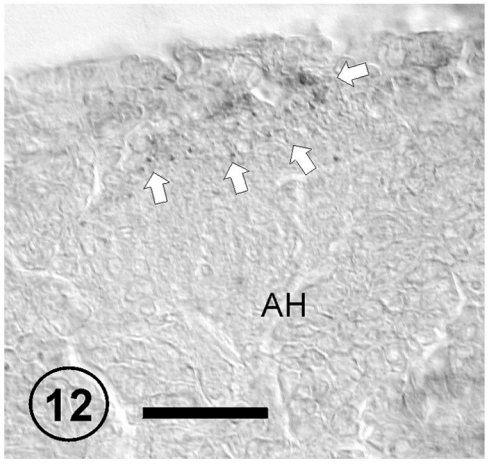


Fig. 11. α-transducin-like immunopositive fibers localised in the neural lobe (arrows). (AH, adenohypophysis). x 500. Calibration bar: 25 μm.

Fig. 12. Some rod-opsin-like immunopositive fibers were also seen in the neural lobe of the hypophysis (arrows). (AH, adenohypophysis). x 650. Calibration bar: 25 μm.

the photoreceptive outer segments (OSs) of the pinealocytes. Furthermore, the Rho4D2 antibody (also anti-rod-opsin) labelled the whole cell, including some axonal processes projecting towards the pretectum (Fig. 3). Some Rho4D2-positive fibers were observed sparsely in the interhabenular and posterior commisures, and also in a region located dorsally to the ependymal subcommisural organ. These cells projected towards the ventricular cavity with a short bulb-like process (not shown). Furthermore, some liquor-contacting processes were seen sparsely among the ependymal cells.

The CERN-9412 and CERN-886 antisera (against α-transducin and rod-opsin, respectively) also labelled a small number of cells in a dorsal-lateral region of the habenulas (Fig. 4). These two antibodies recognised some small groups of cells in the parapineal organ (Figs. 5, 6).

Hypothalamus

The immunopositive somata and fibers observed in this area belonged exclusively to cells of the preoptic nucleus (NPO).

α-transducin-like immunoreaction was found in numerous magnocellular as well as parvicellular neurons at the subependymal area (Fig. 7) of the NPO, from the preoptic recess to the most caudal area of this neurosecretory nucleus. Labelling filled the cytoplasm of

Table 1. Summary of the phototransductory proteins immunopositive areas in the brain of *Phoxinus phoxinus*.

ANTIBODY	SPECIFICITY	EPITHALAMUS			HYPOTHALAMUS	
		Pineal	Parapineal	Habenula	NPO	POHT
CERN-886	rod-opsin	+	+	+	-	+
Rho4D2	rod-opsin	+	-	-	-	-
CERN-9412	α-transducin	+	+	+	+	+
SA	arrestin	-	-	-	-	+

NPO, nucleus preopticus; POHT, preoptic-hypophyseal tract.

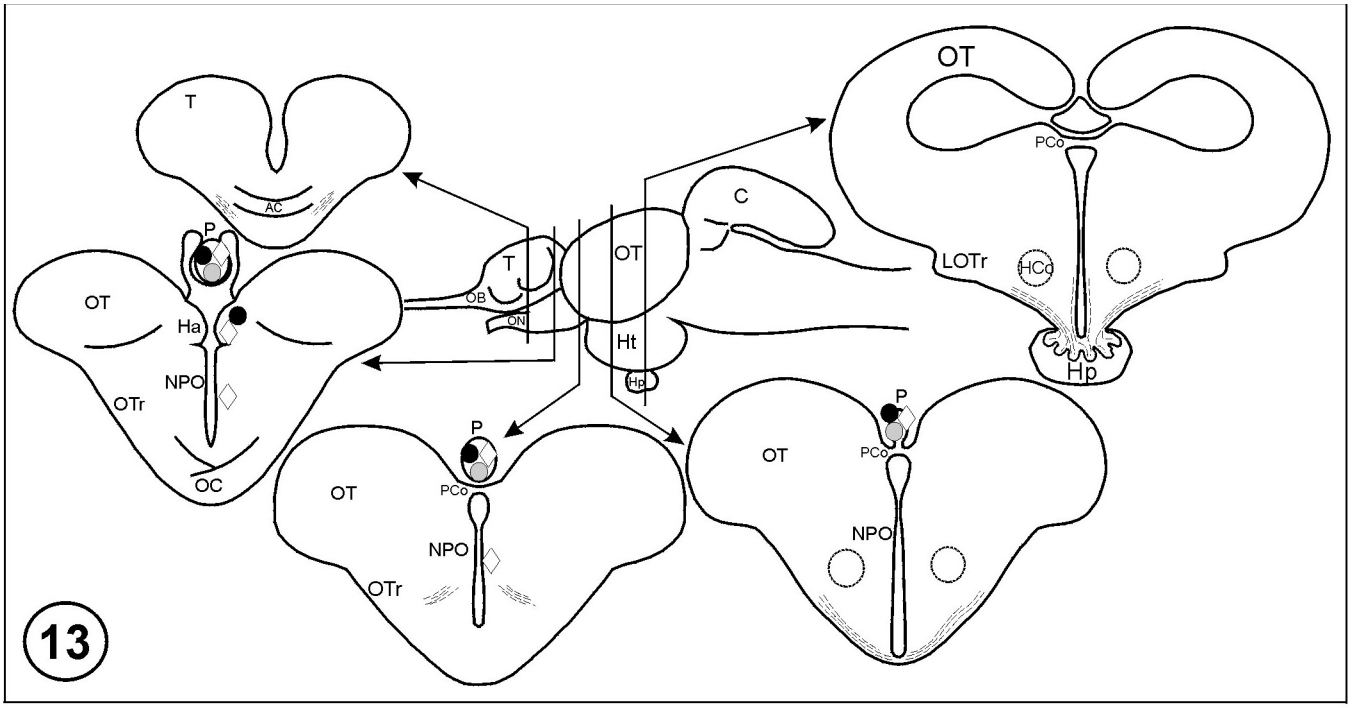


Fig. 13. This diagram shows the immunolabelling at the encephalic areas of *Phoxinus phoxinus*. Cellular somata are indicated as follows: black circle, rod opsin-like (CERN 886); grey circle, rod opsin-like (Rho4D2); white rhomb, α-transducin-like (CERN 9412). Positive fibers are represented by fine discontinuous lines (see text for further details). C, cerebellum; Ha, habenula; HCo, horizontal commisure; Ht, hypothalamus; Hp, hypophysis; LOTr lateral optic tract; OB, olfactory bulb; ON, optic nerve; OT, optic tectum; OTr, optic tract; P, pineal gland; PCo, posterior commisure; NPO, nucleus preopticus; T, telencephalon).

the cells and allowed to distinguish the morphology of these neuronal somata. Both the small-sized ones, which were rounded, and the large ones, which were piriform, showed a thick process that projected laterally (Fig. 7). However, no intraventricular processes were observed at these areas. The number of immunopositive cells decreased gradually towards the caudal part of this nucleus, where only a few cells were labelled (Fig. 8).

Cone-opsin-like, rod-opsin-like, α-transducin-like and arrestin-like immuno-positive fibers were also seen in both the anterior and the medial hypothalamic regions. The number of fibers and the intensity of immunolabelling varied depending on the antibody used. The location of such elements was lateral and caudal with regard to the position of the labelled neuronal somata. The fibers, which joined together in a ventromedial position (Figs. 9, 10) and projected towards the neurohypophysis (Figs. 11, 12), belong to the preoptic-hypophyseal tract.

Vibratome sections showed fibers labelled with the CERN-9412 (against a-transducin) at the preoptic-hypophyseal tract, like those described above. Furthermore, some fibers running rostrally and laterally with regard to the anterior commisure, projected towards the telencephalon.

A summary of the findings described above can be found in both the Table 1 and the Figure 13.

Discussion

Since almost a century ago, the teleost *Phoxinus phoxinus* proved to be an excellent model for studies of extraretinal photoreception. Nevertheless, the exact location of some of these enigmatic photoreceptor cells remained hidden until recent years. In the present study we have found that the pineal gland of this species is rod-opsin- and α-transducin-like immunopositive. The antibodies against these two components of the retinal phototransduction have already demonstrated to be good markers of pineal photoreceptors in other vertebrates (Foster et al., 1993, García-Fernández and Foster, 1994). Our results agree with those of Ekström et al. (1987), who worked in the pineal organ of both *Salmo gairdneri* and *P. phoxinus* and found that some of the antibodies. They used labelled only the OSs and some others the whole soma of the pinealocytes. Such variations in labelling could be explained by the different specificity of the antibodies used. Projection of fibers from the pineal gland towards other epithalamic areas were reported even in some mammals such as the hamster (Korf et al., 1986), suggesting that evolution has preserved well the neural relationship between the pineal and the encephalon in the vertebrates. As is known, the pineal of non-mammalian vertebrates is directly sensitive to light, which can regulate the production of melatonin in this gland (Ekström and Meissl, 1997). Therefore, the immunolabelling described here must be undoubtedly related to this process.

Opsin-like and arrestin-like immunolabelled cells have been reported in the habenular nucleus and also in pretectal areas (Ekström et al., 1987, Korf et al., 1986, 1990) both in teleosts and mammals. In teleosts, these cells were considered as photoreceptor cells that might have migrated from the parapineal organ; however, in mammals they seem to be pinealocytes which have migrated from the pineal and intermingle with neurons at the medial nucleus of the habenula (Korf et al., 1986). This region could thus be considered as a transitional area connecting the pineal to other epithalamic areas of the brain.

The fact that immunohistochemistry has revealed the presence of photosensitive proteins in the pineal gland of some mammals does not necessarily imply that phototransduction is taking place at these cells. Moreover, specific retinoid molecules related to this process had not been localised in mammals (Foster et al., 1989) to date and encephalic as well as pineal photoreception can be considered to be exclusive of non-mammalian vertebrates.

In addition to the pineal, some habenular neurons showed rod-opsin- and α-transducin-like immuno-reaction in our study. Apart from Ekström's (1987), some preceding findings in close-related epithalamic areas can also be cited here: García-Fernández et al. (1997) reported cone-opsin- and a-transducin-like positive neurons in sub- and posthabenular regions of the lamprey, and Philp et al. (2000a), using in situ hibridization, found VA-opsin in the subhabenular region of the salmon.

The parapineal, a small asymmetrical region of the teleost epithalamus (Vollrath, 1981), showed to have rod-opsin- and α-transducin-like immunopositive cells in our samples of *P. phoxinus*, although few studies (Rudeberg, 1969; Ekström et al., 1983) had suggested before that this organ present in fish had photosensitive cells. Remarkably parapinopsin has been recently isolated in this part of the encephalon of the catfish (*Ictalurus punctatus*) (Blackshaw and Snyder, 1997); however, the real function of this new opsin, as other recently discovered opsins, is still unclear. Whether or not our anti-rod-opsin antibody is actually recognising parapinopsin or any other rod-like opsins is also unknown.

The hypothalamus was the second prosencephalic labelled area in *P. phoxinus*. Immunopositive magnocellular and parvicellular neurons were seen in the NPO. As Scharrer (1928) demostrated, blinded and pinealectomiced minnows (*Phoxinus phoxinus*) responded to light stimuli, showing swimming and feeding reflexes. He was the first to discover secretory neurons in the NPOM and, on the basis of these and other observations made later in amphibians and reptiles, he proposed that such cells were also photoreceptive (Scharrer, 1964). Our results fully support this hypothesis. Moreover, García-Fernández et al. (1997), who worked with three species of lampreys, reported cone opsin-like immunolabelling in liquor-contacting neurons, which showed intraventricular terminal bulbs at

T5, a nucleus homologous to NPOM. However, we could not observe similar cytoplasmic processes in the subependymal positive neurons found in the minnow.

In some amphibians, such as *Xenopus laevis* (Provencio et al., 1998; Álvarez-Viejo et al., 2003), *Rana catesbeiana* (Yoshikawa et al., 1994), *Bufo japonicus* (Yoshikawa et al., 1998) and *Salamandra salamandra* (Foster et al., 1994) and in another teleost, *Salmo salar* (Philip et al., 2000a), the expression of opsin-like proteins was also reported in the preoptic nucleus. Collectively taken, these data seem to suggest strongly that the hypothalamic neurosecretory NPO possesses photoreceptive neurons. In reptiles and avians, this type of labelling was found curiously in a more rostral periventricular region, the telencephalic nucleus of the Septum Lateralis (Silver et al., 1988; Foster et al., 1993). However, Okano et al. (2000) also reported opsin-like immunoreactivity in the Septum of the amphibian *R. catesbeiana*.

The majority of the fibers described here belong to the hypothalamic-hypophyseal tract. A few fibers that run towards rostral regions through the basal telencephalon could belong to some of these putative hypothalamic photoreceptor neurons that contact to the Gn-RH neurons reported at the basal telencephalon of some teleosts (Bailhache et al.,1994). Such contacts were also demonstrated in avians (Saldanha et al., 1994, 2001). Therefore, the information of the light stimuli towards the reproductive system could also be sent through a pathway other than the hypothalamus-hypophysis axis.

The data collected from different studies suggest that several are the photopigments expressed in encephalic areas such as pineal, parapineal, habenula, NPO and the suprachiasmatic nucleus (García-Fernández et al., 1997; Philp et al., 2000a,b). Two hypotheses can be outlined: these photoreceptors either act independently regulating different photic responses, or on the contrary, they might cooperate in mediating all the photic information from the outside. Philp et al. (2000a) proposed the habenula as a node for integrating information from different photosensory organs. In fact, some retinal inputs end in the lateral habenula and in the subhabenula in some teleosts (Ebbesson et al., 1988). Also, the habenula receives afferent fibers from the pineal complex (Ekström et al., 1987), as well as from the telencephalon (Yánez et al., 1996) and from the NPO (Yánez and Anadón, 1996). This area could thus work as a center for integrating retinal and extraretinal light information. Moreover, the habenula is connected to the hypothalamic regions involved in the regulation of the sexual and reproductive behaviour (Demski and Northcutt, 1983; Peter and Fryer, 1983).

The CNS of the non-mammalian vertebrates thus contain multiple photopigments in different locations: the retina and other non-visual photosensitive regions. Such organs might function in response to light stimulation either independently or in a coordinated manner. An association to different efectory pathways, differentiated either for neural or for endocrine responses, could be suggested for the extraretinal photoreceptive cells.

In conclusion, the present paper corroborates the idea that the pineal (well studied by other authors), the parapineal and the habenula, express phototransductory proteins. Moreover, we like to remark that for the first time we report here that the hypothalamus of the teleost *Phoxinus phoxinus* contains numerous presumptive photosensitive cells as well. Further studies on the exact subcellular location of such protein machinery, which are currently being carried out in our lab, are needed to describe more precisely the way these cells perceive light.

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