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Central projections from the goldfish pineal organ traced by HRP-immunocytochemistry

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Summary. Pineal efferent projections have been traced in the brain of the goldfish (*Carassius auratus*) by administration of a concentrated solution of horseradish peroxidase onto the pineal organ. After different survival times, fish were sacrificed and the administered peroxidase was revealed by immunocytochemistry on paraffin sections using an anti-horseradish peroxidase antiserum. Immunoreactive fibres were seen in the anterior hypothalamus, habenula, dorsal thalamus, ventral thalamus, optic tectum, torus longitudinalis, area pretectalis, torus semicircularis and dorsal tegmentum. No immunoreactive cell bodies were visualized in the central nervous system, thus suggesting the absence of central pinealopetal innervation. Since all areas showing pineal labelled fibres are also known to receive retinal inputs, it can be suggested that an overlapping of information from retinal and extraretinal photoreceptors may be important to processes depending on photic stimulation such as entrainment of circadian rhythms or photoneuroendocrine responses.

Key words: Pineal organ, Efferent projections, Horseradish peroxidase, Goldfish (*Carassius auratus*), Immunocytochemistry

Introduction

It is thought that extraretinal photoreception in lower vertebrates may play a role in the entrainment of endogenous circadian rhythms (Underwood and Gross, 1982). However, whereas retinal projections are generally well known in all vertebrate classes, the afferences and efferences of the pineal photoreceptor system are poorly understood in lower vertebrates. Teleost pineal organ possesses well-characterized neurons that connect it with the brain via a prominent pineal tract (Holmgren, 1918; Wake, 1973; Korf, 1974; Falcon and Mocquard, 1979; Matsuura et al., 1979). Studies on pineal efferent projections to the central nervous system have been carried out in teleosts using cobalt chloride iontophoresis (Hafeez and Zerihun, 1974), and administration of horseradish peroxidase (HRP) (Ekström and van Veen, 1983, 1984; Ekström, 1984) as tract tracing methods. These investigations have revealed that pinealofugal fibres reach brain regions known to be main retinal targets, such as area pretectalis, dorsal and ventral thalamus, hypothalamus and mesencephalic tegmentum, thus suggesting that both retinal and pineal systems behave as neural mediators of photoperiodic events in the teleostean circadian system (Ekström, 1984). The present study was designed to investigate the connections between the pineal organ and the brain in the goldfish *Carassius auratus*, a species that possesses a well-differentiated retinohypothalamic tract (Springer and Gaffney, 1981).

HRP is a tracer widely used in studies on central nervous system pathways. Up to now, the presence of the exogenous-administered HRP has been revealed by histochemistry on frozen sections. In the present study, HRP was localized by PAP immunocytochemistry on paraffin sections, using a specific antiserum against HRP followed by intensification methods. This procedure allows the identification of nerve profiles containing minute amounts of the tracer.

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Materials and methods

Animals

Fifty-one adult goldfish (*Carassius auratus* L) of both sexes (2-5 g body weight) were obtained from a commercial supplier and used for HRP immunocytochemistry on paraffin sections. They were kept in well-aerated fresh water aquaria for at least one week before experimentation under constant temperature (20 ºC) and photoperiod (L:D, 12:12). Handling, care and processing of the experimental animals were exercised according to national laws (B.O.E. num. 67, 1988, Spain).

HRP administration

During tracer administration, animals stayed in water under anaesthesia (MS-222 006 g/l; Sigma, St. Louis, MO) for a total time of 30-60 min at 5 ºC, with the top of the cranium exposed to air. Under a dissecting microscope, a small incision was made with a razor blade just over the pineal stalk and the distal pineal vesicle. Then, 0.5 µl of 30% horseradish peroxidase (HRP; Type VI, Sigma) diluted in 0.9% NaCl was gently and slowly applied, via a Hamilton syringe, onto the pineal vesicle and pineal tract for 15-30 min. After 1 h, the opening was covered with a piece of aluminium foil and sealed with Cyanoceys (Ceys, Barcelona, Spain); then the fish were placed back in the aquaria.

Fish were again anaesthetized 8 h (n= 3), 16 h (n=9), 24 h (n=30) and 48 h (n=9) after surgery, brain with crania were dissected out, and fixed by immersion in bouin's fluid for 24 h. Then crania were decalcified and embedded in paraffin.

HRP-Immunocytochemistry

Seriated sections of crania containing the brain, 10 µm thick, were immunostained according to the peroxidase-antiperoxidase (PAP) method of Sternberger (1986) using, as primary antibody, a rabbit anti-HRP serum raised in our laboratory. In order to block endogenous peroxidase activity, the sections remained for 10 min in aqueous 10% hydrogen peroxide before immunostaining. Then, they were sequentially incubated in the primary antiserum for 18 h (dilution 1:6000), the secondary antiserum (goat anti-rabbit antiserum; from E.M. Rodríguez, Valdivia, Chile) for 30 min (dilution 1:40) and the PAP complex (Sigma) for 30 min (dilution 1:75). finally, the PAP complex was visualized by incubation with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) containing 0.05% hydrogen peroxide in Tris buffer. All incubations were performed at 20 ºC.

The antisera and the PAP complex were diluted in Tris buffer, pH 7.8, containing 0.7% non-gelling seaweed gelatin lambda carrageenan (Sigma) as saturating agent. Coplin jars were used for the incubation in the first and second antibodies, whereas the PAP incubation was carried out in a moist chamber. In order to test the specificity of the immunoreaction, adjacent sections were processed as described above, but incubation in the primary antisera was omitted. Other sections were incubated in the anti-HRP antiserum previously absorbed with an excess of HRP. No immunoreaction appeared in any of the control sections. To enhance the immunoreaction, three intensifying methods called the bridge PAP (Vacca et al., 1980), silver-methenamine (Rodríguez et al., 1984) and DAB-nickel (Adams, 1981) were used. In some sections the bridge PAP was followed by DAB-nickel or silver methenamine thus significantly increasing the sensitivity of the method.

In the sections the exact place of application of the tracer was detected in all experimental animals. Surrounding tissues, including meninges and parapineal organ, were thoroughly examined to see if nerves other than the pineal tract were labelled. No other nerves were labelled in any specimen. Immunostained fibres of the pineal tract were traced in consecutive brain sections.

Histochemistry on frozen sections

In order to compare the results obtained using HRP-immunocytochemistry with those after HRP histochemistry, three goldfish were prepared for HRP histochemistry on frozen sections. Briefly, 24 h after HRP administration, the brains were dissected out and fixed by immersion in 2.5% glutaraldehyde in 0.1M phosphate buffer for 4 h at 4 ºC. Then the brains were transferred to 30% sucrose at 4 ºC. Cryostast sections (20 µm thick) were processed for peroxidase histochemistry using DAB as chromogen.

Nomenclature of the brain nuclei

In this study, the nomenclature for the goldfish brain nuclei given by Peter and Gill (1975) was used for the description of pineal fibre localization

Results

HRP-immunocytochemistry

The heaviest labelling of the axons was observed in animals killed 24 h after HRP administration. In most specimens, the ventricular lumen as well as the adjacent tissues to the pineal fibres were almost devoid of peroxidase. Other nerves different to the pineal tract were not labelled. The HRP antiserum immunoreacted selectively with the exogenous administered peroxidase, thus providing a sharp profile of the pineal nerve fibres containing the tracer.

The thick frozen sections submitted to histochemistry showed the peroxidase in many of the locations that were also revealed by immunocytochemistry on paraffin sections. However, faintly stained fibres in some regions were only visualized by immunocytochemistry. The following description will be largely based upon the results of paraffin sections submitted to HRP immunocytochemistry.

Distribution of labelled pineal fibres in the brain

The distribution of labelled nerve profiles is schematized in the drawings of sagittal and transverse sections of the goldfish brain shown in Figs. 1, 2. The pineal tract, coursing over the diencephalic roof, divided and penetrated the brain at two points: (i) a group of fibres diverged from the pineal tract and penetrated through the habenular commisure, this group will be called anterior bundle; (ii) the remaining fibres entered

the brain in an area between the rostral border of the posterior commissure and the subcommissural organ, this group will be called posterior bundle. The labelled pineal tract was clearly distinguishable among the unlabelled fibres of the habenular and posterior commissures.

The anterior bundle divided and coursed bilaterally through the habenular region. Most fibres appeared to end in the rostral portion of the nucleus dorsolateralis thalami (Fig. 2c). Other fibres ran ventrally and reached the nucleus anterior tuberis (Fig. 2b). In addition, in intensified sections, faintly stained profiles were observed in the vicinity of the nucleus preopticus and nucleus preopticus periventricularis (Figs. 2a,b).

The posterior bundle penetrated the brain and divided into two distinct fascicles that ran bilaterally over the subcommissural organ having a close spatial relationship with the basal portion of the ependymal cells of the organ, mainly at its rostral half (Figs. 2d, 3). Each fascicle gave out branches that reached several

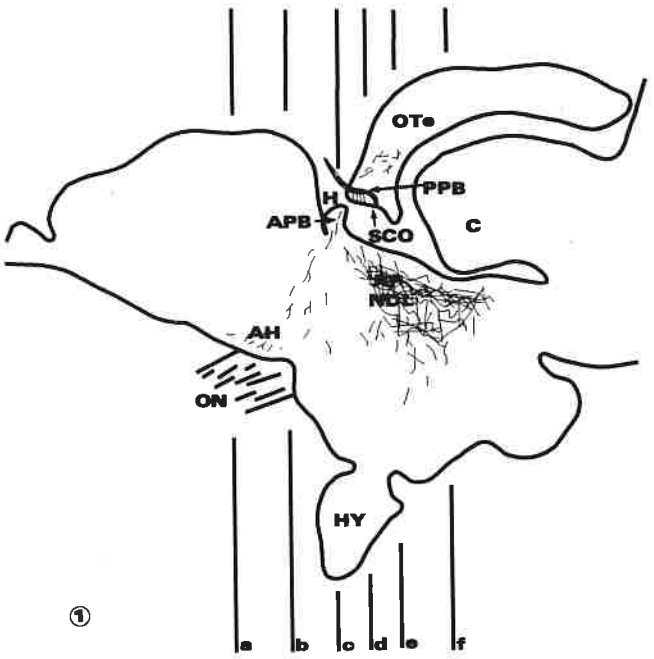


Fig. 1. Schematic representation of a midsagittal section through the goldfish brain showing the distribution of pineal projections. Anterior pineal bundle (APB) enters the habenular region (H), and posterior pineal bundle (PPB) enters the posterior commissure. Levels a-f correspond respectively to the transverse sections shown in Fig. 2. AH: anterior hypothalamus; AP: area pretectalis; C: cerebellum; HY: hypophysis; NDL: nucleus dorsolateralis thalami; ON: optic nerve; OTe: optic tectum; SCO: subcommissural organ.

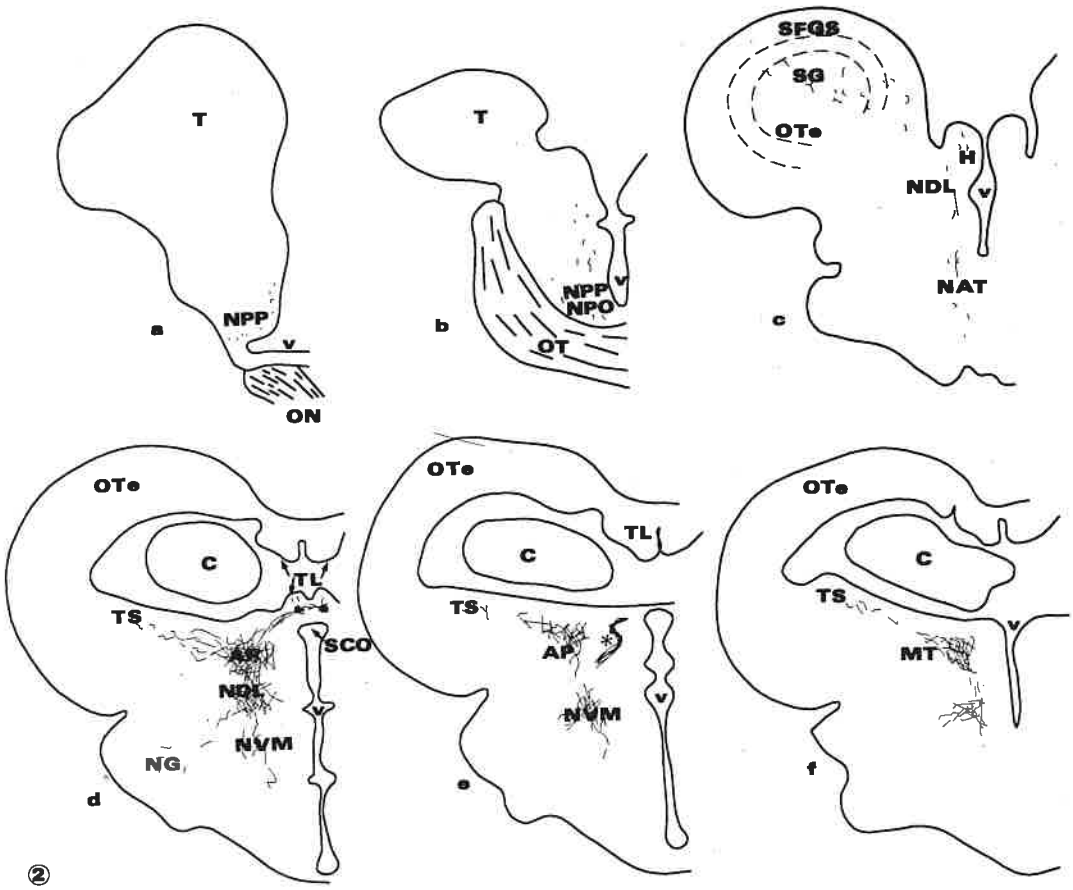
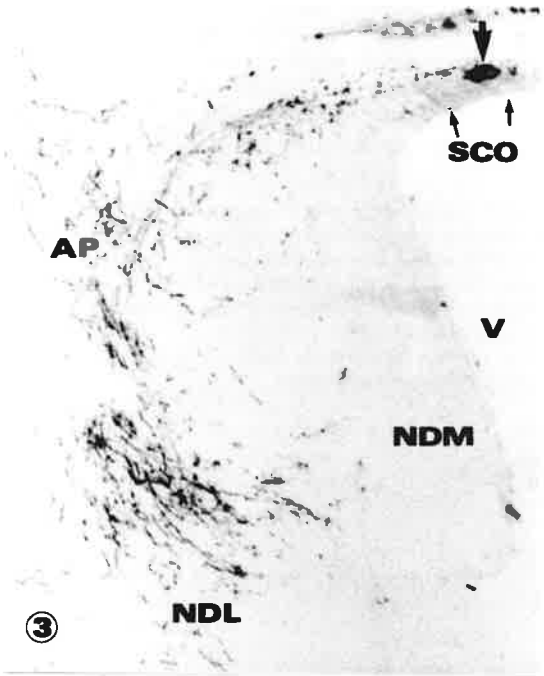


Fig. 2. Charts of HRP traced immunoreactive fibres in representative rostrocaudal transversal levels of the goldfish brain shown in Fig. 1. AP: area pretectalis; C: cerebellum; H: habenula; MT: midbrain tegmentum; NAT: nucleus anterior tuberis; NDL: nucleus dorsolateralis thalami; NG: nucleus glomerulosus; NPO: nucleus preopticus; NPP: nucleus preopticus peri-ventricularis; NVM: nucleus ventromedialis thalami; ON: optic nerve; OT: optic tract; OTe: optic tectum; SCO: subcommisural organ; SFGS: stratum fibrosum et griseum superficiale; SGC: stratum griseum centrale; T: telencephalon; TL: torus longitudinalis; TS: torus semicircularis; V: third ventricle. Asterisk: tractus habenulo-peduncularis.

brain areas namely: i) torus longitudinalis, ii) optic tectum, iii) area pretectalis and the nucleus dorsolateralis thalami, and iv) midbrain tegmentum (Figs. 2d-f).

i) In the region of the subcommissural organ, few labelled profiles appeared to cross the posterior commissure from the pineal fascicles to end among the clustered cells of the anterior region of the torus longitudinalis (Fig. 2d). ii) Some labelled fibres joined the unlabelled fibres of the tractus opticus dorsomedialis, and finally appeared to spatter in the stratum fibrosum et griseum superficiale and stratum griseum centrale and the rostral optic tectum (Figs. 2c, 4). iii) Many labelled fibres left the pineal fascicles and reached the area pretectalis, nucleus dorsolateralis thalami and nucleus ventromedialis thalami (Figs 2d,e 3). In addition, scarce labelled profiles were found in the nucleus glomerulosus (Fig. 2d). iv) Caudally, the remaining fibres of each fascicle of the posterior pineal bundle left the subcommissural organ region and proceeded toward the midbrain tegmentum surrounding the tractus habenulo-peduncularis (Figs. 2e, 5). These fibres reached the



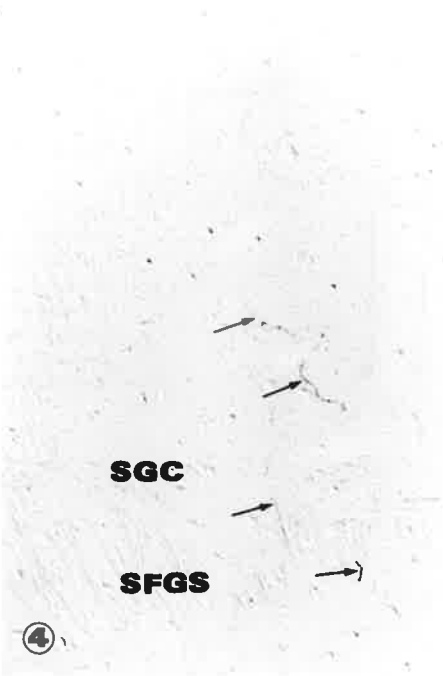
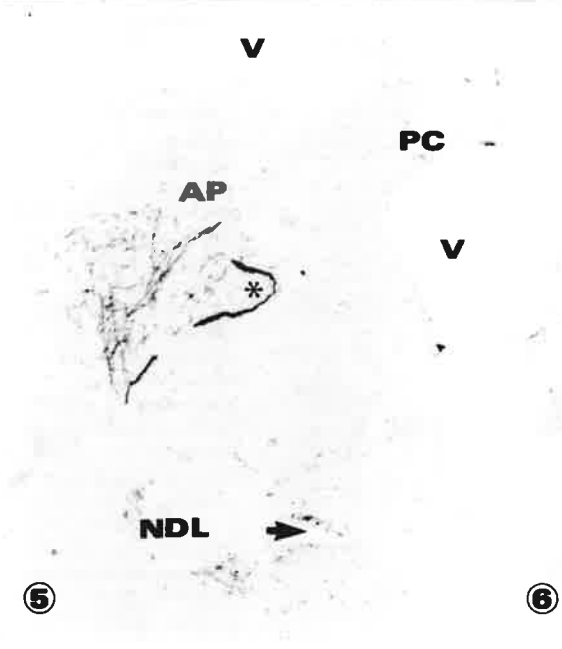


Fig. 3. Labelled pineal fibres leave the posterior pineal bundle (arrow) and form a conspicuous plexus in the area pretectalis (AP) and nucleus dorsolateralis thalami (NDL). NDM: nucleus dorsomedialis thalami; SCO: subcommissural organ; V: third ventricle. x 60



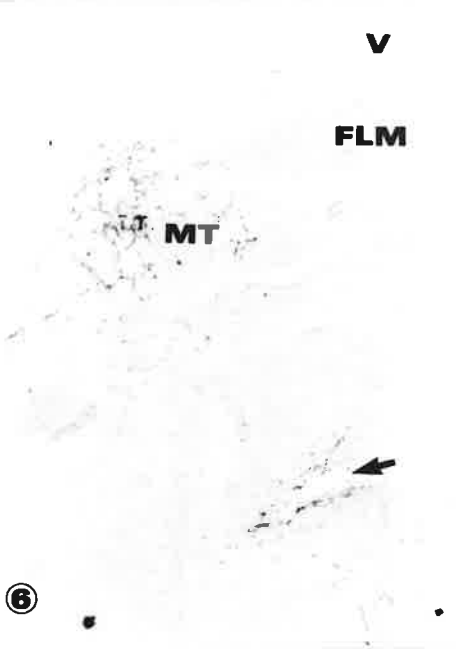


Fig. 4. Labelled fibres (arrows) reach different layers of the optic tectum. SFGS: stratum fibrosum et griseum superficiale; SGC: stratum griseum centrale. Nomarski optic. x 60

Fig. 5. Labelled fibres of the posterior pineal bundle leave the posterior commissure and surround the tractus habenulo-peduncularis (asterisk) among the plexus formed in the area pretectalis (AP) and the nucleus dorsolateralis thalami (NDL). PC: posterior commissure; V: third ventricle; arrow: blood vessel x 40

Fig. 6. In the mesencephalic tegmentum, immunostained fibres form a dense plexus in the nucleus tegmentalis dorsalis (MT) and the ventromedial area where they appear in the vicinity of blood vessels (arrow). FLM: fasciculus longitudinalis medialis; V: ventricle. x 40

dorsomedial area of the midbrain tegmentum, and were distributed in an area occupied by the nucleus tegmentalis dorsalis, nucleus of Edinger-Westphal and nucleus lateralis valvulae, lateral to the fasciculus longitudinalis medialis and the oculomotor nucleus. In the medial area of the mesencephalon, some beaded fibres were frequently found in the vicinity of large blood vessels (Figs. 5, 6).

Discussion

In this study we have traced pineal efferent projections of *Carassius auratus* by applying a concentrated solution of HRP onto the pineal organ. As we have reported before in an ultrastructural study (Jiménez et al., 1993; Pérez-Fígares et al., 1993), after applying the HRP solution, virtually all fibres of the pineal tract of *Carassius auratus* appeared filled with the label.

Revealing the administered HRP by means of immunocytochemistry using an anti-HRP antiserum has proven to be a useful method to study goldfish pineal projections. In some regions pinealofugal fibres could only be visualized by immunocytochemistry but not by histochemistry. Also unspecific labelling of structures containing endogenous peroxidase, such as erythrocytes and endothelial cells of the brain vessels, was avoided (see Pérez-Fígares et al., 1993).

Detailed descriptions of the routes followed by the pineal tract in teleost fish and larval and adult lamprey have been reported before using different tracing methods (Hafeez and Zerihun, 1974; Ekström and van Veen, 1983, 1984; Ekström, 1984; Puzdrowski and Northcutt, 1989; Yañez et al., 1993). Pineal fibres were described in the area pretectalis, dorsal thalamus, ventral thalamus, habenula and dorsal tegmentum. In all these areas convergence of pinealofugal and retinofugal projections exist. Hence authors suggested that an overlapping of retinal and pineal inputs may play a role in processes depending on photic information such as entrainment of circadian rhythms or photoneuroendocrine responses. Our study shows that the goldfish displays pinealofugal fibres in these same locations, and a number of other regions also containing retinal projections.

In *Salmo gairdneri*, Hafeez and Zerihun (1974) suggested the possibility of a pineal innervation of the magnocellular preoptic nucleus. In the lizard *Lacerta sicula*, Korf and Wagner (1981) demonstrated a parietal innervation of the hypothalamus including the magnocellular paraventricular and supraoptic nuclei, thus supporting the previous assumption by Aron et al. (1960) and Vullings (1979) that the pineal complex of lower vertebrates may be involved in the control of the magnocellular neurosecretory system. Our present description of pineal projections to the anterior hypothalamus of *Carassius auratus*, near the nucleus preopticus and the nucleus preopticus periventricularis could support the assumption of Hafeez and Zerihun (1974). However, only occasionally labelled profiles appeared inside the preoptic nucleus proper. This is very weak evidence for a pineal innervation of the neurosecretory preoptic nucleus of the fish. On the other hand, retinal fibres have been demonstrated in the rostrolateral hypothalamus of goldfish (Springer and Gaffney, 1981). Since this area also receives pineal inputs, an overlapping of both photoreceptor systems, retinal and pineal, seems to occur in this region.

The optic tectum is a main retinal target in the goldfish (Sharma, 1972; Springer, 1981; Springer and Gaffney, 1981) and other teleosts (Ebbesson and O'Donel, 1980; Vanegas and Ito, 1983; Ekström, 1984; Springer and Mednick, 1985). In the present HRP tracing study and in studies using the carbocyanine DiI as a tracer on paraformaldehyde fixed goldfish brains (unpublished observations by the authors), scattered labelled pineal fibres were detected in the inner layers of the optic tectum. Their presence suggests synchronization of retinal and extraretinal photoreception in this principal area of photic stimuli integration.

Torus semicircularis is another area receiving retinofugal fibres in *Gasterosteus aculeatus* (Ekström and van Veen, 1983) and *Astronotus ocellatus* (Springer and Mednick, 1985). The presence of pineal fibres in the torus semicircularis of *Carassius auratus* indicates that in this area as in the rostral thalamus and the optic tectum, pineal and retinal inputs could concur.

Puzdrowski and Northcutt (1989) demonstrated in silver lamprey *Ichthyomyzon unicuspis* pinealofugal projections to the torus semicircularis and the optic tectum. Since these projections were not described in reports dealing with teleost species these authors suggested that pineal projections to these two areas could represent a differential character between jawed fishes and lampreys. However, our study, carried out in a jawed fish, the goldfish, evidences pineal fibres in the torus semicircularis and optic tectum.

In conclusion, our work in the goldfish agrees and extends brain areas containing pineal fibres in teleosts. Most of the pineal projecting areas described in this study have been reported to also receive retinal projections. Thus, it seems that an overlapping between retinal and pineal inputs is an extended phenomenon in the central nervous system of *Carassius auratus* and could be of importance for entrainment of circadian rhythms and photoneuroendocrine activities.

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