

## DISTRIBUTION OF HYPOTHALAMIC, HIPPOCAMPAL AND OTHER LIMBIC PEPTIDERGIC NEURONAL CELL BODIES GIVING RISE TO RETINOPETAL FIBERS: ANTEROGRADE AND RETROGRADE TRACING AND NEUROPEPTIDE IMMUNOHISTOCHEMICAL STUDIES

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**Abstract**—In our present work utilizing the retrograde or anterograde transport of tracers (biotinylated dextran amine and Fluorogold, respectively) we have provided direct evidence for the cells of origin of the limboretinal pathway in rats and their termination in the retina using light microscopic approach. Administration of biotinylated dextran amine into the vitreous body resulted in nerve cell body labeling in several structures: the supraoptic and paraventricular nuclei, the hippocampus (CA1, CA3), the dentate gyrus, the indusium griseum, the olfactory tubercle, and the medial habenula, all of them belong to the limbic system. We estimated that the total number of retrogradely labeled cells is  $1495 \pm 516$ . We have seen fiber labeling in the retinorecipient suprachiasmatic nucleus and in the primary visual center, the lateral geniculate body, but labeled nerve cell bodies in these structures were never seen. Iontophoretic application of Fluorogold into the hippocampal formation, where the major part of the biotinylated dextran amine-labeled cell bodies was observed, resulted in labeled fibers in the optic nerve and in the retina indicating that the retrogradely labeled cells in the hippocampus and the dentate gyrus among others are the cells of origin of the centrifugal visual fibers. Sections showing biotinylated dextran amine labeling were stained for vasoactive intestinal polypeptide, pituitary adenylate cyclase activating polypeptide or luteinizing hormone-releasing hormone immunoreactivity using immunohistochemistry. Some biotinylated dextran amine-labeled cells also showed vaso-

active intestinal polypeptide, pituitary adenylate cyclase activating polypeptide or luteinizing hormone-releasing hormone immunoreactivity. We conclude that the limboretinal pathway exists and that the cells of origin are partially vasoactive intestinal polypeptide, pituitary adenylate cyclase activating polypeptide or luteinizing hormone-releasing hormone immunoreactive. © 2006 Published by Elsevier Ltd on behalf of IBRO.

**Key words:** limboretinal pathway, tracing, VIP, PACAP, LHRH.

It is well established that the retinal ganglion cells, other than the primary visual center, send their axons or axon collaterals to the hypothalamic nuclei. These fibers form the retinohypothalamic pathway (Hendrickson et al., 1972; Moore and Lenn, 1972). The major neurotransmitter of this pathway is glutamate, although other transmitters and neuropeptides have also been demonstrated including pituitary adenylate cyclase activating polypeptide (PACAP) (Köves et al., 1996; Hannibal et al., 1997). The major part of these fibers terminates in the suprachiasmatic nucleus (SCH); however, other retinorecipient areas in the hypothalamus have been demonstrated as well (Mai and Junger, 1977; Mai, 1979; Silver and Brand, 1979; Kita and Oomura, 1982; Levine et al., 1994).

Since the description of the olivocochlear bundle by Rasmussen (1946), both anatomical and physiological evidence appears to demonstrate the existence of centrifugal fibers within most of the major sensory pathways. Previous to the use of tracing techniques the presence of these fibers in the avian retina and optic nerve was reported by Ramon y Cajal (1893) and Dogiel (1895). Later, retinopetal projections were described in all vertebrates, including human (Wolter and Knoblich, 1965; Brooke et al., 1965; Wolter, 1978; Repérant et al., 1989; Uchiyama, 1989; Ward et al., 1991). Several structures were proposed to be the origin of these fibers: the reticular formation of the brain stem (Granit, 1955), the superior colliculus (Polyak, 1957) and the lateral geniculate nucleus (Wolter and Lund, 1968).

In lower vertebrates many data have been accumulated about the existence of the centrifugal visual fibers during the last century. The major source of these fibers in birds is the isthmo-optic nucleus (Cowan and Powell, 1962). In snakes the nucleus of the ventral supraoptic decussation is known to send centrifugal visual fibers to the retina, a higher number to the contralateral than to the

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**Abbreviations:** BDA, biotinylated dextran amine, 10,000 MW; CA1, CA1 region of the hippocampus; CA3, CA3 region of the hippocampus; DAB, diaminobenzidine tetrahydrochloride; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; DG, dentate gyrus; FB, Fast Blue; FG, Fluorogold; H, habenular complex; HRP, horseradish peroxidase; IG, indusium griseum; INL, inner granular layer; IPL, inner plexiform layer; KPBS, potassium phosphate-buffered saline; LGB, lateral geniculate body; LHRH, luteinizing hormone-releasing hormone; PACAP, pituitary adenylate cyclase activating polypeptide; PFA, paraformaldehyde; PV, paraventricular nucleus; SCH, suprachiasmatic nucleus; SO, supraoptic nucleus; Tu, olfactory tubercle; VIP, vasoactive intestinal polypeptide.

0306-4522/06/\$30.00 + 0.00 © 2006 Published by Elsevier Ltd on behalf of IBRO.  
doi:10.1016/j.neuroscience.2006.02.081

ipsilateral eye (Halpern et al., 1976). In chameleon, Hassni et al. (1997) described retinopetal nuclei in the ventromedial tegmental region of the mesencephalon and the ventrolateral thalamus of the diencephalon. In their experiments, rhodamine and horseradish peroxidase (HRP) were used as retrograde tracers. In turtles, after intraocular injection of wheat-germ agglutinin (WGA) or HRP Snyder and Kunzle (1983) described retrogradely labeled neurons in the mesencephalic reticular area lying between the trochlear and isthmus nuclei. Their number was small and they were predominantly found contralateral to the injected side.

In mammals centrifugal visual fibers have also been observed. With the use of large molecule retrograde tracers such as HRP injected into the vitreous body of rats, Itaya (1980) and Itaya and Itaya (1985) demonstrated neurons in the pretectal area and in the periaqueductal gray matter which send their axons to the retina, although they did not mention labeled cells in the hypothalamus or in other extrahypothalamic structures. Other researchers have found retrogradely labeled cells in the caudal mesencephalon, in the medial pretectal area and in the oculomotor nucleus of the same species (Hoogland et al., 1985; Villar et al., 1987; Labandeira-Garcia, 1988). In another experiment after an injection of HRP into the vitreous body of dogs, retrogradely labeled cells were observed in the ventral hypothalamus (Terubayashi et al., 1983), while in monkey a fluorescent tracer, Fast Blue (FB), was transported from the vitreous body to the SCH and the arcuate nuclei (ARC) (Bons and Petter, 1986).

The termination of centrifugal visual fibers in lower vertebrates has been exhaustively studied by researchers (Dowling and Cowan, 1966; Repérant et al., 1989; Uchiyama, 1989; Vesselkin et al., 1989; Miceli et al., 1999). On the bases of their results it is clear that the number of centrifugal visual fibers is limited; however, in the retina they show a rich arborization. With the use of electron microscope Dowling and Cowan (1966) described the termination of centrifugal visual fibers of pigeon. The endings are most often found along the outer margin of the inner plexiform layer (IPL) and between the lowermost cells of the inner nuclear layer. The majority of the terminals have contact with the basal part of the amacrine cells. Their observation is very similar to that of Ramon y Cajal (1911) using silver impregnation. It was also demonstrated that the major part of the centrifugal fibers is glutamatergic, GABA-ergic (Rio et al., 1993, 1996, 2003) or serotonergic (Villar et al., 1987), although neuropeptides were also observed in these fibers. Luteinizing hormone-releasing hormone (LHRH) fibers were seen in fish (Münz et al., 1982; Stell et al., 1984; Grens et al., 2005), frogs (Wirsig-Wiechmann and Basinger, 1988), crocodile (Medina et al., 2005) and in rats (Santacana et al., 1996). In fish the LHRH fibers belong to the olfactoryretinal nerve whose mammalian counterpart is the terminal nerve. In rats the origin of these fibers has not been demonstrated.

In our previous study (Fógel et al., 1997) we described bilateral fiber bundle immunoreactive to vasoactive intestinal polypeptide (VIP) in rat projecting into the optic chi-

asm and the optic nerves from the hypothalamus. We supposed that these fibers may originate from the forebrain, partially from the supraoptic (SO) and the paraventricular (PV) nuclei. This was based on the observation that after removal of the eyes (enucleation), a few VIP immunoreactive cell bodies appeared in these nuclei, although in intact rats VIP cells were never seen in these locations. The accumulation of VIP immunoreactive material in these cell bodies might be caused by the interruption of their axons by the enucleation. Five to six months after enucleation, the fibers originating from the retina are completely degenerated. In these animals *Phaseolus vulgaris* leucoagglutinin (PhA-L) was administered iontophoretically into the SO or the PV region and transported into the optic nerve by the surviving fibers; however, the number of the labeled fibers was very limited. This observation suggested that some of the cells sending centrifugal visual fibers to the eye may reside in these regions, but the majority have to be looked for in other parts of the CNS.

The main aim of the present work is to provide direct evidence for the localization in the forebrain of the cell bodies that give rise to the centrifugal visual fibers. To do this we utilized the retrograde transport of a tracer injected in the vitreous body and the anterograde transport of an iontophoretically applied tracer to the locations where the majority of these cells were expected.

The presence of LHRH, VIP and PACAP immunoreactive fibers in the optic chiasm and optic nerves is well established. There is also evidence that PACAP immunoreactive fibers may belong to the retinal ganglion cells (Köves et al., 1996; Hannibal et al., 1997), therefore they are retinofugal, although some fibers may also be retinopetal. In paddy-birds, LHRH immunoreactivity was also observed in the ganglion cells (Fukuda et al., 1982); however, LHRH immunoreactivity was also found in retinopetal fibers of various species as described above. VIP immunoreactive fibers in the optic nerve seem to be retinopetal because the retinal ganglion cells are not VIP immunoreactive (Casini and Brecha, 1991).

On the basis of the abovementioned results we propose that a part of the PACAP, VIP and LHRH immunoreactive fibers belongs to cells that give rise to retinopetal (centrifugal visual) fibers and that these cells reside in the forebrain. To demonstrate this, forebrain sections containing BDA labeled cell bodies were also stained for PACAP, VIP or LHRH immunoreactivity showing the chemical character of the cells sending the centrifugal visual fibers to the eyes.

## EXPERIMENTAL PROCEDURES

### Experimental animals

Adult Sprague–Dawley male rats (2–4 months old) were used in our experiments. The animals were kept in a light- and temperature-controlled vivarium (lights on at 06:00 h and off at 18:00 h, temperature 22 °C±2). They were provided with standard laboratory chow and water *ad libitum*. All interventions, including the injection of the tracer into the vitreous body of the eye, the iontophoretic administration of the tracer into the forebrain regions and the final perfusion, were carried out under general anesthesia using ketamine-hydrochloride (75 mg/100 g bw) (Sigma, St. Louis,

MO, USA). Treatment of the animals was in accordance with the rules of the European Communities Council Directive of 24 November, 1986 (86/609/EEC). Our permission number from the Local Council of Animal Experiments: 27-37/99. We made every effort to minimize the number of experimental animals and their postoperative suffering.

### Selection of tracers

We chose tracers that were too large to pass through gap junctions, were not trans-synaptic, and could not be picked up from the blood at the injection site. Four tracers were tested before use: a) 10  $\mu$ l of 4% biotinylated dextran amine (BDA, 10,000 MW) (Sigma), b) 10  $\mu$ l of 5% FB (Sigma), c) 10  $\mu$ l of 4% Fluorogold (FG) (Fluorochrome, Denver, CO, USA) dissolved in distilled water, d) modified Aujeszky-virus strain (KaVHInc) dispersed in 10  $\mu$ l physiological saline. The anterograde spreading of the virus was slowed down (Boldogkői et al., 2002). Each tracer was first injected i.v. FB, FG and the virus were taken up from the blood by the terminals of the supraoptico-paraventriculo-hypophyseal tract in the posterior pituitary, where the blood–brain barrier is missing, and showed labeling in the magnocellular cell groups in 24 h. FB and FG labeled the hypophyseotropic parvocellular system as well, entering the hypothalamus through the median eminence where the blood–brain barrier is also missing. BDA did not enter the nervous system from the blood when injected i.v. In a further experiment, the retrograde transport of BDA was observed after injection into the vitreous body. We have established that there is no possibility of false labeling due to leakage of BDA into the blood when injected intravitreally. FG was used as an anterograde tracer by iontophoretic administration in the expected location of the cells sending their axons to the retina. Preliminary experiments have shown that this location is the hippocampal formation. By administering a very small amount of FG far from the places where the blood–brain barrier is missing, there is no danger of the tracer being transported by the blood to the retina.

### Experimental groups

Group 1: 10  $\mu$ l of 4% BDA was injected into the vitreous body of the eye of five animals.

Group 2: 0.1  $\mu$ l of 4% FG was applied iontophoretically to the hippocampal formation of five animals. The parameters of administration were the following: 3.0 mm behind the bregma, 2.5 mm right to the midline and 3.5 mm below the dura according to stereotaxic atlas of Paxinos and Watson (1986), 5.5 mA, 7 min on-off periods, 20 min.

Group 3: One control animal received distilled water into the vitreous body. The other control animal was sham-operated (the iontophoretic cannula without any tracer was lowered into the hippocampal formation). These two controls served to test the specificity of BDA and FG labeling, respectively.

At the end of a 2 day-survival time, the animals receiving BDA injection or injection of distilled water in the vitreous body were perfused by 4% paraformaldehyde (PFA) (Merck, Darmstadt, Germany) containing 2.5% acrolein (EM grade) (Polysciences, Warrington, PA, USA) in potassium phosphate-buffered saline (KPBS) (pH 7.2, 0.05 mol/l) (Sigma). The animals receiving FG administration or the sham operated animals were perfused by 4% PFA in KPBS. The brains from all animals and the eyes from FG-injected animals were removed, immersed in 4% PFA solution containing 2.5% acrolein for two hours, and postfixed in 4% PFA solution for 24 h. After washing the brains and eyes in KPBS they were placed in ascending sucrose solution (10–20–30%) for one day.

The entire forebrains were sectioned in sagittal plane. Six series of 25  $\mu$ m-thick sagittal sections of brains from the animals that received BDA were prepared on cryostat (Shandon, Pittsburgh, PA, USA), collected in a solution containing KPBS, glycerol, ethylene glycol (Sigma) and distilled water (1:3:3:3 volume

ratio) and stored at  $-20^{\circ}\text{C}$  until use. Watson and coworkers (1986) demonstrated that the sections could be stored for a long time in ethylene glycol without impairing any immunoreactivity in the tissues.

The brain and eyes of the animals that received FG were embedded in cryomatrix (Shandon). One series of 20  $\mu$ m-thick sagittal sections of the brains and horizontal sections of the eyes were prepared and mounted on gelatinized slides and the staining procedure was started immediately.

### Immunohistochemistry

**Fluorescent visualization of BDA.** Sections were rinsed free of cryoprotectant with KPBS. Goat anti-biotin antiserum (Vector Laboratories, Burlingame, CA, USA) was applied to three series of sections at a dilution 1:35,000 in KPBS+0.4% Triton X-100 for 24 h at room temperature. We purchased Tyramide Signal Amplification Kit (Molecular Probes, Eugene, OR, USA) and followed the protocol described in detail by Berghorn et al. (1994) for tyramide amplification, employing the reagents from an ABC Elite Kit (Vector Laboratories) after the biotin tyramide, we then used streptavidin Alexa Cy2 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) to detect the deposited biotin. Sections were rinsed in saline, mounted onto glass slides, dried overnight and dehydrated briefly with ascending ethanol series. Sections were cleared in xylene and coverslipped with Krystalon (EM Science Harleco, Kansas City, MO, USA). The results were evaluated using a Biorad 2100 Rainbow Confocal System equipped with Nikon Eclipse 800 microscope (Hemel, Hempstead, UK).

**Nickel–diaminobenzidine tetrahydrochloride (DAB) visualization of BDA.** In one series of sections, instead of fluorescent staining, BDA was visualized using ABC immunoperoxidase method and nickel intensification of DAB (Sigma) reaction product as described previously (Hoffman et al., 1992). The sections were rinsed free of cryoprotectant in KPBS, incubated in 1% borohydrate solution (Sigma), and rinsed until bubbles were eliminated. The sections were placed in goat anti-biotin antiserum at a dilution 1:70,000 in KPBS+0.4% Triton X-100 for 24 h at room temperature. After multiple rinses in KPBS, the samples were exposed to biotinylated anti-goat antiserum for one hour at room temperature at 1:600 dilution in KPBS+0.4% Triton X-100. After multiple rinses with KPBS, the sections were incubated with ABC solution (45  $\mu$ l each A and B in 10 ml of KPBS+0.4% Triton X-100, Vector Laboratories) for 1 h. The samples were rinsed again several times and placed into nickel–DAB chromogen solution (250 mg nickel sulfate and 2 mg DAB and 8.3  $\mu$ l of 3%  $\text{H}_2\text{O}_2$ /10 ml of 0.175 mol/l sodium acetate solution, Sigma). Staining was performed for 10 min and then terminated by transfer of the sections into sodium acetate solution. Sections were rinsed in KPBS, mounted onto glass slides, dried overnight and dehydrated briefly with ascending ethanol series. Sections were cleared in Histoclear and coverslipped with Histomount (Raymond A Lamb, Apex, NC, USA). The results were evaluated using Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany), equipped with Olympus PP50 digital camera (Olympus, Tokyo, Japan).

**Double labeling immunohistochemistry for detection of neuropeptides in BDA labeled cells.** Three series exhibiting fluorescent BDA labeling were further stained for VIP, PACAP or LHRH immunoreactivities using immunofluorescent localization of the second antigen with a direct tagged secondary antibody. The second antigens were detected by applying polyclonal anti-VIP (raised in rabbit and generous gift of T. J. Görcs and characterized by Gulyás et al., 1990) at a dilution of 1:10,000, anti-PACAP (raised in rabbit and generous gift of A. Arimura and characterized by Köves et al., 1990, 1991) at a dilution of 1:300 or anti-LHRH (raised in rabbit and generous gift of J. Horváth and characterized

by [Merchenthaler et al., 1980](#)) at a dilution of 1:10,000 to the slides and incubating with the tissue for 48 h at 4 °C. Antigen–antibody complex was visualized using an Alexa 546-tagged secondary antiserum against rabbit immunoglobulin incubated for 3 h at 37 °C. To be sure that the labeled structures were nerve cell bodies a nuclear staining was also carried out using 4,6-diamidine-2-phenylindole dihydrochloride (DAPI) (Molecular Probes). Sections were rinsed in saline, mounted onto glass slides, dried overnight and dehydrated briefly with ascending ethanol series. Sections were cleared in xylene and coverslipped with Krystalon (EM Science Harleco). The results were evaluated using a Biorad 2100 Rainbow Confocal System equipped with Nikon Eclipse 800 microscope (Hemel).

**Fluorescent visualization of FG.** Rabbit anti-FG antiserum (Fluorochrome) was applied to a series of sections of eyes, optic nerves and brains at a dilution 1:3000 for 24 h at room temperature. We followed the protocol described above for fluorescent BDA visualization using tyramide signal amplification kit.

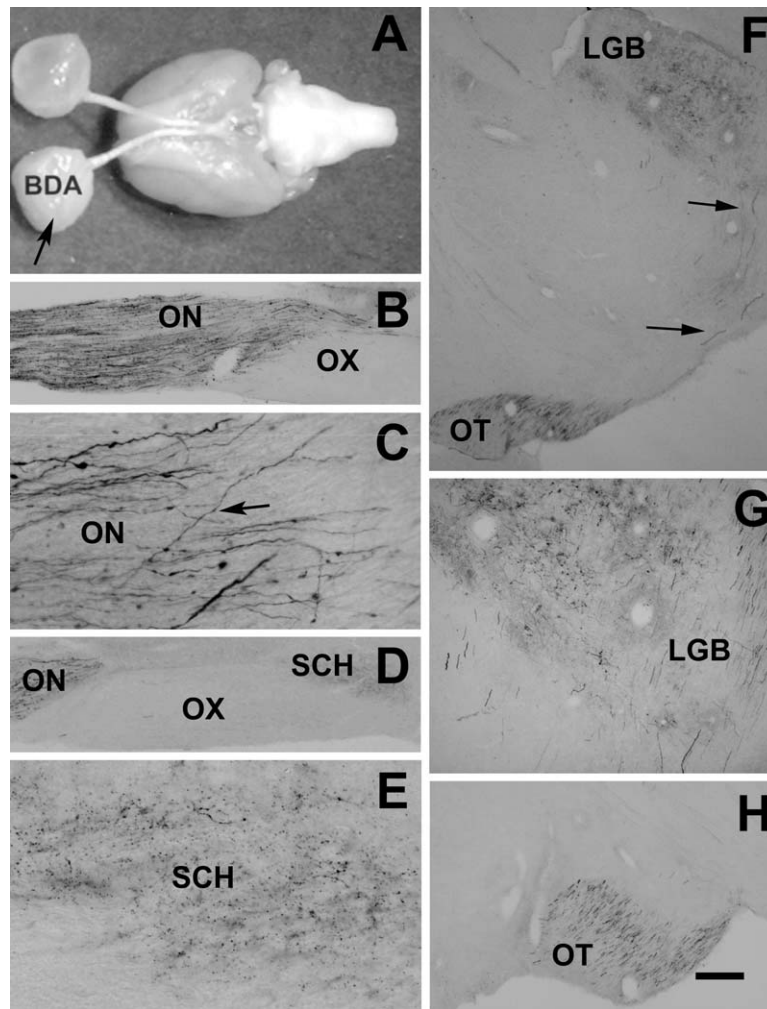
**Specificity of antisera.** PACAP, VIP and LHRH antisera in a working dilution were preabsorbed with an excess amount of

peptides before use: 1 µg LHRH (Sigma), 1 µg VIP (Sigma) or 20 µg PACAP (Sigma) was added to 1 ml of the corresponding antiserum. Parallel sections were stained with the preabsorbed and non-preabsorbed antisera and compared. Staining was not observed with any of the preabsorbed antisera.

The sections stained were rinsed and mounted onto glass slides. Then we followed the protocol described in the section of nickel–DAB visualization of BDA.

### Estimation of the number of BDA-labeled cells

Both hemispheres of rat brains were used for analysis. In one out of six series of sections per animal, BDA was visualized using ABC immunoperoxidase method and nickel intensification of DAB reaction product and the labeled cells were counted. In all sections, the BDA-labeled cells in the hippocampal formation, including the dentate gyrus (DG), CA1 and CA3 regions of the hippocampus, were counted separately in the contra- and ipsilateral sides. Stereologic analysis was not performed because the number of labeled cells was not robust ([Schmitz and Hof, 2000](#)). Instead, all BDA cells were counted individually in every sections on the left



**Fig. 1.** Distribution of anterograde BDA labeling in the sagittal sections of the forebrain. (A) Injection site of BDA (arrow). (B) BDA-labeled fibers in the ipsilateral ON. (C) High power detail of B shows two sets of fibers. Thin fibers exhibit beaded appearance, one of these is indicated by an arrow. (D) In the SCH nuclei (main retinorecipient areas) there was a terminal fiber network labeled by BDA. Ipsilateral side is shown. E is a high power detail of D. F shows BDA-labeled fibers in the contralateral OT and LGB (primary visual center). Arrows show ascending fibers from the OT to the LGB. G and H are high power detail of F. Abbreviations: ON, optic nerve; OT, optic tract; OX, optic chiasm. Scale bar=3 mm in A, 500 µm in B, D and F, 100 µm in C and E, 250 µm in G and H.

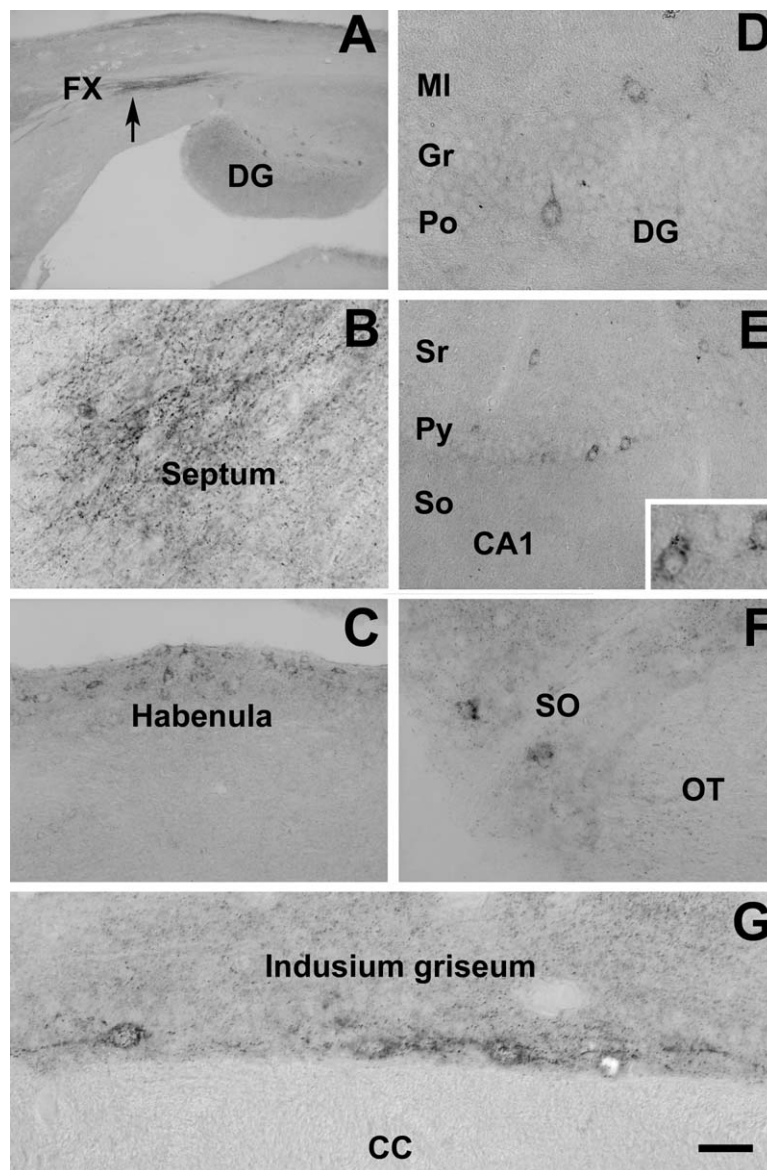
and right sides. The hippocampus and its subregions were identified using Zeiss Axiophot microscope equipped with 5× Zeiss objective and the labeled cells were counted under 40× Plan NeoFluar objective. The number of cells was multiplied by six because six parallel series of sections were prepared. The mean number of BDA-labeled cells of five animals was calculated.

## RESULTS

### BDA labeling

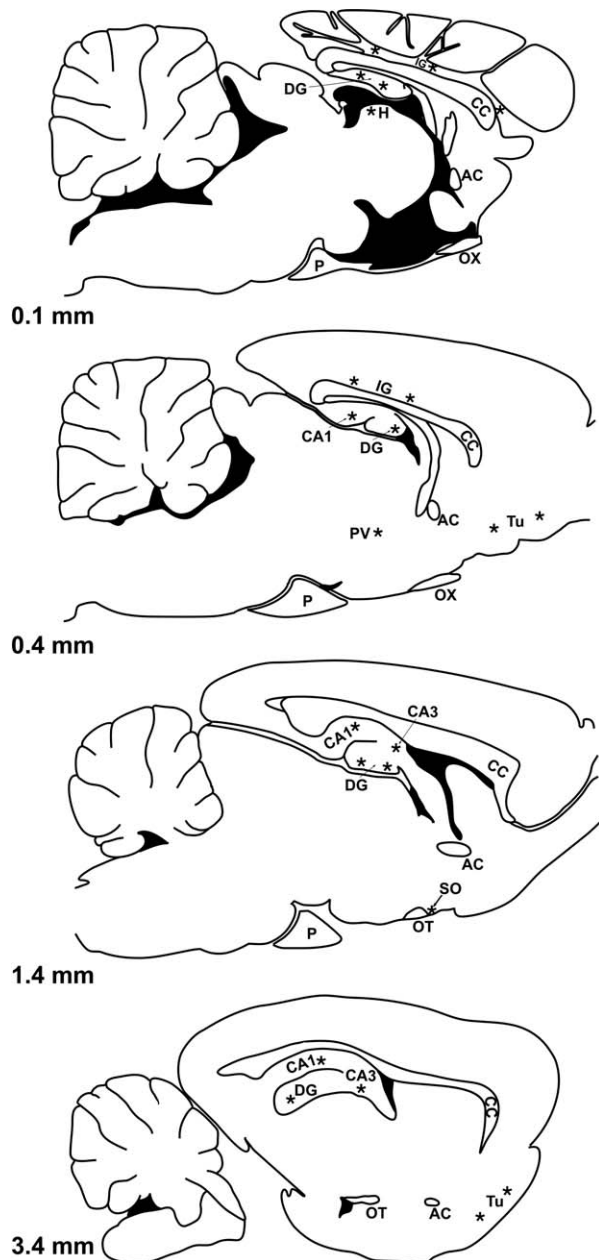
BDA injected in the vitreous body of the eye was transported both ante- and retrograde manners. In group 1,

many BDA-labeled nerve fibers were observed ipsilateral to the BDA injection in the optic nerve, in the optic chiasm and in the optic tract mainly contralateral to BDA injection. Fig. 1A shows the site of BDA administration. Fig. 1B, C and D show labeled fibers in the optic nerve. Fig. 1F and H show labeled fibers in the contralateral optic tract. Fig. 1C is a high power detail of B. It shows two sets of BDA-labeled fibers in the optic nerve. Thick fibers exhibited sharp contour and thin fibers showed beaded appearance. Terminal-like fiber labeling was observed bilaterally in the SCH nucleus (Fig. 1D and E) and in the contralateral



**Fig. 2.** Distribution of retrograde BDA labeling in sagittal sections of the forebrain. A shows BDA-labeled fiber bundle in the dorsal FX (arrow) and labeled cell bodies in the DG. B shows thin beaded fibers labeled by BDA in the septum. (C) Labeled cell bodies are seen in the medial habenula lying on the dorsal aspect of the thalamus. D demonstrates labeled cell bodies in the DG. E shows labeled cells in the CA1 region of the hippocampus, the inset shows two labeled cells with a higher magnification. F shows labeled cells in the SO. (G) Elongated labeled cells are seen in the IG along the dorsal surface of the CC. Abbreviations: CC, corpus callosum; FX, dorsal fornix; Gr, granule cell layer; MI, molecular layer; OT, optic tract; Po, polymorph layer; Py, pyramidal cell layer; So, stratum oriens; Sr, stratum radiatum. Scale bar=250  $\mu$ m in A, 100  $\mu$ m in C and E, 50  $\mu$ m in B, D and F, and 25  $\mu$ m in the inset of E and in G.

lateral geniculate body (LGB) (Fig. 1F and G). The labeled fibers from the optic tract can be followed up to the LGB (Fig. 1F). The appearance of the terminal-like fiber labeling in the SCH and in the LGB indicates that the tracer was transported anterogradely. In these structures labeled cell bodies were never seen (Fig. 1E and G). Fig. 2 shows BDA labeling in structures contralateral to the injection site. A solid bundle of BDA-labeled fibers was seen in the dorsal fornix below the corpus callosum (Fig. 2A), and thin,



**Fig. 3.** Schematic illustration of the distribution of nerve cell bodies retrogradely labeled by BDA. The drawings of sections derive from stereotaxic atlas by Paxinos and Watson (1986). The labeled cells were observed in structures belonging to the limbic system. Asterisks indicate the occurrence of labeled cells, not their relative amount. Abbreviations: AC, anterior commissure; CC, corpus callosum; OT, optic tract; OX, optic chiasm; P, pituitary gland.

**Table 1.** Total number of BDA-labeled cells in the hippocampal formation after injection into one eye

| Number of BDA cells       | CA1     | CA3     | DG      | Total in hippocampus |
|---------------------------|---------|---------|---------|----------------------|
| Ipsilateral side (right)  | 168±159 | 110±104 | 340±86  | 618±177              |
| Contralateral side (left) | 258±240 | 80±43   | 556±290 | 877±391              |
| Total                     | 426±399 | 190±119 | 896±323 | 1495±516             |

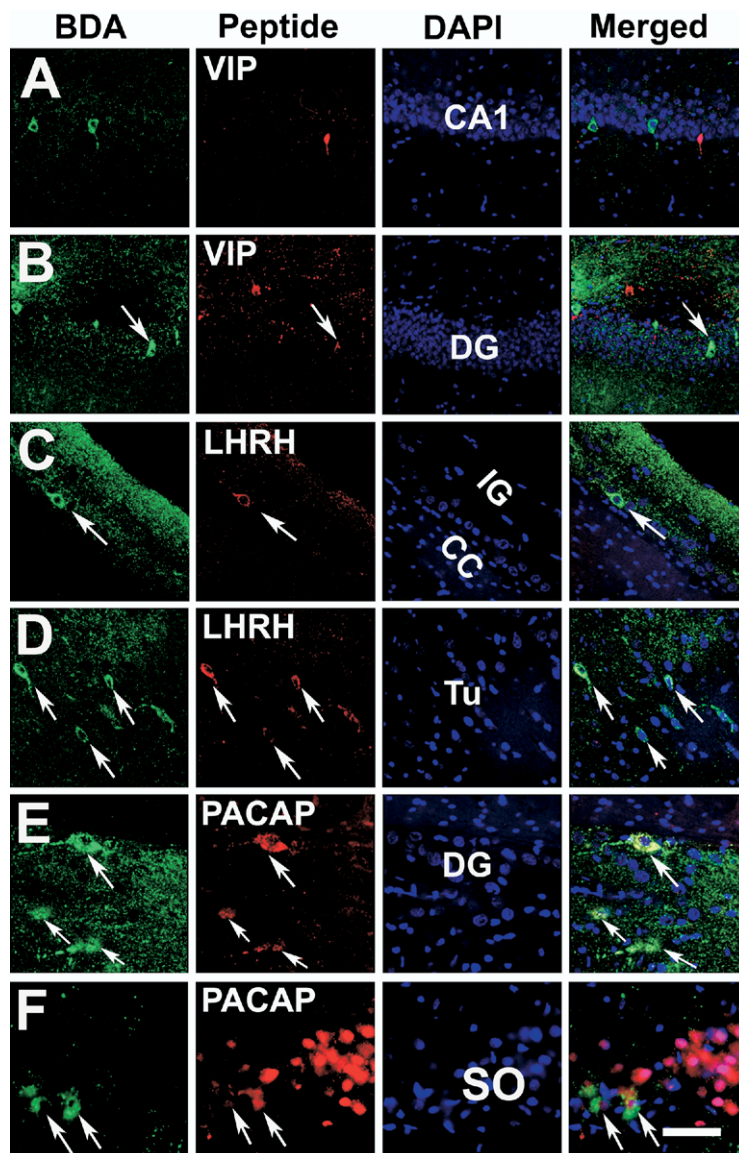
In all sections, the BDA-labeled cells were counted separately in the contra- and ipsilateral sides (results are expressed as mean±standard error,  $n=5$ ).

loosely arranged, beaded fibers were observed in the septum (Fig. 2B) and a considerable number of labeled nerve cell bodies were seen in several structures. They were observed in the DG (Fig. 2A and D), CA1 (Fig. 2E) and CA3 (not shown) regions of the hippocampus, the SO (Fig. 2F) and the PV (not shown) nuclei, the habenular complex (H) (Fig. 2C), the indusium griseum (IG) (Fig. 2G) and the olfactory tubercle (Tu) (not shown). The labeled cells were mostly multipolar, but those present in the IG were elongated bipolar. In the cytoplasm of labeled cells we had seen fine labeled granules. These observations, demonstrated in Fig. 2, show that the tracer was also very well transported retrogradely. The injection of tracer to one eye resulted in cell body labeling at both sides of the forebrain. Fig. 3, according to the stereotaxic atlas by Paxinos and Watson (1986), schematically illustrates the structures where the labeled cell bodies were observed.

We found that the total number of BDA labeled cells in various structures after injection into one eye is relatively low. In the hippocampus and DG we calculated that the total number of labeled cells was  $1495\pm516$  (see details in Table 1). The presence of labeled cells was not restricted to any specific layer of the DG or CA1 and CA3 regions. In the DG, labeled cells were observed in all three layers (molecular, granule cell or polymorph layers). In CA1 and CA3 regions the labeled cells were mostly in the pyramidal cell layer, although some were also seen in the stratum radiatum and oriens (Fig. 2E). In other regions the number of labeled cells was very low and not included in Table 1.

### Colocalization of BDA and peptide immunoreactivities

Double labeling immunohistochemistry revealed the presence of BDA in VIP immunoreactive cells of the DG (Fig. 4B), but not in the CA1 (Fig. 4A) or CA3 (not shown) regions of the hippocampus. BDA appeared in LHRH immunoreactive cells of the DG (not shown), the IG and the Tu (Fig. 4C and D). BDA also appeared in PACAP immunoreactive cells of the DG (Fig. 4E), the SO nucleus (Fig. 4F) and in the medial habenula (not shown). Our observations concerning the double labeling are summarized in Table 2.



**Fig. 4.** Confocal microscopic image of double-labeled cells containing retrogradely transported BDA and showing peptide immunoreactivity. The first vertical row of photos shows BDA labeled cells (green fluorescence), the second row shows peptide immunoreactive cells (red fluorescence), the third row shows nuclear staining by DAPI (blue fluorescence) and the name of the structure, the fourth row is a merged image demonstrating double-labeled cells (green+red) with blue nucleus. Same view fields can be seen in a horizontal row. BDA and VIP colocalize in the DG (B), but not in the CA1 region (A). BDA and LHRH colocalize in IG (C) and Tu (D). BDA and PACAP colocalize in DG (E) and SO (F). Arrows show the cells in that BDA and one of peptides coexist. Abbreviations: CC, corpus callosum. Scale bar=50  $\mu\text{m}$  in A, B, C and D, 30  $\mu\text{m}$  in E and F horizontal rows.

### FG labeling

Iontophoretic administration of FG into the hippocampal formation resulted in labeled fibers in the optic nerves and in the retina of the eyes at both ipsi- and contralateral sides. Fig. 5A shows the localization of iontophoretic administration. FG was taken up by the cell bodies of the DG and CA3 region of the hippocampus indicated by a bright fluorescence. Fig. 5B shows labeled fibers in the contralateral optic nerve. There were only a few labeled fibers in the optic nerve whereas we observed many fibers in the retina (Fig. 5C). The labeled fibers first appeared in the optic nerve layer. Then traversing the layer of ganglion cells,

they entered the inner plexiform layer (IPL). A few fibers reached the inner region of the inner granular layer as well (INL). In this layer they sometimes formed loops around unlabeled cells. Labeled ganglion cells were not seen in these animals.

### DISCUSSION

We were able to provide direct evidence for the origin of a limboretinal pathway using anterograde and retrograde tracing techniques. The present results support the previous observation that there are neuronal cell bodies in the hypothalamic magnocellular nuclei that send their axons

**Table 2.** Double labeling immunohistochemistry revealed the presence of BDA in VIP, PACAP or LHRH immunoreactive cells in various structures of the limbic system

| BDA-labeled cell | VIP | PACAP | LHRH |
|------------------|-----|-------|------|
| CA1              | –   | –     | –    |
| CA3              | –   | –     | –    |
| DG               | +   | +     | +    |
| IG               | –   | –     | +    |
| H                | –   | +     | –    |
| Tu               | –   | –     | +    |
| SO               | –   | +     | –    |
| PV               | –   | –     | –    |

toward the eyes. We have also revealed cell bodies in several other limbic structures which send axons to the eye.

In this present experiment we have found that BDA is a suitable tracer for demonstrating the origin of retinopetal fibers arising in the hypothalamic and various extrahypothalamic structures, all of which belong to the limbic system. This molecule is small enough to be taken up by thin fibers but too large to pass through gap junctions. And it did not enter the brain when it was administered i.v. In our previous study (Köves et al., 2002), cobaltic lysin was applied to the transected optic nerve after removal of the eye. The interrupted nerve fibers were able to take up and transport the tracer toward the hypothalamus. This tracer could neither pass gap junctions nor pass transsynaptically. It did not enter the brain from the blood where the blood–brain barrier is missing but, unfortunately, this tracer is hardly suitable for double labeling. There are only two research groups (Duve et al., 1983; Antal, 1984; and Nagy et al., 1994) that have published the colocalization of cobalt labeling and peptide immunoreactivity. The double labeling for BDA and neuropeptides using the above described protocol (Berghorn et al., 1994) was elaborated in Hoffman's laboratory (Hoffman et al., 1992). This method is very sensitive, specific and highly suitable for demonstrating a low amount of BDA and neuropeptides in nerve cells.

It is possible that the retinopetal terminals in rats cannot take up large molecule tracers injected into the vitreous body. This may have been the reason why Itaya (1980) and Itaya and Itaya (1985) did not observe retrogradely labeled cells in the hypothalamus, the hippocampal formation or other limbic structures of rats; although, in the pretectal area they did find labeled cells. We have also observed labeled cells in the pretectal area using Co-ly, BDA or other tracers.

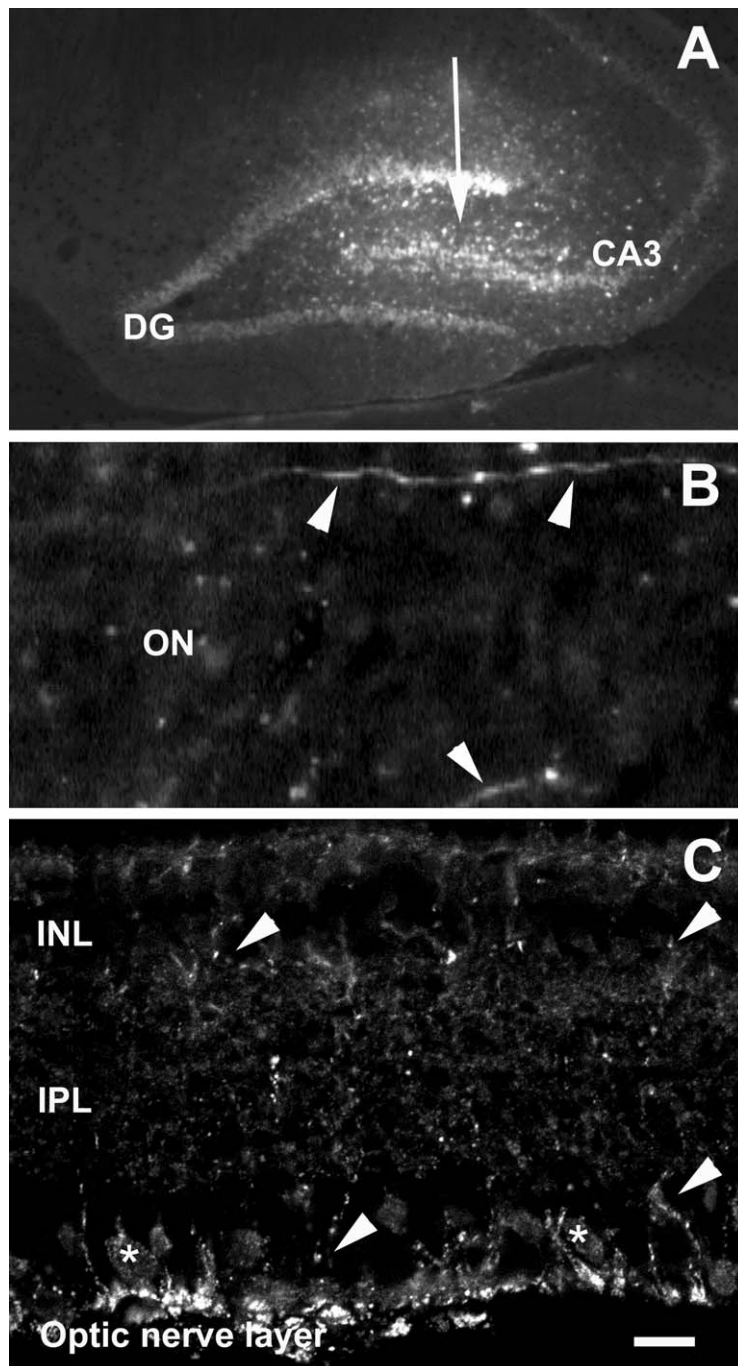
Though the cells sending fibers to the retina are scattered in several structures, their total number is considerable (about 1000–1500/animal). In the auditory efferent pathway, the olivocochlear band is composed of about 1500 fibers (Bishop and Henson, 1987) and the number of LHRH neurons in rats is 600–700 (Funabashi and Kimura, 1995). Therefore, the efferent retinal fibers must play an important role in modifying the transmission of neural impulses within the retina. It is interesting that labeled cells

were never seen in the LGB, which is the primary visual center, or in the major retinorecipient area in the SCH.

In our previous experiments, when we examined the distribution of PACAP and VIP in the forebrain, we found PACAP and VIP immunoreactive cells in several limbic structures including the hippocampal formation (Köves et al., 1991). Other researchers have also observed PACAP immunoreactive cells in the hippocampal formation (Hannibal, 2002). In the DG, PACAP was mainly present in cell bodies at the border of the granule and the polymorphic cell layers. As expected, double labeling for BDA and PACAP revealed that PACAP immunoreactivity is present not only in retinal afferent (Hannibal et al., 1997; Köves et al., 2002) but in efferent fibers as well. In the present experiment, PACAP-ergic cells sending retinopetal fibers were observed in the medial habenular and SO nuclei and in the DG. Double labeling for BDA and VIP revealed that the retrogradely transported BDA appeared in some VIP immunoreactive cell bodies in the DG indicating that a part of the retinopetal fibers may contain VIP as well. VIP immunoreactive neurons in the DG and CA1 and CA3 hippocampal regions were described by Sloviter and Nilaver (1987).

In our experiment there was colocalization of BDA labeling and LHRH immunoreactivities. This was expected because LHRH fibers have been observed in the optic nerves of many species including rats (Santacana et al., 1996). Medina and coworkers (2005) demonstrated LHRH fibers in the IPL of crocodile retina. They were able to follow these fibers to the preoptic–septal junction. The forebrain origin of these fibers was not demonstrated by either research group. In our material we have also seen LHRH fibers in the optic nerve and we have found LHRH cells in limbic structures (Tu, DG and IG) that send fibers to the eye. We have proven this using the combination of BDA labeling and peptide immunohistochemistry. This observation correlates with previous results published by Merchenthaler and coworkers (1984) who found LHRH immunoreactive cells in limbic structures including the IG and the Tu.

Our hypothesis on how axons arising in limbic structures can reach the optic chiasm, optic nerve and the retina is schematically illustrated in Fig. 6. The limbic structures project to the septum. The majority of these fibers travel through the fornix. Both septum and fornix showed retrograde BDA labeling (Fig. 2A and B) indicating that the retinopetal fibers may leave the hippocampal formation through the dorsal fornix and then the septum. When FG was iontophoretically applied into the hippocampal region, where the majority of cell bodies belonging to the limbo-retinal pathway reside, we found anterogradely labeled fibers in the retina indicating that our hypothesis is accurate. The distribution and termination of visual centrifugal fibers that we observed are very similar to that of Dowling and Cowan (1966) who placed a lesion in the isthmo-optic nucleus of pigeons and observed degenerated centrifugal fibers in the inner plexiform and INLs in the vicinity of the amacrine cells. The observations of Dowling and Cowan (1966) and our observations correlate with the early de-

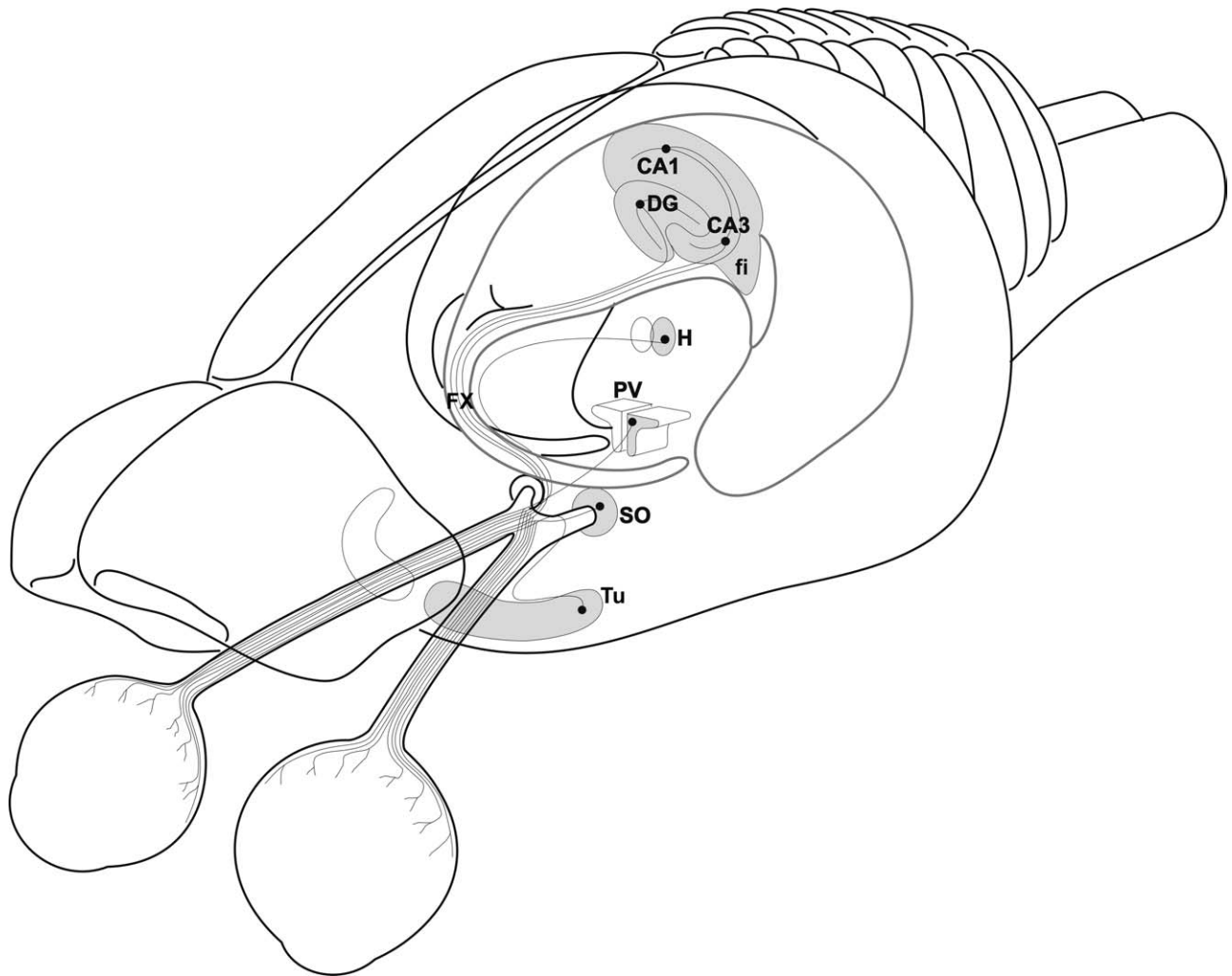


**Fig. 5.** Microphotographs demonstrating the localization of iontophoretic administration of FG into the hippocampal formation and fibers anterogradely labeled by FG in the ON and in the retina of the eye contralateral to the injection site. FG was taken up by many cells adjacent to the application site shown by arrow (A). FG-labeled fibers in the ON are shown by arrowheads. The lower fiber is out of focus (B). Many labeled fibers can be seen in the innermost layer of the retina: the optic nerve layer. Leaving this layer the fibers take a perpendicular course then enter deeper layers of the retina. Arrowheads show fibers traversing the ganglion cell layer and then entering the IPL. Some of them enter the inner part of INL. Asterisks show unlabeled ganglion cells (C). Abbreviations: ON, optic nerve. Scale bar=125  $\mu\text{m}$  in A, 10  $\mu\text{m}$  in B, and 25  $\mu\text{m}$  in C.

scription of centrifugal visual fibers in the avian retina by Ramon y Cajal (1889, 1911) and Dogiel (1895).

This purely morphological study does not give any evidence for the physiological role of the limboretinal pathway. Dowling and Cowan (1966) supposed the following on the basis of their morphological evidence concerning the centrif-

ugal fibers from the isthmo-optic nucleus of birds: 1. Because the majority of the centrifugal fibers end near the origin of main amacrine cell processes, this indicates that they have a dominant role in amacrine cell activity; 2. amacrine cells are interneurons in the inner nuclear layer interposed between bipolar and ganglion cells on the one side, and centrifugal



**Fig. 6.** Schematic illustration of the hypothetical course of the rat limboretinal pathway. Gray profiles show the structures giving rise to retinopetal fibers. From the hippocampal formation (CA1, CA3 and DG) the fibers run through the dorsal fornix (FX) and then the septum. From the habenula the fibers may run through the stria medullaris to the septum. The fibers from the septum, PV and SO nuclei and from the Tu may join and enter the optic chiasm and both optic nerves. Finally, the fibers terminate in the retina, more abundant at the contralateral side. In the retina, the fibers richly arborize. The scheme does not show any correct amount and proportion of fibers leaving the forebrain or entering the retina. Abbreviations: Fi, fimbriae hippocampi.

fibers on the other side; 3. centrifugal fibers should be capable of sensitively regulating intra-retinal transmission and excitability; 4. the apparent abundance of amacrine to amacrine synapses allows the centrifugal effects to be widely dispersed within the retina.

What may be the functional consequences of the change in the intra-retinal transmission upon stimulation by centrifugal fibers? [Cervetto and coworkers \(1976\)](#) proposed that the retinopetal fibers modulate the responses of retinal ganglion cells to light while others suggest that they influence the responses of retinal cells to hormonal cycle ([Stell, 1985](#)) or attention mechanism ([Van Hasselt, 1972](#)). [Rogers and Miles \(1972\)](#) concluded that the centrifugal system mediates visual guidance of motor behavior which could prevent confusion between self-produced movements and motion exterior to the animals. Retinopetal fibers may also be involved during food selection among static stimuli at a short viewing distance ([Weidner et al.,](#)

[1987](#)) or in facilitating the change from panoramic vision to binocular focusing in lateral eyed animals ([Schutte and Weiler, 1988](#)).

Taken together, we can suggest the existence of a distinct pathway. The cells involved in this pathway originate from various hypothalamic and extrahypothalamic regions, all of which belong to the limbic system. For this reason, it is appropriate to identify this pathway as the limboretinal pathway.

*Acknowledgments*—We are grateful to Mrs. Anna Takács, Dr. Flóra Szabó and Dr. Wei Wei Le for their help and advice, to Dr. Tamás J. Görcs for VIP antiserum, to Dr. Akira Arimura for PACAP antiserum, to Dr. Judit Horváth for LHRH antiserum, to Endre Nemcsics for computerizing the schematic illustrations and to Erin C. Fitzgerald (Native American from Massachusetts) for proof-reading of the manuscript. This work was supported by OTKA grant T34429 and ETT grant 437/2003 to K.K.

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