The centrifugal visual system of rat

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INTRODUCTION

The sensory organs receive stimuli from the external environment and transmit them to the central nervous system. The retinal ganglion cells, besides the primary visual center, send axons or axon collaterals to hypothalamic retinorecipient areas forming the retinohypothalamic tract. This pathway mainly terminates in the suprachiasmatic nucleus (the biological clock) and regulates biological rhythms. It has been supposed for a long time that pathways of the opposite direction also exist. Centrifugal visual fibers were described in the retina of bird at the end of the nineteenth century, before tracing techniques were developed. Rasmussen described the olivocochlear bundle in 1946 and since then many data have been accumulated about the existence of centrifugal fibers within most of the major sensory pathways. With the aid of experimental tracing techniques and immunohistochemical methods the existence of retinopetal projections was described in all vertebrates including human.

The major source of the centrifugal visual fibers of bird is the isthmo-optic nucleus, in snake the nucleus of the ventral supraoptic decussation, in chameleon the ventromedial tegmental region of the mesencephalon and the ventrolateral thalamus and in turtle the mesencephalic reticular area. In mammals the origin of the centrifugal visual fibers are contradictory. Injections of tracers into the eye resulted in labeled neurons in the caudal mesencephalon, the medial pretectal area and the oculomotor nucleus of rats, in the ventral hypothalamus of dog and hamster, in the lateral geniculate nucleus of Mongolian gerbil and in the suprachiasmatic and arcuate nuclei of monkey suggesting that the labeled neurons are the origin of centrifugal visual fibers. Some authors query these results. They suppose that neuronal labeling could be the product of technical artifact due to transneuronal labeling or the
leakage of some tracers. In our laboratory, it was previously observed that there are vasoactive intestinal polypeptide (VIP) immunoreactive fibers in the dorsal aspect of rat optic nerve and optic chiasm. It was supposed that these fibers may be centrifugal. Enucleation (axotomy of the optic nerve) induced the appearance of VIP immunoreactive neurons in the supraoptic (SO) and paraventricular (PV) nuclei, where in intact rats no VIP immunoreactive perikarya can be found. We hypothesised that these neurons send VIP immunoreactive fibers to the eye. Ten days after the unilateral injection of Phaseolus vulgaris leucoagglutinin (PhA-L) into the PV or SO nuclei, anterogradely labeled fibers were observed in the contralateral optic nerve of rats enucleated five-six months previously (Fögel et al., 1997). In these animals the retinofugal fibers had to be degenerated five-six months after enucleation; the fibers that survived and picked up the tracer had to be retinofugal. The abovementioned data led us to provide direct evidence for the origin of retinopetal fibers of rat with the use of a very sensitive tracing method.

**AIMS OF THE EXPERIMENTS**

**Aim 1.**

With the use of a tract tracing technique, our aim was to elucidate whether retinopetal neurons in the forebrain of rat exist.

**Aim 2.**

Previous data show that VIP, PACAP and LHRH fibers occur in the optic nerve, which may be centrifugal. With the use of double labeling immunohistochemistry we wanted to characterize the retinopetal neurons of the forebrain, which were identified by the tracer. We looked for colocalization
between BDA and VIP or PACAP or LHRH immunoreactivities.

**Aim 3.**
We wanted to examine whether PACAP and VIP are present in the same cells of the SO and PV, since both PACAP and VIP-ergic retinopetal projections may arise from these nuclei.

**Aim 4.**
We wanted to verify the observation that neurons, retrogradely labeled from the eye, do really terminate in the retina. FG was applied iontophoretically to those structure, where the greatest number of retrogradely labeled cells were seen and we looked for anterogradely labeled fibers in the retina.

**MATERIALS AND METHODS**

**Experimental animals and tissue preparation**
Adult Sprague-Dawley male rats (2-4 month-old) were used in our experiments. The animals were kept in a light and temperature controlled vivarium (lights on at 6:00 h and off at 18:00 h, temperature 22°C±2). They were provided with standard lab chow and water ad libitum. At the end of the survival time the animals were perfused by 4% buffered paraformaldehyde (PFA) solution containing 2.5% acrolein and postfixed in 4% PFA for 24 hours. After cryoprotection six series of 25 µm-thick sagittal sections were prepared on cryostate, collected in phosphate buffer containing ethyleneglycol and stored in deep freezer until use.
Selection of tracers
We chose tracers that were too large to pass through gap junctions, they 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 µl of 4% biotinylated dextran amine (BDA, 10,000 MW), b) 10 µl of 5% fast blue FB, c) 10 µl of 4% Fluorogold (FG). BDA, FB and FG were dissolved in distilled water. d) modified Aujesky-virus strain (KaVHInc) dispersed in 10 µl physiological saline. Each tracer was first injected intravenously. 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 hours. 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 intravenously. In further experiments, the BDA was injected into the vitreous body. We accepted that there was no possibility of false labeling due to leakage of BDA into the blood when injected intravitreously and it was never reported that BDA could be transported transsynaptically. The BDA of 10,000 MW, due to its size, can not pass through gap junctions. FG was used as an anterograde tracer by iontophoretic application into the expected location of the cells sending their axons to the retina. Preliminary experiments have shown that this location is the hippocampal formation. By applying 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.

Immunocytochemistry
For single immunohistochemistry in the free floating sections the antigen-antibody complex was visualized by conventional
ABC technique using Nickel intensified DAB reaction product or Streptavidin Alexa fluorescent conjugate as described previously by Hoffman et al., (1992).

For fluorescent double labeling immunohistochemistry the sections were processed by ABC technique. To intensify the final fluorescence signal tyramine amplification was used as previously described by Berghorn et al., (1994).

Primary antibodies:
1. Goat anti-biotin antibody was used for demonstrating the transported BDA. Dilution for Ni-DAB was at 1:70,000, for tiramin amplified fluorescent visualization at 1:35,000.
2. Rabbit anti-VIP antibody in 1:10,000.
3. Rabbit anti-LHRH antibody was used at 1:10,000 dilution.
4. Rabbit anti-PACAP antibody was used at 1:300 dilution.
5. Rabbit anti-flurogold antibody was used at 1:3,000 dilution.

In situ hybridization for PACAP combined with immunohistochemistry for VIP

For this experiment we have used rats bearing pituitary stalk section. This intervention induced the appearance of PACAP and VIP immunoreactivities in SO and PV nuclei where it can not be stained in intact rats.

First the in situ hybridization was performed using PACAP riboprobe labeled radioactively with S35-UTP then the immunoreaction was visualized using biotin-tyramine amplification and avidin-biotin-peroxidase method and DAB chromogen.

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, where the BDA was visualized using ABC immunoperoxidase method and nickel intensification of DAB reaction product. The labeled cells were separately counted in the contra- and ipsilateral sides of the hippocampal formation, including the dentate gyrus (DG), CA1 and CA3 regions of the hippocampus. Sterologic analysis was
not performed because the number of labeled cells was not robust. Instead, all BDA cells were counted individually in every sections on the left and right sides. The hippocampus and its subregions were identified using Zeiss Axiopt microscope equipped with 5x Zeiss objective and the labeled cells were counted under 40x 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

1. BDA labeling
BDA injected in the vitreous body of the eye was transported by both ante- and retrograde manners. Many BDA labeled nerve fibers were observed ipsilateral to the BDA injection in the optic nerve and contralateral to BDA injection in the optic chiasm and optic tract. Terminal-like fiber labeling was observed in the SCH nucleus and in the lateral geniculate body (LGB) indicating that the tracer was transported anterogradely. In the latter regions labeled cell bodies were never seen. A solid bundle of BDA labeled fibers were seen in the dorsal fornix below the corpus callosum and thin beaded fibers in the septum and a considerable number of labeled nerve cell bodies were seen in several structures: in the DG, CA1 and CA3 regions of the hippocampus, the SO and the PV nuclei, the habenular complex (H), the indusium griseum (IG) and the olfactory tubercle (Tu). The labeled cells were mostly multipolar and we had seen fine labeled granules in their cytoplasm. Our results show that the tracer was transported retrogradely as well. The injection of tracer to one eye resulted in cell body labeling at both sides of the forebrain. We found that the total number of labeled cells in the various structures after injection of BDA into one eye was relatively low. In the
hippocampus and DG we calculated that the total number of labeled cells was 1495±516. In other regions the number of labeled cells was very low and was not included in the cell counting. 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.

2. Colocalization of BDA and peptide immunoreactivities

Double labeling immunohistochemistry revealed the presence of BDA in VIP immunoreactive cells of the DG. PACAP immunoreactivity colocalized with BDA in nerve cells in the DG, the SO and in the medial habenula. BDA also appeared in LHRH immunoreactive cells of the DG, the IG, and the Tu.

3. PACAP and VIP do not colocalize in the hypothalamic magnocellular nuclei

With the combination of in situ hybridization for demonstrating PACAP mRNA and immunohistochemistry for demonstrating VIP, we were not able to find double labeled nerve cells in the magnocellular nuclei of rats bearing pituitary stalk section.

4. FG labeling in the retina

Iontophoretic application of FG into the hippocampal formation, where we have seen the major part of retrogradely labeled cells, resulted in labeled fibers in the optic nerves and in the retina of the eyes at both ipsi- and contralateral sides. The number of the labeled fibers was very few in the optic nerves, however, in the retina they were numerous. The labeled fibers first appeared in the optic nerve layer. Then traversing
the layer of ganglion cells, they entered the inner plexiform layer. A few fibers reached the inner region of the inner granular layer as well. In this layer they sometimes formed loop around unlabeled cells. FG 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 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 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 intravenously. The double labeling for BDA and neuropeptides using the above described protocol (Berghorn et al., 1994) was elaborated in Hoffman’s laboratory. 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 his coworkers (2005) demonstrated LHRH fibers in the inner plexiform layer 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 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 his 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. 1. 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 limboretinal 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 pigeon and observed degenerated...
Fig. 1. Three dimensional illustration of the hypothesis of the course of the limboretinal pathway. Grey 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, paraventricular (PV) and supraoptic (SO) nuclei and from the olfactory tubercle (Tu) may join and enter the optic chiasm and both optic nerves. Finally, the fibers terminate in the retina, more abundantly on the contralateral side. In the retina the fibers richly arborize. This scheme does not show correct amount and proportion of fibers leaving the forebrain or entering the retina. Abbreviations: CA1 = CA1 region of the hippocampus; CA3 = CA3 region of the hippocampus; DG = dentate gyrus; fi = fimbria; FX = fornix; H = habenular complex; HC = hippocampus; PV = paraventricular nucleus; SO = supraoptic nucleus; Tu = olfactory tubercle.
centrifugal fibers in the inner plexiform and inner granular layers in the vicinity of the amacrine cells. The observations of Dowling and Cowan (1966) and our observations correlate with the early description 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 centrifugal 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 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 his 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 connection as the limboretinal pathway.

NEW ORIGINAL OBSERVATIONS

1. In the forebrain the retrogradely labeled cells, following intraocular injection of BDA, were observed in various structures of the limbic system.
2. The retrogradely labeled neurons are partially PACAP or VIP or LHRH immunoreactive.
3. PACAP and VIP do not colocalize in the magnocellular nuclei of hypothalamus, therefore the retinopetal fibers, which arising from SO and PV, PACAP or VIP-ergic, and they belong to different cell population.
4. The iotophoretic application of FG into the hippocamppal formation resulted in anterogradely labeled fibers in the inner plexiform and inner nuclear layers of the retina confirming our observation that a retinopetal system arising in limbic structures.

On the basis of the above observations we suggest to call this newly described connection as limboretinal pathway.

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Publications related to the dissertation
IF: 3.456
2. Vereczki V, Köves K, Tóth ZE, Baba A, Hashimoto H, Fogel K, Arimura A, Kausz M. Pituitary adenylate cyclase-activating polypeptide does not colocalize with vasoactive
IF: 1.608


IF: 1.381

**Other publications**

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IF: 2.180

**Summerized IF: 14.298**

**Book chapter:**


Oral Presentations


Poster presentations related to the dissertation


Poster presentations not related to the dissertation

3. V. Vereczki, R.E. Rosenthal, P.R. Hof, C.C. Sherwood, W. Hu, G.E. Hoffman, G. Fiskum. Protection against canine hippocampal neuronal damage by normoxic compared to
