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Glucagon-related peptides in the mouse retina and the effects of deprivation of form vision

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Abstract *Background:* In chickens, retinal glucagon amacrine cells play an important role in emmetropization, since they express the transcription factor ZENK (also known as NGFI-A, zif268, tis8, cef5, Krox24) in correlation with the sign of imposed image defocus. Pharmacological studies have shown that glucagon can act as a stop signal for axial eye growth, making it a promising target for pharmacological intervention of myopia. Unfortunately, in mammalian retina, glucagon itself has not yet been detected by immunohistochemical staining. To learn more about its possible role in emmetropization in mammals, we studied the expression of different members of the glucagon hormone family in mouse retina, and whether their abundance is regulated by visual experience. *Methods:* Black wildtype C57BL/6 mice, raised under a 12/12 h light/dark cycle, were studied at postnatal ages between P29 and P40. Frosted hemispherical thin plastic shells (diffusers) were placed in front of the right eyes to impose visual conditions that are known to induce myopia. The left eyes remained uncovered and served as controls. Transversal retinal cryostat sections were single- or double-labeled by indirect immunofluorescence for early growth response protein 1

(Egr-1, the mammalian ortholog of ZENK), glucagon, glucagon-like peptide-2 (GLP-2), glucose-dependent insulinotropic polypeptide (GIP), peptide histidine isoleucine (PHI), growth hormone-releasing hormone (GHRH), pituitary adenylate cyclase-activating polypeptide (PACAP), secretin, and vasoactive intestinal polypeptide (VIP). In total, retinas of 45 mice were studied, 28 treated with diffusers, and 17 serving as controls. *Results:* Glucagon itself was not detected in mouse retina. VIP, PHI, PACAP and GIP were localized. VIP was co-localized with PHI and Egr-1, which itself was strongly regulated by retinal illumination. Diffusers, applied for various durations (1, 2, 6, and 24 h) had no effect on the expression of VIP, PHI, PACAP, and GIP, at least at the protein level. Similarly, even if the analysis was confined to cells that also expressed Egr-1, no difference was found between VIP expression in eyes with diffusers and in eyes with normal vision. *Conclusions:* Several members of the glucagon super family are expressed in mouse retina (although not glucagon itself), but their expression pattern does not seem to be regulated by visual experience.

Keywords Glucagon · Retina · Emmetropization · Mouse · Myopia

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Introduction

Myopia is the most common ocular growth disorder of the human eye in the industrialized world, with about a third of the population affected (e.g., [19]). Although genetic background modulates the probability of becoming myopic (e.g., [25, 41]), visual experience, probably related to the heavy loads of near work in early childhood, appears a major factor (e.g., [24]). Because the myopia-inducing visual experience cannot be changed much during education, there is much interest in the possibility of pharmacological inhibition of myopia development. In particular, muscarinic antagonists have been promising and were successfully tested in animal models [18, 35, 37] and children (e.g., [25, 31, 32]). However, they have side effects like mydriasis (e.g., [21]), cycloplegia (atropine is the most common and powerful cycloplegic drug), corneal dryness [32] and tend to lose their effects after extended periods of application [25, 32]. Linked to cycloplegia, the children treated also need to wear reading glasses.

Therefore, it is necessary to consider alternative pharmacological approaches. One alternative target is the glucagon family [2, 12, 39, 40]. In the chick retina, glucagon is a rare transmitter that is expressed exclusively by the glucagon amacrine cells, at least two types of which have recently been identified [33]. They nevertheless make up about only 1% of the total amacrine cell population. Glucagon amacrine cells in the central and near peripheral retina express the transcription factor ZENK in correlation with the sign of imposed defocus [2] after only about 15 min of exposure [20]. In addition, direct application of glucagon [9, 39] and a superagonist has shown that it may act as a stop signal for axial eye growth [9]. Conversely, an antagonist of glucagon was found to inhibit selectively hyperopia development in the chick [9], which can otherwise be induced by positive lens wear. Therefore, glucagon and/or other glucagon-like peptides could be a promising and relatively selective target [40].

Unfortunately, so far, it has not been detected immunohistochemically in the mammalian retina, although early growth response protein 1 (Egr-1), the mammalian ortholog of ZENK, is expressed in monkey retina and is also regulated by the sign of imposed defocus. Here, it is localized to glutamic acid decarboxylase-65 immunoreactive amacrine cells (GAD65) rather than to glucagon [42]. Recently, receptors of GLP-2 were localized by immunohistochemical staining in lens and cornea of the mouse [17], and Feldkaemper et al. [10] found the mRNA of both the glucagon receptor and of preproglucagon in retina. Therefore, it is possible that another member of the glucagon super family may have taken over the role of glucagon itself in the mechanisms of visual control of eye growth in mammals.

The abundance of glucagon-related peptides in the mammalian retina has not yet been thoroughly studied. The glucagon superfamily includes nine bioactive peptides,

eight of which are found in the brain and, hence, are properly classified as neuropeptides. They are closely related in structure, distribution, function, and receptors (Fig. 1).

We have therefore studied their distribution in mouse retina, and whether they are altered by visual conditions that induce myopia in avian and mammalian models of myopia (including the mouse [26]).

Materials and methods

Animals

Black C57BL/6 wildtype mice were obtained from Charles River GmbH, Sulzfeld, Germany, and bred in the animal facilities of the Institute. Age-matched untreated control animals, and the animals that were later treated with diffusers, were initially housed in groups of 6–8 in standard mouse cages under a 12 h light/dark cycle. Light onset was at 8:00 am. Ambient illuminance was provided by incandescent lights and was about 500 lux on the cage floor (measured with a calibrated photo cell [United Detector Technology, USA] in photometric mode). The strains were completely inbred, and with the exception of sex chromosome differences and exceedingly rare spontaneous mutations, all individuals were isogenic. In total, 45 animals were studied. For the experiments with the diffusers, 5–6 animals were used for each of the five different treatment durations, with 28 animals treated in

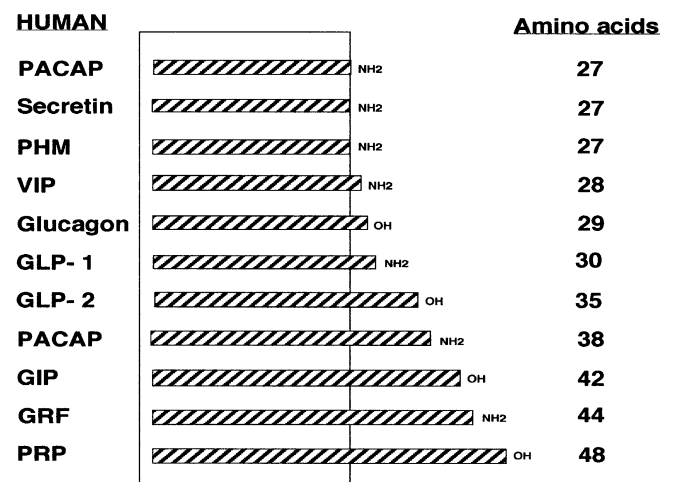


Fig. 1 The glucagon superfamily members that are present in humans are arranged by length. Nine peptides are bioactive, but PACAP-related peptide (PRP) has not been shown to be bioactive to date. The N-terminal region (first 27 amino acids) is the main part of the bioactive core for these peptides. PACAP pituitary adenylate cyclase polypeptide, PHM peptide histidine methionine, VIP vasoactive intestinal polypeptide, GLP-1 glucagon-like peptide-1, GLP-2 glucagon-like peptide-2, GIP glucose-dependent insulinotropic polypeptide, GRF growth hormone releasing factor. Adapted from Sherwood et al. [30]

total. Note that different retinal sections from the same animals could be stained for different epitopes. The remaining 17 animals served as controls.

The treatment of the mice was approved by the University Commission of Animal Welfare (reference AK3/02) and was in accordance with the ARVO resolution for care and use of laboratory animals.

Imposing visual conditions that induce deprivation myopia

Diffusers were hand-made hemispherical thin plastic shells with frosted surfaces as previously described [3]. They acted as severe low pass filters on the spatial frequency spectrum, and reduced contrast over a wide range of spatial frequencies. The visual condition imposed by the diffusers is traditionally referred to as “deprivation of form vision.” The diffusers also attenuate light by about 0.3 log units [3], reduce the abundance of the Egr-1 transcript and the protein in the retina [3], and induce axial eye elongation after 2 weeks [27]. These observations show that the diffusers effectively change retinal metabolism. Their rims, about 1 mm wide, were attached to the fur around the right eye with cyanoacrylic glue (instant glue “Sekundenkleber,” UHU company, Buehl, Germany) during light ether anesthesia. The rims of the diffusers were placed far enough from the eyelids not to interfere with their function. Collars made from thin plastic were fitted around the neck to prevent mice from removing their diffusers during cleaning behavior (Fig. 2). In all experiments, the right eyes were covered with the diffusers for various lengths of time, whereas the left eyes remained uncovered and served as internal “controls.”

Animals were studied between postnatal ages P29 and P40. A previous study [27] has shown that axial eye



Fig. 2 Mouse with translucent hemispherical plastic diffuser in front of the right eye. A collar, made from a ring of plastic foil, was fitted around the neck to prevent the diffuser from being removed during cleaning behavior

elongation can be induced by treating C57BL/6 mice with diffusers from day 26 to 41. During the treatment with the diffusers, the numbers of animals per cage were reduced to 2–3, to reduce stress and the probability of the diffusers getting damaged. During this time, the animals were kept in translucent plastic boxes with wire tops under a laboratory fume hood, illuminated by cool white light (Lumilux 30 W/840; Osram, Munich, Germany). Ambient illuminance was about 120 lux, which is at least 2 log units below the levels that induce retinal degeneration in mice with extended exposure [13]. Food pellets were distributed on the cage floor to facilitate foraging with the collars. Before deprivation experiments started in the morning, the animals were kept in complete darkness, except for in the case of Egr-1 measurements where mice were exposed to light for 60 min before diffusers were attached because Egr-1 is strongly regulated by the diurnal light cycle (see Fig. 6b).

Tissue preparation and immunohistochemistry

Animals were sacrificed by an overdose of diethylether at the end of the deprivation period. Eyes were enucleated and immediately placed into a Petri dish filled with Ringer solution for immediate preparation. They were perforated by a cannula and opened with scissors cutting around the iris. The anterior segment of the eye was discarded and the vitreous gel removed. Fixation occurred by immersion in 4% paraformaldehyde plus 3% sucrose in 0.1 M phosphate buffer (pH 7.4) for 20 min at room temperature. Fixed samples were washed three times (10 min each time) in phosphate-buffered saline (PBS; pH 7.4) and cryoprotected in PBS plus 30% sucrose overnight at 4°C. They were then soaked in embedding medium (Tissue Freezing Medium; Jung, Nussloch, Germany) for 5 min before freezing. Vertical sections 12 μ m thick were cut and thaw mounted onto silane-coated glass slides.

Sections were washed three times in PBS (10 min each time), incubated with blocking buffer (PBS plus 0.3% Triton X-100 [PBST; Sigma-Aldrich, Taufkirchen, Germany] plus 10% normal goat serum [Sigma-Aldrich]), covered with primary antibody solution (200 μ l of antiserum in PBST plus 20% normal goat serum), and incubated for about 20 h at room temperature in the dark. Slides were washed three times in PBS (10 min each time), covered in secondary antibody solution (200 μ l of 1:1,000 Cy3-conjugated goat anti-rabbit IgG [Amersham Pharmacia, Freiburg, Germany] or 1:500 Oregon Green-conjugated goat anti-mouse IgG [Molecular Probes, Leiden, The Netherlands]) and incubated for 2 h at room temperature in the dark. Samples were washed three times in PBS (10 min each time) and mounted under coverslips in 70% Sorbitol (Caelo, Caesar & Loretz, Hilden, Germany) for observation with a fluorescence microscope. Primary antibodies and their working dilutions are listed in Table 1.

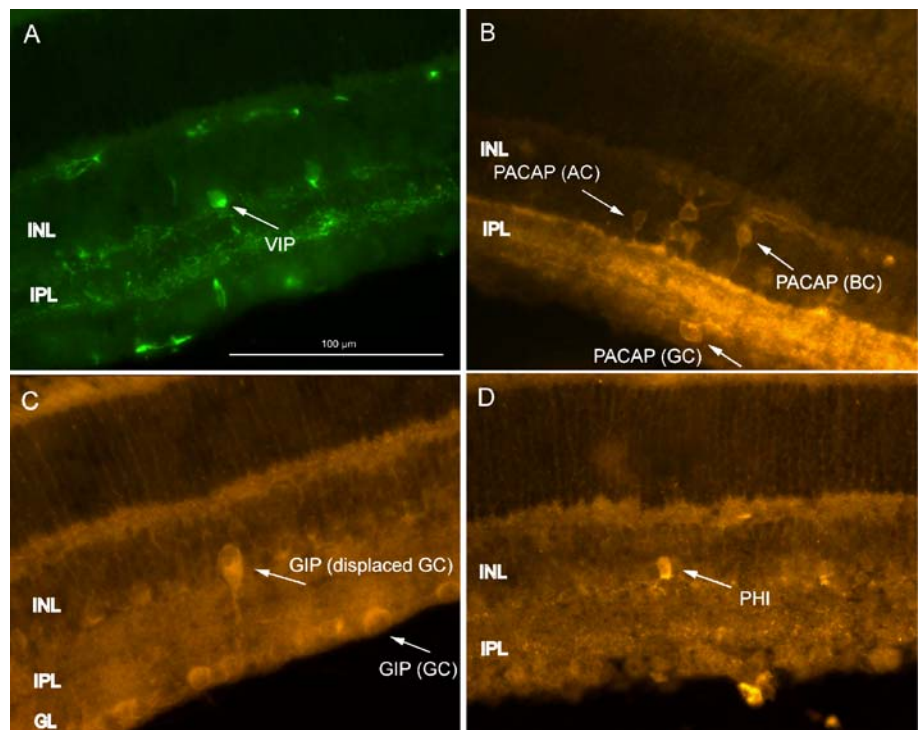
Table 1 List of antibodies, their origin, and their respective working dilutions. EGR-1 early growth response protein 1

Antigen	Antibody	Origin	Working dilution
Egr-1, the mammalian ortholog of ZENK	Anti-Egr-1, anti-rabbit polyclonal, no. 588, sc-110, Lot number L239	Santa Cruz Biotechnology, CA USA	1:1,250
Glucagon	Anti-glucagon, mouse monoclonal	Gordon Ohning, University of California, LA, CA USA	1:400
Glucagon-like peptide-2,	Anti-GLP-2, mouse monoclonal # 7-36	CURE/Digestive Diseases Research Center, Los Angeles, CA, Antibody/RIA Core, NIH Grant #DK41301	1:1,000
Pituitary adenylate cyclase-activating polypeptide	Anti-PACAP 38, rabbit polyclonal # 95129	Same	1:6,700
Glucose-dependent insulinotropic polypeptide	Anti-GIP, polyclonal rabbit # 9102	Same	1:2,000
Secretin	Anti-secretin, rabbit polyclonal # 7846	Same	1:1,000
Vasoactive intestinal polypeptide	Anti-VIP, mouse monoclonal # CURE.v55	Same	1:6,000
Growth hormone-releasing hormone	Anti-GHRH, rabbit polyclonal AB-16AP	Advanced Targeting Systems, San Diego, CA, USA	1:1,500
Peptide histidine isoleucine	Anti-PHI, rabbit polyclonal PA 1246	Biomol, Hamburg, Germany	1:770
Glicentin	Anti-glicentin, rabbit polyclonal No. 11186	PROGEN Biotechnik GmbH, Heidelberg, Germany	1:200

In double labeling experiments, sections were first incubated with a mixture of two primary antibodies and second with a mixture of the above listed secondary antibodies (the respective working dilutions of the antibodies remained unchanged).

Specificity of the staining was assessed as follows: omission of the primary antibody and control stainings in body tissues where the respective peptide is present (pancreas, small intestine).

Fig. 3 **a** Mouse retina labeled for VIP. VIP-like immunoreactivity was found in amacrine cell bodies in the innermost part of the inner nuclear layer. Varicose fibers were distributed in three laminae of the inner plexiform layer. The staining of the blood vessels is due to unspecific staining of the VIP antibody used, which is of mouse origin. **b** PACAP labeling of bipolar (BC) and amacrine cells (AC) in the inner nuclear and ganglion cells (GC) in the ganglion cell layer. Furthermore, PACAP-like immunoreactivity was detected in fibers in the inner plexiform layer. **c** GIP staining in ganglion cells and displaced ganglion cells. **d** PHI could be localized in a population of amacrine cells as well as in sublayers of the inner plexiform layer. Scale bar, 100 μ m. *INL* inner nuclear layer, *IPL* inner plexiform layer, *GL* ganglion cell layer



Cell counts and statistical analysis

Frozen eye cups were vertically cut until the optic nerve became visible. Forty sections, originating from the plane of the optic nerve region, were analyzed per eye. Labeled cells were counted in at least four different sections throughout the entire nasotemporal dimension of the retinas from each animal examined. Because the total dimension was counted in each case, potential confounding effects of regional variations were excluded. The counting was performed in a masked fashion. To compare data from treated and contralateral control eyes, statistical significance was assessed by using an unpaired two-tailed Student's *t* test. Images were recorded by a 12-bit charge-coupled device (CCD) camera and overlaid with software provided by the manufacturer (SIS analysis, version 3.0; Doku software; Soft Imaging Systems, Münster, Germany).

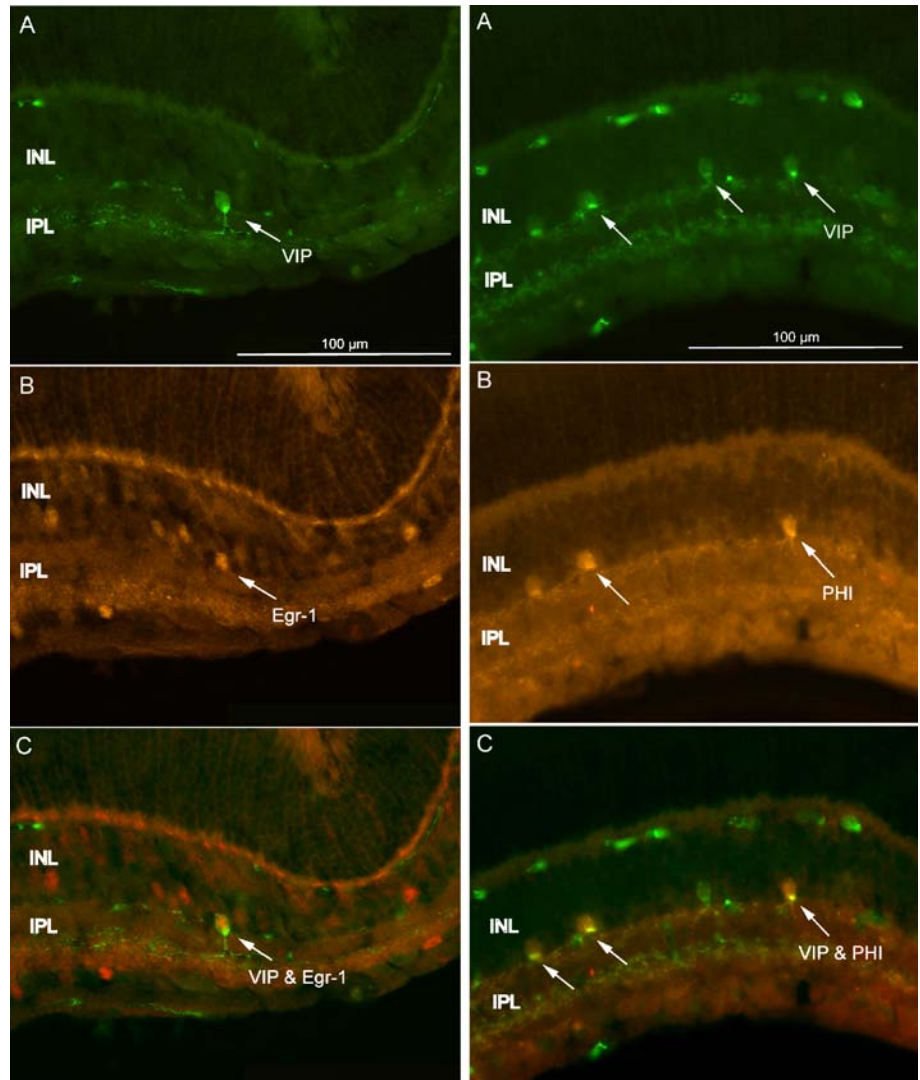
Results

Distribution of glucagon-related peptides in the mouse retina

Glucagon itself was not detected in mouse retina. The same was true for GLP-2, another proglucagon-derived peptide, secretin and GHRH. However, positive stainings were obtained in the pancreas for glucagon, and the small intestine for GLP-2, secretin and GHRH, using the same antibodies.

Vasoactive intestinal polypeptide, PACAP, GIP, and PHI were all identified by indirect immunofluorescence (Fig. 3). As described in previous studies in rat and mouse retina [5, 8, 15, 34, 36], VIP-like immunoreactivity was found in rounded or pear-shaped cell bodies of scattered amacrine cells in the inner nuclear layer and occasionally of amacrine cells displaced in the inner plexiform layer or ganglion cell layer. VIP immunoreactive

Fig. 4 Colocalization of neuropeptides in amacrine cells. Left: **a** VIP, **b** Egr-1, **c** VIP and Egr-1. Right: **a** VIP, **b** PHI, **c** VIP and PHI. Note that each PHI-positive cell is also VIP-positive, but not vice versa. *Egr-1* early growth response protein 1. Scale bars denote 100 μ m



processes had a varicose appearance and were labeled in three narrow bands in the inner plexiform layer (Fig. 3a). PACAP was detected in two different cell types in the inner nuclear layer as well as in cells in the ganglion cell layer (Fig. 3b).

The cells of the inner nuclear layer were identified as bipolar and amacrine cells respectively by their relative positions. The extensive staining of amacrine cells with the PACAP antibody made it impossible to count those cells under various deprivation conditions (see below). At the same time, PACAP immunoreactivity was found in fibers in the inner plexiform layer. These findings are consistent with PACAP investigations in the rat retina by Seki et al. [28, 29]. GIP and PHI were identified for the first time in the mouse retina (Fig. 3c,d). GIP was detected in ganglion cells and in displaced ganglion cells, although only 1–3 cells per section. As previously observed for other rodents [1, 4, 22], PHI was labeled in a subpopulation of amacrine cells and in fibers of inner plexiform sublayers.

Colocalization of VIP with Egr-1 and PHI

In double labeling experiments VIP was co-localized with Egr-1 (Fig. 4a), which itself was strongly regulated by retinal illumination (see Fig. 6b). Furthermore, VIP was also co-localized with PHI (Fig. 4b). Note that each PHI-positive cell was also VIP-positive, but not vice versa.

Effects of form deprivation on peptide expression

Form deprivation imposed for various durations of time (1, 2, 6, 24, and 48 h) had no effect on the expression of the neuropeptides studied in the mouse retina (Fig. 5).

No difference was found in the number of VIP-expressing amacrine cells between eyes with diffusers and eyes with normal vision (Fig. 5a), as well as for PHI-expressing amacrine cells (Fig. 5b), for PACAP-expressing bipolar cells and cells in the ganglion cell layer (Fig. 5c), and for GIP-expressing ganglion cells (Fig. 5d). Similarly, even if the analysis was confined to VIP-expressing cells that were also expressing Egr-1, no effect of visual experience was detected (Fig. 6). In this case, the changes

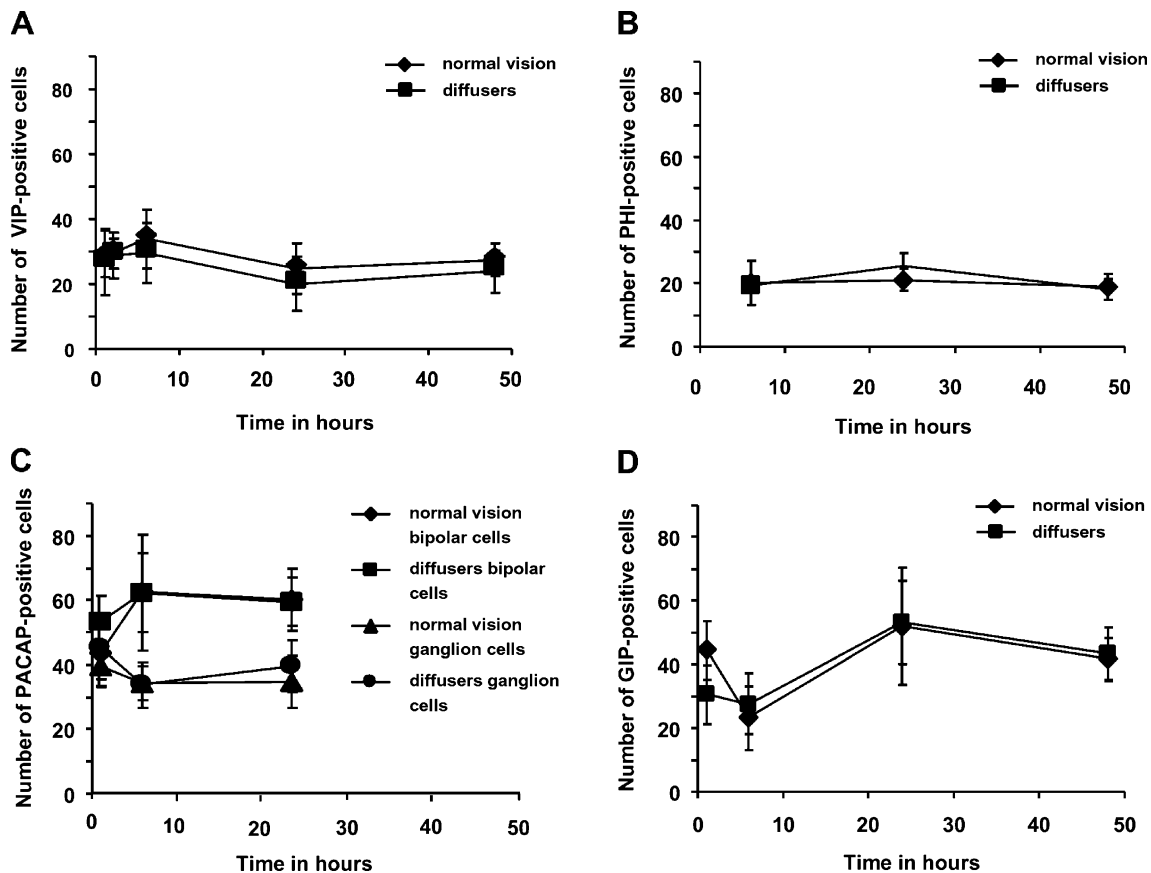


Fig. 5 Numbers of labeled cells, for various durations of form deprivation (1, 2, 6, 24, and 48 h). No significant differences in the numbers of stained cells was found between treated (diffusers) and control eyes (normal vision) for **a** VIP, **b** PHI, **c** PACAP, and **d** GIP. Error bars represent standard deviations. The sample sizes were 5–6 animals per group

in numbers of VIP/Egr-1 co-expressing cells between diffuser-treated and uncovered eyes (Fig. 6a) was due to the diurnal changes in Egr-1 expression itself. The same was true for cells that co-expressed VIP and PHI. Just as VIP and PHI expression on their own show no changes with diffuser wear, the number of cells that co-localize VIP and PHI was not controlled by visual experience.

Discussion

Neither glucagon nor glucagon-like peptide-2 (GLP-2) were detected in mouse retina by immunohistochemical techniques, although positive staining was detected with the same antibodies in pancreas and small intestine. This indicates that the antibodies were functional and should also have detected the peptides in retina if they were present in sufficient amounts. Both peptides are, together with glucagon-related peptide-1, glicentin, and oxyntomodulin, derived from a common precursor molecule (Fig. 7), which is cleaved by tissue-specific endoproteases.

Also, we could not localize glicentin in the retina, although, again, positive staining with the same antibody was observed in the small intestine. Feldkaemper et al. [10] detected small amounts of mRNA for preproglucagon and of the glucagon receptor in the mouse retina, using the more sensitive polymerase chain reaction. On the other hand, preproglucagon could not be detected in the blood [10]. Therefore, it is possible that glucagon is, in fact, produced in retinal cells, but at very low levels, far below the detection limit of our immunohistochemical procedures. It remains then unclear what its role could be because it can probably not elicit significant receptor-mediated responses at these concentrations, given that the IC_{50} and K_d values were determined in a similar range as

for other neuropeptides, with 0.54 nM [38] or between 2.5 and 7.0 nM [11] (all in the rat). The receptors for both glucagon and GLP-2, at least, were recently detected in mouse retina by immunohistochemistry [17].

We did localize VIP, PHI, PACAP, and GIP. VIP and PHI also share a common precursor molecule (e.g., [30]) and they were both localized in the mouse eye. Previous studies suggested that VIP expression is controlled by light exposure [8, 15, 23], as shown by lid suture or dark rearing experiments in primates and rats. Ekman and Tornqvist [7] showed that the retinal glucagon-like immunoreactivity was high in the retinae of birds and cold-blooded animals, but not in mammals. Instead, immunoreactivity to VIP-like epitopes was high. Although VIP seems to be a promising substitute for glucagon, its expression was not affected by form deprivation in our experiments. This also remained true if the analysis was confined to those amacrine cells that also expressed Egr-1. Using realtime PCR, Brand et al. [3] showed that both Egr-1 mRNA and protein expression are strongly regulated by diurnal light cycles. This was confirmed in our deprivation experiments on the protein levels (Fig. 6). Brand et al. [3] also found that the retinal mRNA levels of VIP in mice were not changed by form deprivation, in line with our observations at the protein level.

Pituitary adenylate cyclase-activating polypeptide (PACAP) immunoreactivity was reported in many cell types and their processes in the rat retina [29]. In contrast, Hannibal et al. [14] reported a sparse population of PACAP-positive cells in the rat, which were shown to be involved in the diurnal light response. In our study, the cells that were immunoreactive for PACAP did not show any changes in expression during diffuser wear.

Cho et al. [6] showed that, in the rat, retinal GIP mRNA, and protein are expressed and that their levels are higher in

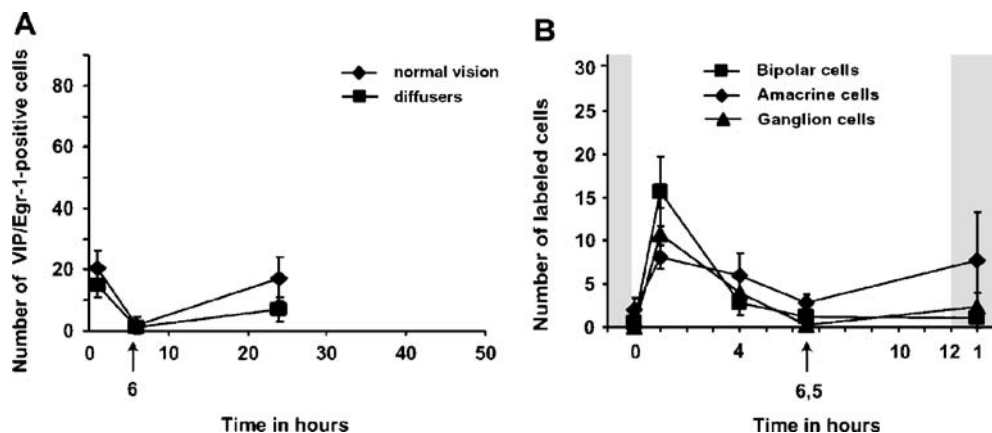
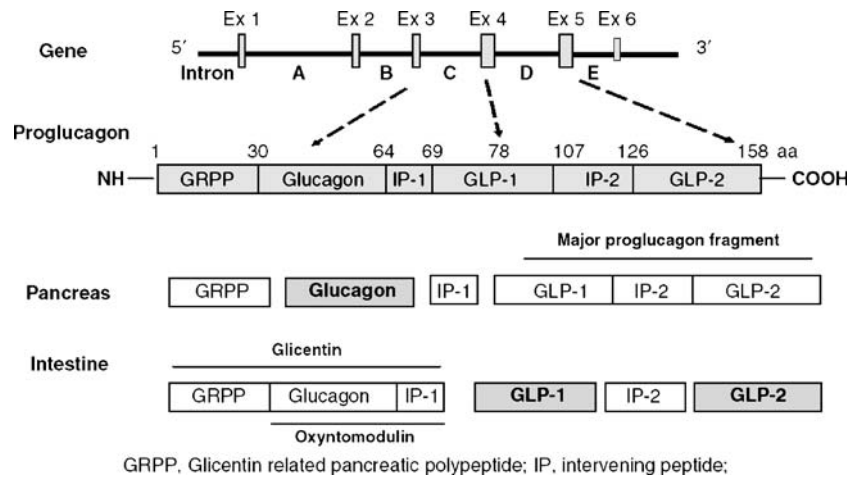


Fig. 6 a Number of cells that co-localized VIP and Egr-1, determined after different periods of diffuser wear (1, 6 or 24 h). The decline in the numbers of immunoreactive cells between 1 h and 6 h in the retinas, both with normal vision and with diffusers, is due to the diurnal change in expression of Egr-1. Egr-1 reached a minimum between 6 and 6.5 h after light onset (arrows). **b** Retinal

Egr-1 expression in amacrine cells, ganglion cell layer, and bipolar cells of untreated animals over the day. The gray bars denote the margins of the dark phase (adapted from [3]). Error bars represent the standard deviations. The sample sizes were 5–6 animals per group

Fig. 7 Human proglucagon undergoes differential processing in pancreatic alpha cells and intestinal L cells. In the alpha cells, the major products released into plasma are glicentin-related polypeptide, glucagon, a minor and a major proglucagon fragment, whereas in the intestinal L cells, the major products released are glicentin, GLP-1 (glucagon-like peptide-1) and GLP-2 (glucagon-like peptide-2). Adapted from [16]



diabetic rats than in normal controls. We localized GIP in mouse retinal ganglion cells and displaced ganglion cells. But, like PACAP, GIP expression was not different in eyes with diffusers and in eyes with normal vision.

In summary, we found that several members of the glucagon super family are expressed in the mouse retina. However, we could not find evidence that any of them take over the role of glucagon in emmetropization, as observed

in avian models, since their abundance was not regulated by visual experience.

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