EXTENDED REPORT

Inhibitory effect of certain neuropeptides on the proliferation of human retinal pigment epithelial cells

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Aims: To define the effect of the neuropeptides substance P, calcitonin gene related peptide, vasoactive intestinal polypeptide, neuropeptide Y, and secretoneurin on the proliferation of human retinal pigment epithelial (RPE) cells.

Methods: ARPE-19 cells were used. The cells were cultured in Dulbecco's modified Eagle's medium. 1000 and 2000 cells were incubated with the peptides for 3 and 5 days, and the effect of the peptides was evaluated by an ATP lite assay dose dependently. Furthermore, specific antagonists at 10^{-6} M were used to find out whether the effect would be reversed.

Results: In brief, each of the peptides tested had an inhibiting effect. This inhibiting effect was weak but highly significant, averaging 10% to 15%, and was most pronouncedly seen at concentrations between 10^{-10} M and 10^{-14} M. Each antagonist reversed the inhibiting effect fully.

Conclusions: These results clearly indicate that RPE cells are under neural control and the low effective concentration of the peptides may be the one physiologically acting on these cells. The results are of important relevance both physiologically and pathophysiologically: physiologically, the inhibitory effect may mean that these peptides cause the cells to remain in a differentiated condition. Pathophysiologically, the findings are relevant in proliferative vitreoretinopathy where RPE cells proliferate in excess. The authors hypothesise that the inhibiting effect diminishes when these cells are swept out and actively migrate from their physiological location and thus, dedifferentiate and begin to proliferate. This hypothesis improves the knowledge of the initial processes in the pathogenesis of the disease as there seems to be a discrepancy between facilitatory and inhibitory influences favouring the former in proliferative vitreoretinopathy. Furthermore, these neuropeptides constitute the first endogenous inhibitors of RPE cell proliferation.

R etinal pigment epithelial (RPE) cells constitute highly specialised cells forming a monolayer interposed between the choroid and the neurosensory retina. There is evidence that these cells have several functions in the eye—namely, they transport nutrients from the choroid to the photoreceptors, phagocytose the shed photoreceptor membranes, and ensure firm retinal attachment by net fluid removal from the interphotoreceptor subretinal space.

Neuropeptides are a group of neurotransmitters and/or neuromodulators widely distributed in the central and peripheral nervous system. There are several families of these peptides, the most important and best characterised peptides in the eye are substance P (SP), calcitonin gene related peptide (CGRP), vasoactive intestinal polypeptide (VIP), and neuropeptide Y (NPY). SP and CGRP are sensory peptides, whereas VIP-ergic nerves are part of the parasympathetic and NPY-ergic nerves part of the sympathetic nervous system (for review, see Stone *et al*[1]). SP, CGRP, VIP, and NPY are neuropeptides which innervate the anterior segment of the eye and there are abundant nerve fibres in the choroid predominantly around blood vessels and in the stroma.[1] Except for CGRP (own observation), these peptides are also present in the retina in various species including humans. In brief, neuropeptides are classic amacrine cell transmitters.[1] Amacrine cells are a group of cells located in the innermost part of the inner nuclear layer and constitute interneurons with dendrites ramifying into the inner plexiform layer. Furthermore, these peptides occur in displaced amacrine cells in the ganglion cell layer and SP at least is also present in ganglion cells in certain animal species.[2–4]

But there are certain neuropeptides which have not been investigated until now. One of these peptides is secretoneurin

(SN). SN is a peptide first found in 1993[5] and constitutes a cleavage product of secretogranin II. Secretogranin II is a member of the chromogranin family comprising chromogranin A, chromogranin B, secretogranin II, and NESP55.[6–9] These families are the acidic proteins of secretory granules and are also widely distributed within neuroendocrine tissues.[6–9] In the eye, SN has been found in the human retina in amacrine cells[10] but in the anterior segment of the eye this peptide has not been explored.

Each of these peptides can influence RPE cells as they can diffuse from both sides to the RPE—from the choroid and from the retina. This study aimed to investigate whether certain neuropeptides influence the proliferation of these cells. This is very important because RPE cells proliferate in definite circumstances. The best example is proliferative vitreoretinopathy (PVR), a disease process, which is characterised by intense proliferation of RPE cells.

MATERIALS AND METHODS
Cell culture
The experiments were performed with a human RPE cell line, ARPE-19 cells, which were obtained from the American Type Culture Collection (ATTC, PO Box 1549, Manassas, VA 20801, USA). The cells were transferred into 25 cm² culture flasks and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 200 mM L-glutamine, 0.1 mg/ml streptomycin, 100 U/ml penicillin, and 10% fetal calf serum until a confluence of 80% was reached. The cells were routinely passaged by dissociation in 0.05% trypsin and 0.02% ethylenediaminetetra-acetic acid followed by replating at a split ratio ranging from 1:3 to 1:6. Cells from passages 3–10 were used for the experiments. For this purpose, the medium

was removed from the culture flasks and 0.05% trypsin was added. The cells then were incubated for 5 minutes followed by scraping off the cells. After centrifugation at 1000 rpm for 10 minutes, the cell pellet was resuspended in DMEM medium.

ATP lite assay

Evaluation of the effect of neuropeptides on cell proliferation was performed with the ATP lite assay, a method standardly used as a measure of cell proliferation.[11][12] ATP is a marker for cell viability because it is present in all metabolically active cells and the concentration declines very rapidly when the cells undergo necrosis or apoptosis. The ATP lite-M assay system is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin. This is illustrated in the following reaction scheme: ATP + D-luciferin + O_2→luciferase, Mg^{2+}→oxyluciferin + AMP + PPi + CO_2 + light. The emitted light is proportional to the ATP concentration within certain limits.

At the beginning of the proliferation assay, cells were estimated in a "Bürker Zählkammer" (cell counter) and 1000 and 2000 cells in 100 µl cell suspension were transferred into a 96 well microtitre plate. After 1 day SP, CGRP, VIP, NPY, and SN were added to the wells. The peptides were purchased from Peninsula Laboratories (Peninsula Laboratories, 601 Taylor Way, San Carlos, CA 94070, USA). The concentrations used were 10^{-6} M, 10^{-8} M, 10^{-10} M, 10^{-12} M, 10^{-14} M, and 10^{-16} M. Incubation of cells without addition of neuropeptides served as controls.

Following incubation of the cells for 3 and 5 days at 37°C and 5% CO_2, the ATP lite assay was performed (obtained from Packard Bioscience BV, Rigaweg 22, 9723 TH, Groningen, Netherlands). For this purpose, 50 µl of mammalian cell lysis solution was added to 100 µl of cell suspension per well of a microtitre plate and the plate was shaken for 5 minutes in an orbital shaker at 700 rpm. This lyses the cells and stabilises the ATP. Then 50 µl substrate solution were added to the wells and the microplate was shaken for 5 minutes in an orbital shaker at 700 rpm. The plate was placed in the dark for 10 minutes and the luminescence was measured in a microplate scintillation counter (Top count, Packard). The experiments were repeated three times so that 7–16 values were obtained for each concentration and each peptide. Furthermore, experiments with highly specific antagonists were performed—namely, Spantide I, CGRP 8-37, VIP antagonist, and BIPB3226NPY. For this purpose, an effective concentration of the peptides (10^{-14} M) was incubated with an excess of the antagonists (10^{-6} M). This high concentration was given to certainly saturate receptors. The antagonists were also purchased from Peninsula Laboratories. For SN, an antagonist is not commercially available at present.

Evaluation of the data

The values obtained by counting on the scintillation counter were processed as percentages: the control values were given as 100% and the data of peptide effects as percentages of controls. Statistical calculation of differences between controls and peptide effects was performed with the Mann-Whitney U test (*** $p<0.001$; ** $p<0.01$; * $p<0.05$).

RESULTS

The results are illustrated in Figures 1 and 2, where the mean values (SEM) are given as percentages.

Effect of SP

SP incubated for 3 days exerted an inhibiting effect on both 1000 and 2000 cells. The effect was seen more pronouncedly with 1000 cells with a maximum at 10^{-12} M (86.7% (SEM 2.5%) of controls; ***), whereas with 2000 cells an 8% inhibition was reached at this concentration (92.2% (1.8%); **).

Experiments for 5 days also had an inhibiting effect which was less pronounced than that for 3 days, but the effect was significant with 1000 cells at 10^{-10} M (91.7% (2.7) of controls; *) and 10^{-12} M (90.3 (3.4); *) and with 2000 cells at 10^{-10} M (90.2% (3.6) of controls; *), 10^{-12} M (92.0% (2.4) of controls; *) and 10^{-14} M (92.8% (3.4) of controls; *).

Spantide reversed the inhibitory effect fully.

Effect of CGRP

CGRP, on the other hand, had a modest decreasing effect 3 days after peptide addition. Significance was only reached at 10^{-14} M with 1000 cells (89.9% of controls (3.4); *), with 2000 cells at 10^{-10} M (93.6% (3.5) of controls; *), and at 10^{-12} M (91.5% (3.1); *).

Five days of culture with the peptide had a more pronounced effect with 1000 cells. Significance was reached at 10^{-6} M (91.1% (3.2) of controls; *), at 10^{-8} M (92.8% (2.3); *), at 10^{-10} M (82.9% (2.9); ***), at 10^{-12} M (73.3% (3.6); ***), and at 10^{-14} M (81.1% (2.7); ***), whereas incubation of 2000 cells with the peptide failed to be statistically significant.

CGRP 8-37 abolished the decreasing effect.

Effect of VIP

VIP had the highest significant effect when 2000 cells were incubated for 5 days with the peptide. There was significance at 10^{-6} M (92.3% of controls (1.9); **), at 10^{-8} M (90.2% (2.3); **), at 10^{-10} M (82.3% (3.8); ***), at 10^{-12} M (90% (1.4);***), and at 10^{-14} M (88.9% of controls (1.8); ***). The peptides with 1000 cells in culture had a decreasing effect at 10^{-10} M (84.0% of controls (4.1); **), at 10^{-12} M (90.7% (1.8); **), and at 10^{-14} M (87% (1.8); ***).

Incubating 1000 cells with the peptide for 3 days had a weaker effect (91.0% (3.4) of controls at 10^{-12} M; * and 89.3% (3.3)at 10^{-14} M; *). But 2000 cells in culture for 3 days with the peptide revealed significance at 10^{-8} M (87% (3.5) of controls; **), at 10^{-10} M (89.7% (3.4); **), at 10^{-12} M (87% (3.4); **) and at 10^{-14} M (86.7% (3.2); **).

Again the antagonist reversed the inhibition.

Effect of NPY

NPY had a decreasing effect on cell proliferation following 3 days in culture both for 1000 (90.6% of controls (2.7) at 10^{-8} M; **; 89.3% (2.8) at 10^{-10} M; **; 89.3% (2.8) at 10^{-12} M; ** and 86.2% (4.0) at 10^{-14} M; ***) and 2000 cells (88.3% of controls (1.9) at 10^{-8} M; ***; 86.1% of controls (2.7) at 10^{-10} M; ***; 85.2% of controls (2.8) at 10^{-12} M; *** and 84.4% (3.5) at 10^{-14} M; ***).

Culturing 1000 cells with NPY for 5 days had an inhibiting effect at 10^{-6} M (90.9% of controls (2.4); **), at 10^{-8} M (91.7% of controls (1.8); **), at 10^{-10} M (89.8% (2.2); ***), at 10^{-12} M (80.2% (3.4); ***), and at 10^{-14} M (85.5% of controls (1.8); ***). Significance was also reached when 2000 cells were incubated with the peptide for 5 days at 10^{-6} M (88.9% of controls (2.4); **). at 10^{-8} M (91.9% (2.7); *), at 10^{-10} M (86.7% (2.4); ***), at 10^{-12} M (87.5% (2.7); ***) and at 10^{-14}M (91.5% (2.3) of controls; **).

The antagonist fully reversed the inhibiting effect.

Effect of SN

Another peptide, SN, also decreased cell proliferation. Incubating 1000 cells and 2000 cells for 3 days respectively, revealed a significant inhibiting effect at 10^{-6} M (88.4% (2.2) of controls; ***), at 10^{-8} M (93.3% (2.4); **), at 10^{-12} M (86.6% (2.4); ***), and 10^{-14} M (91.6% (2.4); **). Cultures with 2000 cells had an inhibiting effect, which was
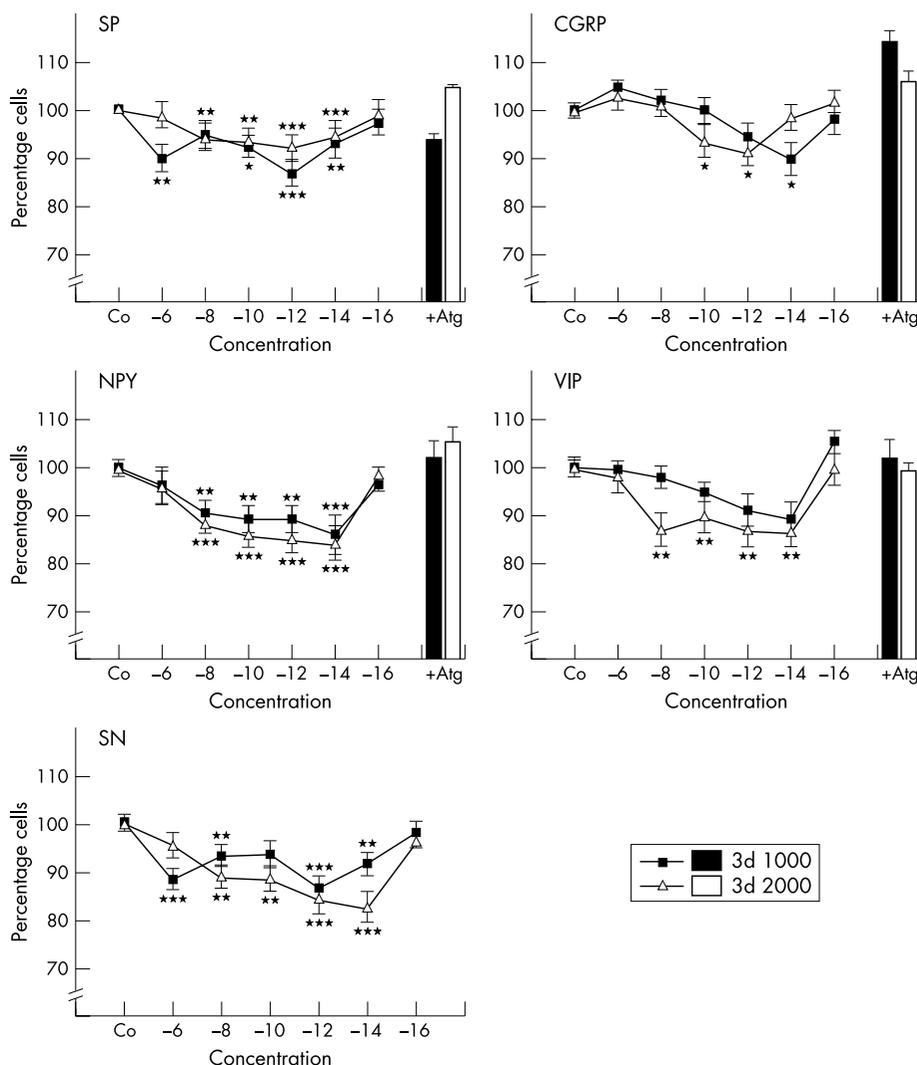
Figure 1 Effect of SP, CGRP, NPY, VIP, and SN on the proliferation of human RPE cells incubated for 3 days. ARPE-19 cells were cultured standardly in DMEM medium and experiments were performed with 1000 cells (solid squares) and 2000 cells (open triangles). The concentrations used were between 10^{-6} M and 10^{-16} M and incubation of cells without addition of neuropeptides served as controls (Co). Furthermore, an effective concentration of each peptide (10^{-14} M) was incubated with a highly specific antagonist at a concentration of 10^{-6} M (+Atg). 1000 cells incubated with the peptides and the antagonists represent solid bars, 2000 cells open bars. Controls (n = 15–24), peptide effects (n = 7–16), and antagonist effects (n = 5–8) were evaluated by an ATP lite assay. The values obtained by counting on a scintillation counter were processed as percentages: the control values were given as 100% and the data of peptide effects as percentages of controls. Mean values (SEM) are given. Statistical calculation of differences between controls and peptide effects was performed with the Mann-Whitney U test (*** p<0.001; ** p<0.01, *p<0.05).

significant at 10^{-8} M (89.2% (2.5) of controls; **), 10^{-10} M (88.6% (2.6); **), 10^{-12} M (84.4% (3.1); ***) and 10^{-14} M (82.8% (3.3); ***).

Results obtained with 1000 and 2000 cells for 5 days in culture also had an inhibitory effect which was very similar. For 1000 cells, significance was reached at 10^{-8} M (93.4% (3.3) of controls; *), at 10^{-10} M (93.1% (2.4); *), at 10^{-12} M (88.4 % (2.3); ***), and 10^{-14} M (90.8% (2.5); **). For 2000 cells, significance was obtained at 10^{-8} M (92.6% (3.5) of controls; *), at 10^{-10} M (89.7% (2.0); *), 10^{-12} M (88.1% (2.0); ***), and 10^{-14} M (81.4% (4.0); ***).

DISCUSSION

This study aimed to investigate whether certain neuropeptides influence the proliferation of RPE cells. Thus, the authors found a weak but significant inhibiting effect averaging 10% to 15%. Concerning SN, this is the first biological effect of this peptide in the eye. Surprisingly, each of the peptides studied acted in an inhibitory manner and the effect was seen most pronouncedly between 10^{-10} M and 10^{-14} M. This concentration is low but taking into account that there is a distance between the RPE monolayer on the one side and the choroid and the retina on the other side, this concentration may be the one being physiologically effective on these cells. The results tend to be highly specific for two reasons: firstly, the authors incubated both 1000 and 2000 cells with the neuropeptides for 3 and 5 days and the

outcome was qualitatively the same. Secondly, the authors used an antagonist to verify whether the effect would be reversed and this was clearly the case. The results are not identical to those of Koh et al who found a stimulating effect of VIP,[13] or those of Kishi et al who found a stimulating effect of SP, CGRP, and β endorphin.[14] The discrepancy can be explained, firstly, by species differences as the present study used human RPEs and, secondly, methodologically. Koh et al and Kishi et al used ^{3}H-thymidine uptake as their method whereas our study preferred an ATP lite assay. Both methods are well established, but the authors chose the latter one, as it constitutes a more modern method and an endogenous parameter in our opinion represents a better indicator for estimation of cell proliferation.

The results are of relevance both physiologically and pathophysiologically. Physiologically, the findings indicate that RPE cells are under neural control: the inhibitory effect of these peptides on the proliferation of RPE cells may mean that those peptides cause the cells to maintain a differentiated condition. Indeed, Koh et al found that VIP promotes the melanogenesis which is in agreement with this hypothesis.[13 15] These peptides may also be among those agents which influence RPE cells to form a monolayer in the ontogenesis and which prevent them proliferating in excess. In the retina, at least SP appears to have a fundamental role in the development of inner retinal circuits.[16] RPE cells are in close proximity to the retina and therefore certain
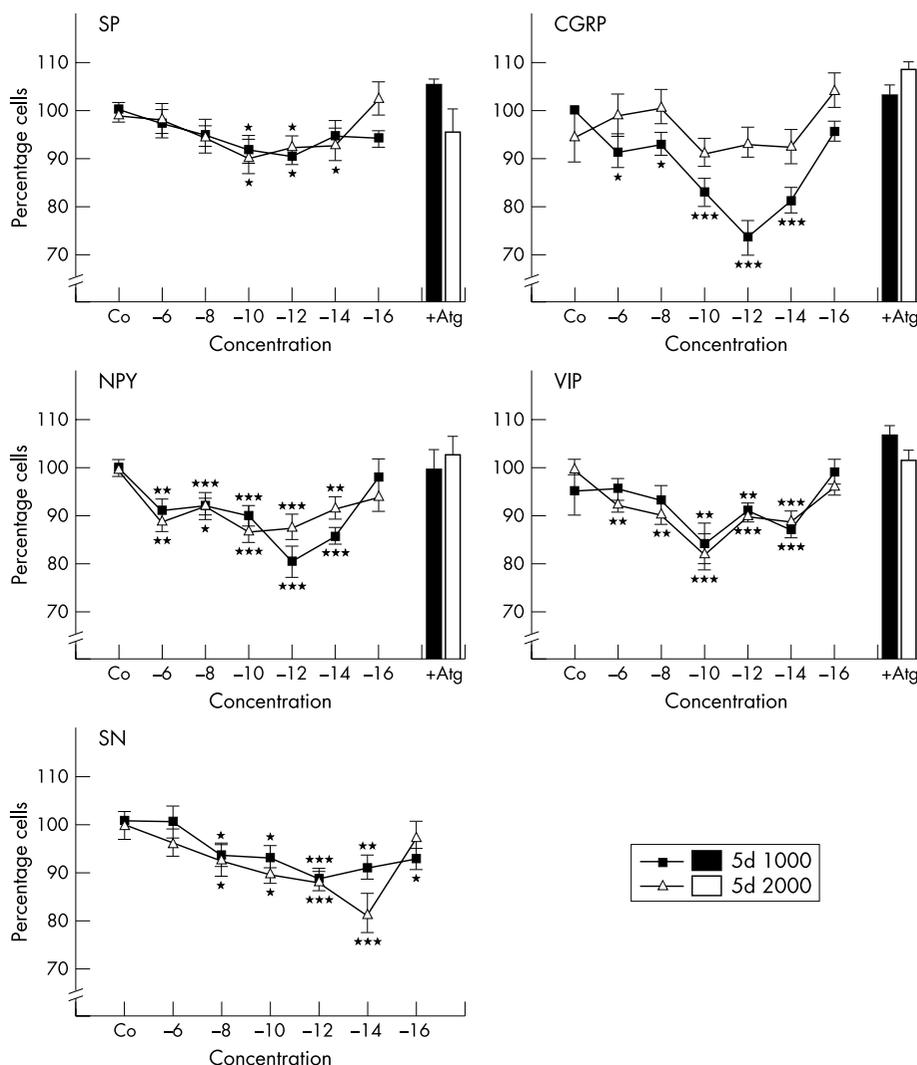
Figure 2 Effect of SP, CGRP, NPY, VIP, and SN on the proliferation of human RPE cells incubated for 5 days. The protocol is the same as described in Figure 1. In brief, open triangles represent 1000 cells and solid squares 2000 cells. The effect was evaluated dose dependently (controls: n = 16–23; peptide effects: n = 9–15; antagonist effects: n = 5–10) and highly specific antagonists at a concentration of 10^{-6} M clearly reversed the peptide effects (+Atg). Solid bars represent incubation of 1000 cells with the antagonists, open bars incubation of 2000 cells. Mean values (SEM) are given (*** $p<0.001$, ** $p<0.01$, * $p<0.05$).

neuropeptides may have an influence on the development of the RPE monolayer too.

The results are also pathophysiologically relevant, as the proliferation of retinal pigment epithelial cells is a hallmark of PVR (reviews, see Machemer,[17] Hiscott et al,[18] Wiedemann et al,[19] and Weller et al[20]). The decisive step in the pathogenesis of PVR is the first phase, in particular the infiltration of mainly RPE cells into the preretinal or subretinal space, or both, where the cells dedifferentiate and proliferate in excess. The exact mechanisms leading to migration and proliferation of RPE cells are still not exactly clear. The inhibiting effect of the neuropeptides found in this study provokes the authors to define the following hypothesis: in the physiological state, RPE cells remain in a differentiated condition where at least certain neuropeptides act in an inhibitory way on cell proliferation. In PVR, where these cells are swept out and actively migrate from their physiological location to the preretinal or subretinal space, this inhibitory effect of the retina and the choroid diminishes. Thus, RPE cells first dedifferentiate and become myofibroblasts and then they begin to proliferate. It must be emphasised that the absence of the inhibiting effect of neuropeptides may not be the main reason for the development of PVR because there are several growth factors and cytokines responsible for evolution of the disease which act in a migratory way and/or have a proliferative potency. This knowledge is based on the finding that certain mediators are present in the vitreous and

subretinal fluids of patients with PVR, in particular PDGF,[21 22] HGF,[23] IL-1, IL-6, γ-IFN,[24–26] IL-8, MCP-1, M-CSF,[27 28] or TNF-α[29] and distinct factors have been detected in epiretinal membranes—that is, EGF,[30] acidic and basic FGF,[31 32] PDGF,[33] VEGF, and TNFα,[34] TGF-β,[35] IL-1β or IL-6.[36] The relevance of growth factors and cytokines should be emphasised by the fact that experimental PVR can be attenuated by inhibiting the platelet derived growth factor receptor[37] and that high vitreous levels of IL-6 have been found to be a risk factor for the development of PVR.[38] Therefore, both the absence of the inhibiting effect of neuropeptides together with an imbalance between adhesive/counteradhesive molecules stimulating RPE cells to dissociate from Bruch's membrane (for review, see Hiscott et al[39]), and the formation of an adequate milieu with synthesis of growth factors and cytokines, may be mainly responsible in the pathogenesis of PVR. Thus, this hypothesis provides a better understanding about the initial processes leading to the formation of epiretinal membranes. However, there seems to be at least a discrepancy between facilitatory and inhibitory influences favouring the former in PVR. It must be emphasised that the effect of single neuropeptides is only weak in vitro. But acting together, the effect may become additive even in vivo and then may indeed be relevant both physiologically and pathophysiologically.

The most important problem concerning PVR is the therapeutic management. Currently, the only effective

therapy is to surgically remove the membranes. Furthermore, most clinicians use systemic cortisone to inhibit cell proliferation although the effect is not always beneficial. Recently, there have been several studies published which aimed to explore whether certain substances have an inhibiting effect on the proliferation of RPE cells in vitro, in particular tranilast,[40] genistein,[41][42] ciprofloxacin,[43] vitamin E,[44] minoxidil,[45] hypericin,[46] cis-hydroxyproline,[47] retinoic acid,[48][49] aclacinomycin A,[50] daunorubicin,[51] N,N-dimethyla-driamycin,[52] or cis retinoic acid.[53] These substances have an inhibiting effect but possible toxic side effects restrict their usefulness as treatment in PVR. The neuropeptides tested in this study are endogenous elements and, therefore, one could recommend employing these peptides as therapeutic targets. Consequently, it would be of interest to investigate whether combinations of certain neuropeptides have an additive effect in vitro as the effect of single peptides is only weak. However, further studies are necessary to evaluate the efficacy of neuropeptides in PVR, in particular to find out whether and which combinations have an additive effect, to find out whether and which neuropeptides act in a migratory way and, if the latter is not the case, to test them finally in PVR animal models by intravitreal application. Nevertheless, it must be emphasised that the neuropeptides in this study constitute the first endogenous inhibitors of RPE cell proliferation. In the literature, to our knowledge, only TGF-β has been described as a potent endogenous inhibitory substance[48] but TGF-β is known as a fibrogenic cytokine and the concentration in the vitreous of patients with PVR correlates well with the extent of fibrosis.[54]

In conclusion, the authors found a weak, but highly significant inhibitory effect of certain neuropeptides on the proliferation of human RPE cells. The results indicate that the cells are under neural control and these neuropeptides constitute the first endogenous inhibitors of RPE cell proliferation.

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