

Secreted key regulators (Fgf1, Bmp4, Gdf3) are expressed by PAC1-immunopositive retinal ganglion cells in the postnatal rat retina

Viktória Dénes,¹ Kármén Kovács,¹ Ákos Lukáts,^{1,2} Adrienn Mester,¹ Gergely Berta,³ Arnold Szabó,⁴ Róbert Gábrriel¹

¹Department of Experimental Zoology and Neurobiology, University of Pécs

²Department of Translational Medicine, Semmelweis University, Budapest

³Institute of Medical Biology, School of Medicine, University of Pécs

⁴Department of Anatomy, Histology and Embryology, Semmelweis University, Budapest, Hungary

ABSTRACT

Identified as a member of the secretin/glucagon/VIP superfamily, pituitary adenylate cyclase-activating polypeptide (PACAP1-38) has been recognized as a hormone, neurohormone, transmitter, trophic factor, and known to be involved in diverse and multiple developmental processes. PACAP1-38 was reported to regulate the production of important morphogens (Fgf1, Bmp4, Gdf3) through PAC1-receptor in the newborn rat retina. To follow up, we aimed to reveal the identity of retinal cells responsible for the production and secretion of Fgf1, Bmp4, and Gdf3 in response to PACAP1-38 treatment. Newborn (P1) rats were treated with 100 pmol PACAP1-38 intravitreally. After 24 h, retinas were dissected and processed for immunohistochemistry performed either on flat-mounted retinas or cryosections. Brn3a and PAC1-R double labeling revealed that 90% of retinal ganglion cells (RGCs) expressed PAC1-receptor. We showed that RGCs were Fgf1, Bmp4, and Gdf3-immunopositive and PAC1-R was co-expressed with each protein. To elucidate if RGCs release these secreted regulators, the key components for vesicle release were examined. No labeling was detected for synaptophysin, Exo70, or NESP55 in RGCs but an intense Rab3a-immunoreactivity was detected in their cell bodies. We found that the vast majority of RGCs are responsive to PACAP, which in turn could have a significant impact on their development or/and physiology. Although Fgf1, Bmp4, and Gdf3 were abundantly expressed in PAC1-positive RGCs, the cells lack synaptophysin and Exo70 in the newborn retina thus unable to release these proteins. These proteins could regulate postnatal RGC development acting through intracrine pathways.

Key words: PAC1 receptor; Fgf1; Bmp4; Gdf3; retinal ganglion cell.

Correspondence: Viktória Dénes, Department of Experimental Zoology and Neurobiology, University of Pécs, 6 Ifjúság str., H-7624 Pécs, Hungary. Tel. +36.72.503600 ext. 2-4444.

E-mail: vdenes@gamma.ttk.pte.hu

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Introduction

Transformation of a certain range of electromagnetic radiation, experienced as light, begins in the retina, where the photic energy is turned into neuronal information (colors, forms, contrasts, *etc.*). The first step of visual processing takes place within the layers and neuronal networks, which are composed of six neural cell types (*i.e.*, rod, cone, bipolar cell, amacrine cell, horizontal cell, ganglion cell). Retinal ganglion cells (RGCs), as the only output neurons of the retina, forward the visual information to the brain. In order to be integrated into functional circuits, RGCs are objects of temporally overlapping, developmental processes (*i.e.*, proliferation, migration, cell death, neurite outgrowth, synapse formation)¹⁻³ that are regulated by numerous intrinsic and extrinsic environmental factors. The latter group includes neuropeptides and a large set of secreted proteins such as fibroblast growth factors (Fgf) or bone morphogenic proteins (Bmp/Gdf). One of the prominent candidates orchestrating RGC development might be the pituitary adenylate cyclase activating peptide (PACAP1-38).⁴⁻⁷

Identified as a member of the secretin/glucagon/vasoactive intestinal peptide superfamily,⁸ the evolutionarily conserved PACAP1-38 has been recognized as a hormone, neurohormone, transmitter, trophic factor, and known to be involved in diverse and multiple physiological processes in nearly every bracket of the mammalian body.⁹ The exceptional omnipresence of PACAP1-38 and its receptors results in a great clinical relevance.¹⁰ Depending on the receptor type, PACAP1-38 affects numerous aspects of neural development: progenitor proliferation,^{11,12} neurite outgrowth,¹³ migration,¹⁴ and synaptogenesis.¹⁵ PACAP1-38 acts *via* three major receptors: PAC1, VPAC1 and VPAC2.¹⁶ Intriguingly, fourteen PAC1-receptor (PAC1-R) isoforms have been distinguished in mammals based on the assembles of the third intracellular loop or/and the N-terminal domain that results in altered receptor functions (*e.g.*, ligand-binding specificity, affinity, signaling).¹⁷

In the adult rat retina, the transcription of PAC1-R gene was detected in cells of the ganglion cell layer (GCL) by *in situ* hybridization technique.¹⁸ Focusing on the developmental impacts of PACAP1-38 in the rat retina, we confirmed that RGCs are certainly one of the PAC1-R expressing elements of the rat retina in the middle-phase of postnatal development (*i.e.*, P5-P10).¹⁹ The only consequent data available from earlier developmental stages are found in our study reporting an abundant expression of PAC1-R among the cells of the GCL without precise identification.²⁰ It is important to point out, however, that the presence of displaced amacrine cells in the GCL has been proved in both developing and matured retina,^{21,22} thus identification of the labelled cells by using RGC specific marker is crucial. We also revealed that intravitreally injected PACAP1-38 at postnatal day 1 (P1) caused changes in the expression of a large set of developmental factors including important morphogens (*i.e.*, Fgf1, Bmp4, Gdf3). Furthermore, applying PAC1 receptor antagonists (*i.e.*, PACAP6-38 and M65), PAC1 signaling was revealed to be responsible for the upregulation of Fgf1, Bmp4 and Gdf3.²³

Pursuing these investigations, we performed in the present study a series of immunohistochemical labelling to provide morphological data that elaborate further insights into the PACAP1-38 effects in the newborn retina. Based on our previous results (see above), we aimed i) to make a precise analysis of PAC1-R expressing cells of the GCL in the early postnatal stage revealing their identity and density; ii) to investigate whether PAC1-R and Fgf1, Bmp4 and Gdf3 are co-expressed in the P1 retina; and iii) to prove whether the RGCs are responsible for the production and secretion of the aforementioned proteins.

Materials and Methods

Experimental animals, treatment

Albino Wistar-Hannover rats at age of P1, equal number of males and females were used for the present study. Animal handling, housing, and experimental procedures followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, also, were reviewed and approved by the ethical committee of University of Pécs and the Animal Health and Animal Welfare Directorate of the National Food Chain Safety Office of the Hungarian State (BA/35/51-58/2016). All efforts were made to minimize pain. Prior to treatment or sacrifice, animals were anesthetized by inhalation of Forane (isoflurane) (Abbott Laboratories, Budapest Hungary). To detect induced Fgf1, Bmp4 or Gdf3 expression, P1 pups were injected intravitreally as described earlier.²³ Briefly, one eye was treated with 2.5 μ L 0.2 μ g/ μ L N-terminally acetylated PACAP1-38 (100 pmol) (Bio Basic Canada Inc., Markham, Canada) while the paired eye was injected with the same volume of 0.9% sterile saline. Both injection and needle removal were progressed slowly and carefully to prevent reflux. After 24 h, eyes were removed and dissected in cold, phosphate-buffered saline (PBS).

Immunohistochemistry on cryosections

Immunolabeling was performed on control and PACAP1-38 treated postnatal day 2 (P2) retinal sections. The eyecups were fixed in 2% phosphate buffered paraformaldehyde for 2 h at room temperature. The fixative was removed by rinsing the eyecups in PBS for 3 \times 10 min. For cryoprotection, the eyecups were sequentially immersed into 10-20-30 % sucrose solution then embedded in tissue embedding medium (Shandon Cryomatrix, Thermo Scientific, UK); 10-15 μ m thick sections were cut and processed for immunohistochemistry. Using the optic disc as reference points, care was taken to compare identical, central parts of both control and treated retinas. For membrane-permeabilization, 0.3% Triton X-100 in PBS was used (Sigma-Aldrich, Budapest, Hungary) for 30 min. To prevent nonspecific binding, sections were incubated in 5% normal goat serum and 1% bovine serum albumin dissolved in PBS for 30 min. All primary antibodies were applied on sections overnight at room temperature. To visualize, sections were incubated in fluorochrome-conjugated secondary antibodies for 4-6 h at room temperature. Detailed information about the primary and secondary antibodies utilized are summarized in Tables 1 and 2, respectively. Sections were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) by mounting with ProLong Gold Antifade Mountant containing DAPI according to the manufacturer's instructions (Life Technologies, Budapest, Hungary). Omission of the primary antibodies in both the single- and double-labeling experiments resulted in no staining. Cross-reactivity of the non-corresponding primary and secondary antibodies was not detected. Specificity of anti-PAC1-R used in our immunolabeling was carefully tested and reported by Schulz *et al.*²⁴ Positive controls (*i.e.*, adult rat retina, rat hypothalamus) were used to exclude false negativity and/or verify antibody specificity in case of Rab3a, synaptophysin, NESP55, and Exo70. Reactions were carried out on tissues derived from at least three different animals.

Double immunolabeling on whole-mounted retinas

Three, untreated P1 animals were used to perform Brn3a and PAC1-R immunostaining on flat-mounted retinas, which allowed an accurate cell counting across the tissue. Fixation was performed as described above. Whole retinas were carefully detached from the eyecup and processed separately thereafter. Four radial inci-

sions were made, then tissues were incubated in dimethyl sulfoxide (VWR International, Budapest, Hungary) for 30 min followed by repeated rinsing in PBS containing 0.4% Triton X-100 (Sigma-Aldrich). To avoid non-specific binding, sections were incubated in 5% normal goat serum and 1% bovine serum albumin dissolved in PBS for 2 h. Retinas were incubated in anti-Brn3a and anti-PAC1-R antibodies (Table 1) for 48 h at room temperature followed by an incubation in Alexa 448 conjugated goat anti-mouse IgG and Alexa 568 conjugated goat anti-rabbit IgG (Table 2) for 24 h. Retinas were flat-mounted onto microscope slides and to preserve signal, covered with ProLong Gold Antifade Mountant with DAPI reagent (ThermoFisher Scientific, Budapest, Hungary).

Confocal imaging

Images were taken by either a Zeiss LSM 780 Confocal System coupled to a Zeiss Axio Imager upright microscope (Carl Zeiss Meditec AG, Oberkochen, Germany) or Olympus FV-1000 laser scanning confocal fluorescence microscope (Olympus, Hamburg, Germany) using a 40x or 60x objective and strictly identical settings.

Cell counting

The density of Brn3a-immunoreactive (IR) and PAC1-R-IR cells was assessed in three whole-mounted preparations. Confocal

Z-stack images were created and the cell number was analyzed in the entire depth (9.6 μm) of the field from evenly distributed regions of both peripheral and central parts of the retinas. In total, 0.10 up to 0.12 mm^2 area were examined in each retina. Cells were counted manually using ImageJ FIJI software.

Results

Nearly 90% of the RGCs express PAC1-R in the newborn rat retina

Previously, we demonstrated that plenty of cells in the GCL layer of the P1 retina expressed PAC1-R,²⁰ which gave the rationale to investigate PAC1-R/Brn3a colocalization in the GCL in order to prove that these cells are actually RGCs and to assess their relative co-distribution. PAC1-R-IR (Figure 1 A1, asterisks) and Brn3a-IR neurons of the GCL (Figure 1 A2, arrows) and their colocalization (Figure 1 A3) were mapped in P1 flat-mounted retinas. Table 3 summarizes the results of cell counting. The vast majority, approximately 89.6% of Brn3a-immunopositive RGCs express PAC1-R and only 10.4% lack PAC1-R expression. Thus, the majority of RGCs are PAC-IR positive and vice versa. The majority of the PAC1-R-immunopositive neurons were indeed proved to

Table 1. List of primary antibodies used for immunohistochemistry.

Name	Host and type	Dilution	Supplier
anti-PAC1 receptor	Rabbit polyclonal	1:1000	ThermoFisher Scientific, Budapest Hungary (PA3-115)
anti-Fgf1	Goat polyclonal	1:1000	RDsystems, Budapest, Hungary (AF232)
anti-Gdf3	Goat polyclonal	1:2000	RDsystems, Budapest, Hungary (AF958)
anti-Bmp4 (N-16)	Goat polyclonal	1:100	Santa Cruz Biotechnology Inc., Heidelberg, Germany (sc-6896)
anti-Brn3a	Mouse monoclonal	1:250	Santa Cruz Biotechnology Inc., Heidelberg, Germany (sc-8429)
anti-Rab3A	Rabbit polyclonal	1:1000	ThermoFisher Scientific, Budapest, Hungary (PA1-770)
anti-Synaptophysin	Mouse monoclonal	1:200	Sigma, Budapest, Hungary (S5768)
anti-Exo70	Mouse monoclonal	1:50	Santa Cruz Biotechnology Inc., Heidelberg, Germany (sc-365825)
anti-NESP55 (GNAS)	Rabbit polyclonal	1:250	ThermoFisher Scientific, Budapest Hungary (PA5-22261)

Table 2. List of secondary antibodies used for immunohistochemistry.

Name	Fluorochrome	Dilution	Supplier
Anti-rabbit IgG	Alexa 568	1:500	ThermoFisher Scientific, Budapest Hungary
Anti-rabbit IgG	Alexa 488	1:500	ThermoFisher Scientific, Budapest Hungary
Anti-goat IgG	Cy3	1:500	ThermoFisher Scientific, Budapest Hungary
Anti-mouse IgG	Alexa 488	1:500	ThermoFisher Scientific, Budapest Hungary
Anti-mouse IgG	NL557	1:500	RDsystems, Budapest, Hungary

Table 3. Density of PAC1-R expressing cells of the GCL in P1 retina.

	Brn3a+ cell density (cell/ mm^2)	Brn3a+/Pac1+ cell density (cell/ mm^2)	Brn3a+/Pac1- cell density (cell/ mm^2)	Brn3a-/Pac1+ cell density (cell/ mm^2)
Retina#1	12 137.49	11 344.55	792.94	1428.86
Retina#2	12 212.32	10 418.61	1 701.51	1676.37
Retina#3	13 798.27	12 403.80	1 394.47	1633.30
Average \pm SD	12 716.03 \pm 709	11 388.99 \pm 702.66	1 296.31 \pm 392.82	1 579.51 \pm 114.01

be RGCs: only 8.2% of the total PAC1-R positive population did not show Brn3a positivity in the GCL (Figure 1 A3, asterisks). The identity of this latter population is yet unknown. These cells may be displaced amacrine or belong to the Brn3a negative RGC population.

GCL neurons express key regulators in the newborn rat retina: Fgf1, Bmp4 and Gdf3

We also reported that exogenous PACAP1-38 upregulated Fgf1, Bmp4, and Gdf3 transcription as well as protein expression.²³ To follow up, we revealed the distribution of Fgf1, Bmp4, and Gdf3-expressing elements of the P1 retina. In congruence with result of Catalani *et al.* reported in mouse retina,²⁵ Fgf1-IR cells were observed in the GCL, in the proximal as well as distal neuroblast layer (NBL) and RPE in both control and intravitreally PACAP1-38 injected tissues (Figure 2 A,A'). Similarly, Bmp4-immunoreactivity was detectable in the GCL, in the proximal NBL, and at last but not least, in the RPE cells (Figure 2 B,B'). Furthermore, for the first time, we identified the Gdf3-expressing elements of the mammalian retina revealing Gdf3-immunoreactivity also in RGCs and proximal NBL (Figure 2 A,B). It is noteworthy that intravitreal PACAP1-38 injection did not alter their expression patterns.

Fgf1, Bmp4, Gdf3 are co-expressed with PAC1-R in RGCs

Next, we examined if the morphogen-expressing RGCs bore PAC1-R. Considering that 90% of RGCs expressed PAC1-R, the majority of the protein/PAC1-R co-labeled neurons in the GCL could be considered as RGC. In Figure 3, the double-labeling clearly shows that most, if not all Fgf1-IR cells express PAC1-R (Figure 3 A1,A2,A3). However, the distribution of the two signals were overlapping only partially. Fgf1-immunostaining (Figure 3A1, arrows) appeared in cytoplasm as well as in the nucleus whereas PAC1-R-immunopositivity was not seen in the nuclei (Figure 3 A2, asterisks). Figure 2 B1,B2,B3 pictures demonstrate that Bmp4-IR neurons (arrows) were also PAC1-R-immunopositive (asterisks). In Figure 3 C1,C2,C3 Gdf3-immunoreactivity (arrows) displays the same cytoplasmic distribution as PAC1-immunoreactivity (asterisks), although a very few Gdf3-IR neurons were also found that did not express PAC1-R.

RGCs are not prepared yet for vesicle release at P1

Fgf1, Bmp4 and Gdf3 are well-known secreted signaling pro-

teins,^{26,27} thus we hypothesized that the three proteins might be secreted either through synaptic or neuroendocrine vesicle release from the ganglion cells. Therefore, we intended to examine if the proteins colocalize with vesicle markers (*i.e.*, Rab3a, synaptophysin, and NESP55) or a member of the vesicle-release machinery (*i.e.*, Exo70).

Intense Rab3a-immunoreactivity was detected in most cell bodies in the GCL (Figure 4 A1,C1, asterisks). It is important to note that Rab3a seemed to have a cytoplasmic distribution and the axons were immunonegative. Furthermore, the inner plexiform layer (IPL) and plenty of unidentified fibers in the outer NBL appeared to be Rab3a-IR (Figure 4B1, arrows). In P20 retina, used as a positive control, a faint staining was visible in the GCL cells (Figure 4D, arrow), while a bright Rab3a-immunopositivity appeared in amacrine cells (Figure 4D, asterisk), as well as in both synaptic layers (*i.e.*, IPL and OPL) (Figure 4D). Synaptophysin-immunoreactivity was spotted in sparse, distinct fibers of the IPL (Figure 4A2, thick arrows) and in the outer NBL (Figure 4B2). Colocalization could not be observed between Rab3a and synaptophysin in the inner retina (*i.e.*, IPL, GCL) (Figure 4 A3,A4), nevertheless, spatial overlap was revealed in the processes of the NBL (Figure 4 B3,B4). In P20 retina (positive control), presynaptic terminals were seen as immunopositive puncta in both IPL and OPL (Figure 4E).

Having found Rab3a-immunopositivity, we intended to investigate if active exocytosis is taking place in ganglion cells. Therefore, co-occurrence of Exo70, one of the eight subunits composing the exocyst and Rab3a immunolabeling was examined. A very few Exo70-IR cells were found in the GCL. Interestingly, no colocalization was found between Exo70 and Rab3a. Rab3a-IR ganglion cells did not show Exo70 positivity (Figure 4 C1,C3, asterisk). Likewise, none of those few Exo70-IR cells seen in the GCL displayed Rab3a-immunoreactivity (insert in Figure 4 C1,C2,C3, arrow). However, there were quite a few endothelial cells in capillaries that seemed to be Exo70-IR (Figure 4C2, double asterisks). In contrast, rich Exo70-immunopositive terminals were observed in the adult retina used as positive control (Figure 4F).

Finally, expression of NESP55, a vesicle protein considered as a marker of neuroendocrine activity was examined. No NESP55 immunoreactivity was found in either developing (P1) or mature (P20) retinal tissues. In rat hypothalamus, used as positive control, numerous cell bodies with NESP55-IR membrane appeared (Figure 4G, arrow).

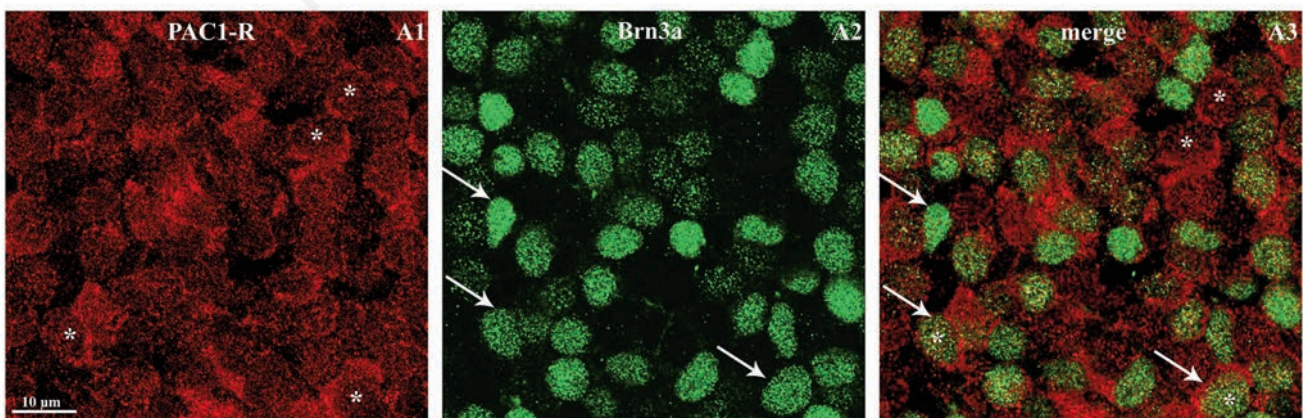


Figure 1. Colocalization of PAC1-R and Brn3a in P1 retinal whole-mounted preparation. PAC1-R-IR neurons are shown in A1 (asterisks). Brn3a-positive ganglion cells are labeled with arrows in A2. A3) Neurons co-expressing PAC1-R and Brn3a are labeled by both arrow and asterisk. PAC1-R-/Brn3a+ neurons are marked with asterisks whereas PAC1-R-/Brn3a+ neuron is labeled by an arrow (A3).

Discussion

Assembling of the retinal layers by six neuronal cell types, the formation of their functional networks including dendrites and neurites, electrical and chemical synapses is a result of an intriguing, complex process governed by a diverse and enormous set of extrinsic factors.

We have previously demonstrated that Fgf1, Bmp4, and Gdf3 were upregulated in the PACAP1-38 treated retina through PAC1-R-mediated mechanism.²³ Given the evidence of PACAP1-38 effects on morphogens expression, we revealed in the present study that most RGCs are synthesizing these key regulatory pro-

teins. Co-labeling with Brn-3a, a marker that is known to recognize most of the RGCs, we showed that the vast majority (90%) of the RGCs were PAC1-R-IR and *vice versa* – most PAC1-R positive neurons in the GCL were actually ganglion cells. Immunohistochemistry helped us to elucidate the expression of Fgf1, Bmp4 and Gdf3 in the retina, where numerous cells expressed them, most importantly, PAC1-R-IR cells in the GCL. All things considered, PAC1-R expressing RGCs emerged as one of the most likely candidates that could very well release these morphogens.

A large amount of published data has proved the physiological significance of Fgf receptors in retinal development (progenitor

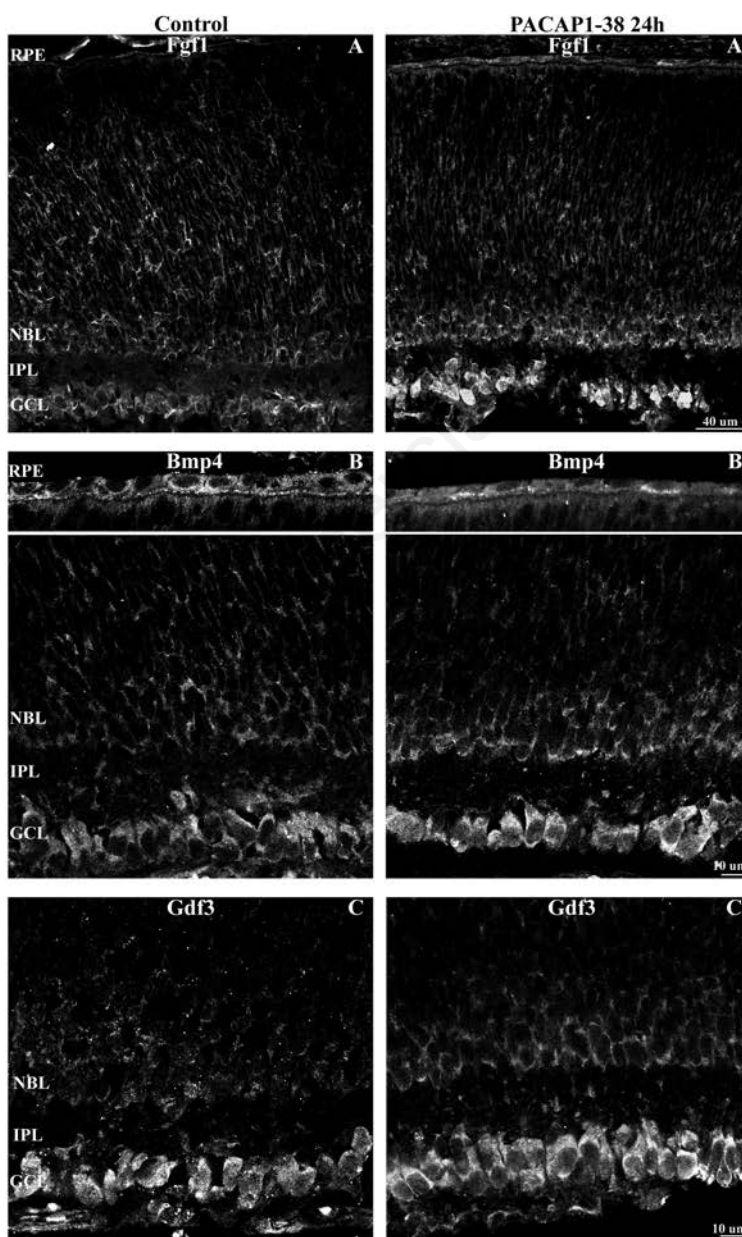


Figure 2. Fgf1, Bmp4 and Gdf3-IR structures in P1 retinal cross-section. A,A') Fgf1, immunopositive neurons in control and PACAP1-38 treated retina, respectively. B,B') BMP4-immunopositive layers of the P1 retina are depicted. C,C') Gdf3-immunopositive cells are shown in control and PACAP1-38 injected retina. RPE-retinal pigment epithelium; NBL, neuroblast layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars: A,A') 40 μ m; B,B',C, C') 10 μ m.

proliferation, RGC differentiation, photoreceptor survival).²⁸⁻³⁰ However, accurate studies revealing the precise identity of Fgf1-IR retinal cells in the postnatal period are strikingly sparse, especially in rat. In mice, Catalani and co-workers²⁵ provided a detailed analysis concluding that Fgf1 was expressed by RGCs and putative amacrine cells of the proximal NBL in the newborn (P0) mouse retina. Likewise, we demonstrated here that RGCs produced Fgf1 in the postnatal rat retina. In addition, we also revealed that Fgf1-expression displayed both cytoplasmic and nuclear pattern in the developing rat retina supporting the old observation of various studies spotting Fgfs in the nucleus.^{31,32} As an underlying molecular mechanism, Fgf1 has a signal peptide essential for secretion but also contains a nuclear localization sequence, which enables cells to transfer the protein into the nucleus and directly stimulate DNA synthesis.^{33,34} Cell-surface Fgf receptors are directly targets for internalization and translocation to the nucleus.³⁵ Interestingly enough, the source of Fgf1 molecule does matter because the effect of extracellular Fgf1 could differ from that of the endogenous Fgf1.³⁶

Numerous reports have proved the crucial roles of Bmp4 in the embryonic and early postnatal stage of RGC development in mouse as well as in chick embryo.^{5,6,37,38} Bmp4 was reported to be persistently expressed in the RGCs including their axons from P1 through adulthood but no immunoreactivity was detected in the NBL and no description was found about a Bmp4 expression in the RPE.^{39,40} Our finding is partially consistent with these studies. We observed Bmp4-immunopositivity in the majority of the RGC population, nevertheless, excluding their axons. In addition, a remarkable number of NBL cells and the RPE appeared to be Bmp4-IR.

Likewise Bmp4, Gdf3 belongs to the BMP/GDF branch of the Transforming Growth Factor-beta superfamily (TGF- β) implicated in embryonic cell fate decision and stem cell maintenance.⁴¹ To date, no data are available on either expression or function of Gdf3 in retinal tissues. To the best of our knowledge, this is the first study that reports an abundant expression of Gdf3 by RGCs in postnatal retina positively controlled by PAC1-R. However, the study is restricted to the description of mere appearance of Gdf3, meaning that the function still remains to be unveiled.

Apart from the morphological identification of the retinal cells expressing Fgf1, Bmp4 and Gdf3, we examined if the PACAP1-38-dependent protein expression could have an overall impact on the postnatal retinogenesis as all three investigated proteins are known to be secreted. Therefore, we proposed that RGCs may exert secretory activity at this early postnatal stage either through synaptic transmission or paracrine release. Either way, for vesicle secretion, RGCs need to express a large branch of factors responsible for transportation, association, tethering, and fusion of vesicles. Rab3a, a member of the large Ras family of small GTPases is expressed exclusively in the plexiform layers of the adult retina (OPL and IPL) where conventional as well as ribbon synapses are formed.⁴² Bound to the vesicle membrane, Rab3a is evidently essential for vesicle delivery,⁴³ thus Rab3a can be used as a reliable marker of synaptic vesicles. Although conventional synapses with synaptic vesicles have not been described until P3 in rat retina,⁴⁴ the IPL of P1 rat retina appeared to be immunoreactive for Rab3a. More surprisingly, cell bodies of numerous RGCs were also immunostained for Rab3a, which might indicate the presence of

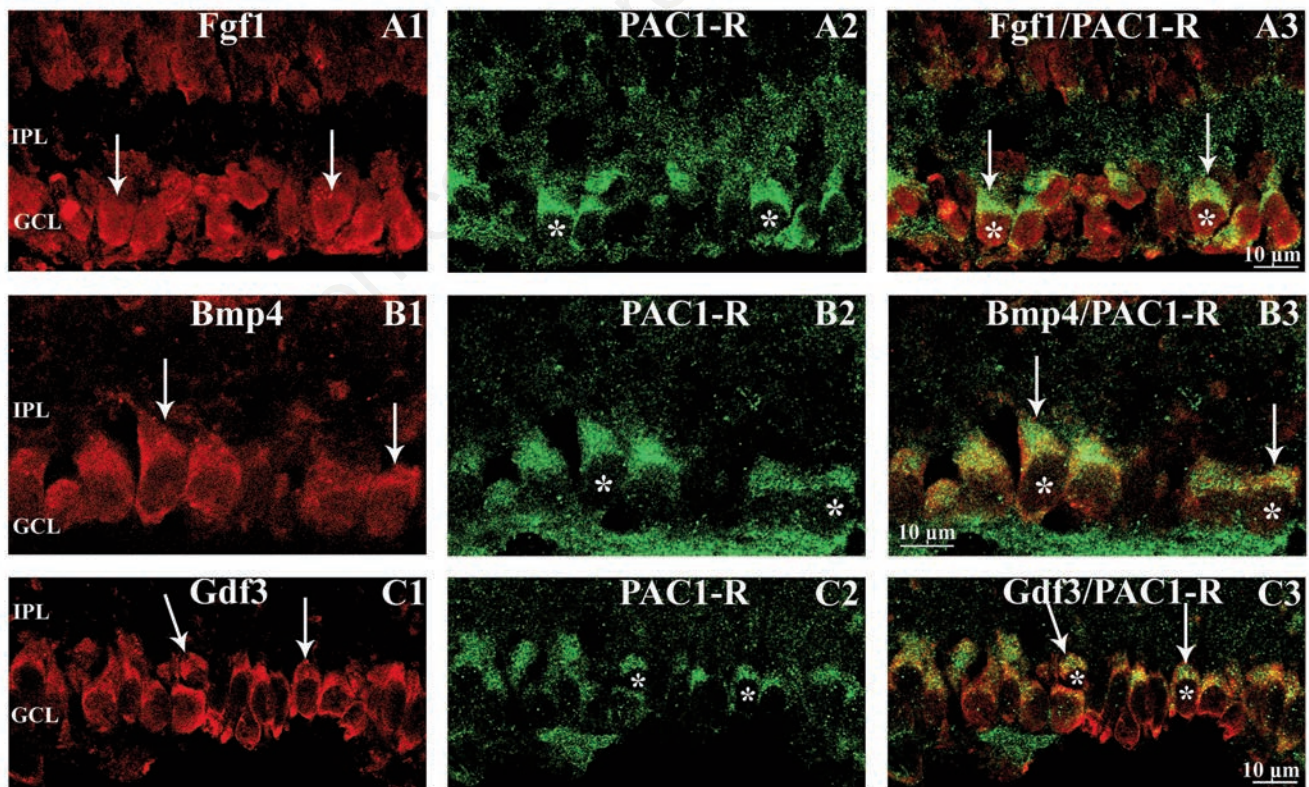


Figure 3. Co-expression of Fgf1, Bmp4, and Gdf3 with PAC1-R in P1 retinal cross-section. A1-A3) Fgf1 and PAC1-R-immunoreactivity are colocalized in RGCs. B1-B3) Bmp4-IR ganglion cells also express PAC1-R. C1-C3) Gdf3 and PAC1-R are co-expressed in the majority of the ganglion cell population as well. IPL, inner plexiform layer; GCL, ganglion cell layer.

synaptic vesicles in the soma. However, the axons of RGCs were not labeled thus our result can be interpreted as a sign of intense synthesis of Rab3a in this early postnatal period, particularly, in light of the fact that the other key component of a synaptic vesicle, synaptophysin was absent in RGCs. At the same time, bouton-like synaptophysin-IR structures, fine processes and intensely labeled thick fibers were observed in the IPL and the outer NBL, respectively. The literature provides quite incoherent data on the developmental expression of synaptophysin. Our description does not match with an early report of Dhingra *et al.*,⁴⁵ who found a diffuse synaptophysin staining in the IPL of P2 rat retina but no more. While synaptophysin expressing fibers were detected before synaptogenesis (*i.e.*, embryonic day 17) in mice,⁴⁶ it appeared in

the human retina at the onset of synaptogenesis.⁴⁷ Based on our result, synaptophysin-expression precedes synaptogenesis in rat retina as well. Furthermore, it does not colocalize with Rab3a thus vesicle maturation very probably is not complete in RGCs in the early postnatal stage of rat retinogenesis.

To test the provisional neuroendocrine activity of RGCs, the presence of another vesicle marker, NESP55 was examined. As a member of chromogranins, NESP55 was identified and considered as neuroendocrine-specific secretory protein in neurons as well as in neuroendocrine cells.⁴⁸ Seemingly, RGCs did not exert neuroendocrine activity because NESP55-immunoreactivity could not be revealed in any retinal cell type including RGCs. To confirm the lack of vesicle release, the expression of Exo70, a marker of active

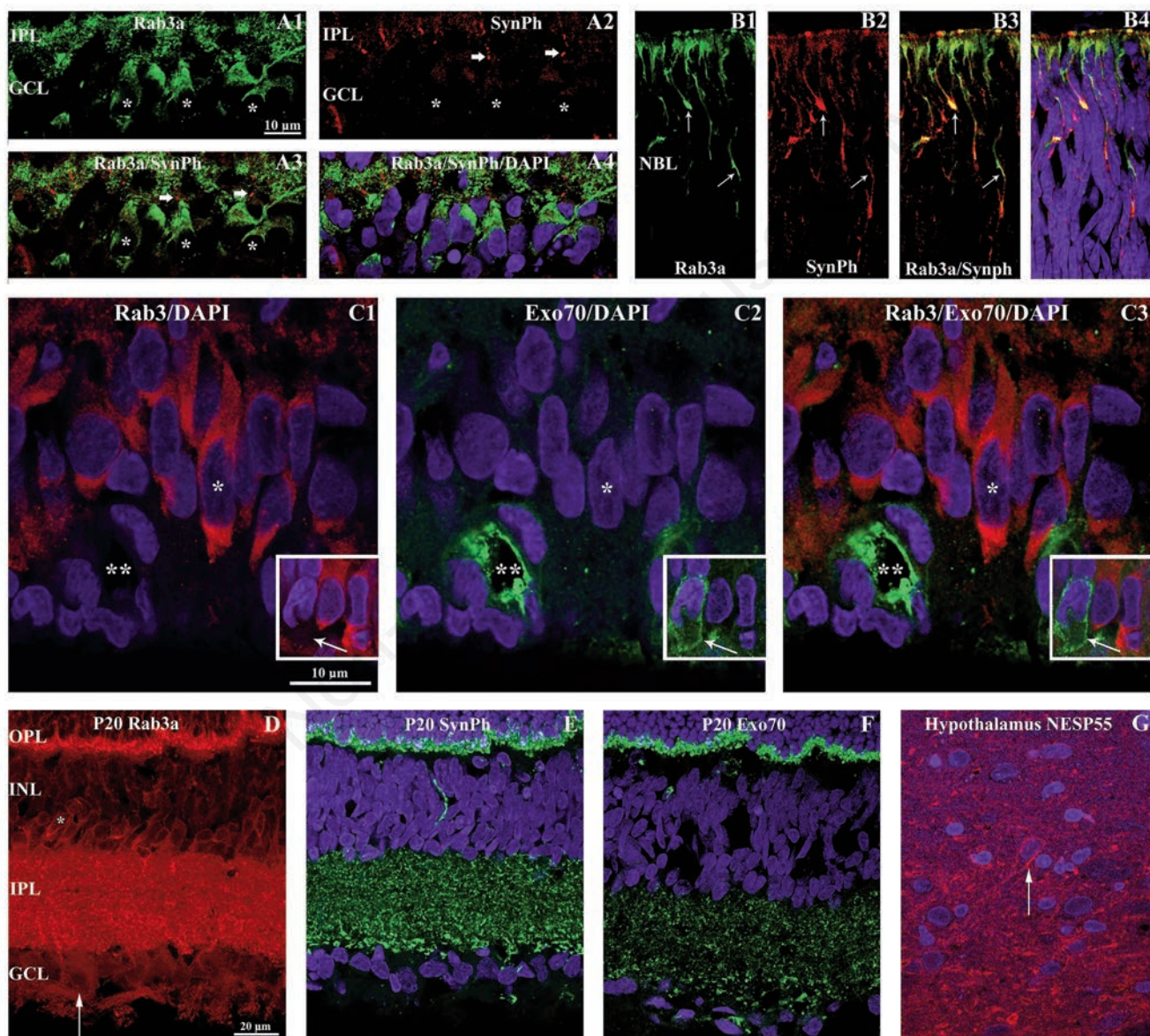


Figure 4. Expression of Rab3a, synaptophysin, and Exo70 in the GCL of P1 retinal cross-section. A1-A4) Rab3a-IR ganglion cell bodies (asterisks) in the GCL do not express synaptophysin (thick arrows). B1-B4) Rab3a-IR processes in the outer NBL contain synaptophysin (arrows). C1-C3) Rab3a-IR neurons (asterisk) do not express Exo70, which was detected rather in capillaries (doubled asterisk) and sparse, RAB3a-immunonegative cell bodies in the GCL (arrow in inserted panel). D-G) Positive controls: Rab3a-IR (D), synaptophysin-IR (E) and Exo70-IR (F) structures of the P20 adult rat retina. NESP-55-IR neurons in rat hypothalamic section (arrow). NBL, neuroblast layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

exocytosis, was also investigated in the P1 retina.⁴⁹ Although very few Exo70-IR cells were seen in the GCL, none of those cells were Rab3a-immunopositive indicating that Rab3a-expressing RGCs missed one of the main components of an exocyst, consequently RGCs were not prepared for exocytosis at P1. Nevertheless, Exo70 expression was spotted in blood vessels thus we can speculate that those single Exo70-IR cells among RGCs might have been migrating endothelial cells forming new vessels as the presence of the exocyst complex directing sprouting was reported in endothelial cells.⁵⁰

In conclusion, PACAP1-38 can induce RGCs *via* PAC1-R to upregulate Fgf1, Bmp4 and Gdf3 expression and synthesis in P1 rat retina. However, lacking crucial component of synaptic docking (synaptophysin) and release (Exo70), RGCs do not seem to be capable of Fgf1, Bmp4 or Gdf3 secretion yet. Given their intense expression, one can speculate that RGCs might be synthesizing these crucial regulators ahead and store them for future applications or the proteins could affect RGC physiology through an intracrine pathway, an important regulatory process to consider. In addition to endocrine, paracrine or autocrine regulation, a large set of secreted regulatory agents, brain derived neurotrophic factor, vascular endothelial growth factor, transforming growth factors just to name a few prominent ones, acts as intracellular signaling molecules. In the case of intracrine regulation, cells either internalize the extracellular molecules or they take actions in the synthesizing cell upon their synthesis.⁵¹ Of all the three proteins we had examined, Fgf1 has been reported to be internalized and to then traffic to the nucleus hence exerts effects within the synthesizing cells.^{36,52} As far as we are concerned, no data are available in respect of the intracrine action of Bmp4 or Gdf3. Apart from the intracrine action, there is another possibility to mention. It is a well-documented yet puzzling phenomenon that classic messenger molecules (*e.g.*, acetylcholine, glutamate, gamma-aminobutyric acid) are expressed, moreover, released⁵³ well before clustered receptors, transporters, synaptic proteins are assembled.^{44,54-56} However, the mechanism of this neurotransmitter release in the absence of functioning machinery is unclear. Therefore, the existence of another alternative pathway to release the aforementioned proteins cannot be ruled out and remains to be elucidated along with the potential involvement of PAC1-R in this process.

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