UNIVERSITY OF PÉCS

Biological Doctoral School

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Anti-apoptotic and ontogenetic effects of the pituitary adenylate cyclase-activating peptide in the postnatal rat retina

PhD Thesis

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1. Introduction and aims

The retinas of vertebrates include five distinct histological layers: photoreceptor layer, outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL) and ganglion cell layer (GCL). The mature retina is a uniquely laminated structure composed of six neuronal cell types (ganglion, amacrine, horizontal, bipolar, rod and cone photoreceptor cells) and one major glial element, the Müller cell (Bagnoli et al., 2003). Each of the neuronal components of the retina has a distinctive location, form and synaptology, as results of several developmental processes patterned in space and time: cell proliferation, cell fate commitment, migration and morphological as well as neurochemical differentiation (Reese and Colello, 1992; Rapaport et al., 2004).

As a pleiotropic hormone/neurotransmitter, the pituitary adenylate cyclase-activating polypeptide (PACAP1-38) is responsible for a variety of physiological actions in the developing nervous systems (e.g. cell proliferation, differentiation, migration, neurite outgrowth, synaptic development and plasticity) (Vaudry et al., 2009, Blechman and Levkowitz, 2013). PACAP1-38 signals via three G protein-coupled receptors: (1) PAC1-R, which binds PACAP1-38 more potently than VIP and (2) VPAC1 and (3) VPAC2 receptors, which do not discriminate between PACAP1-38 and VIP (Gottschall et al., 1990). In addition, PAC1-R is an object of alternative splicing that generates numerous isoforms. At present, sixteen PAC1-R splice variants have been described in mammals; these isoforms are distinguished by alterations in the N-terminal domain or the third intracellular loop or different combinations of both. Consequently, PAC1-R isoforms differ with respect to ligand-binding specificity/affinity and G-protein coupling resulting in the PACAP1-38 signal being transduced through multiple intracellular pathways (Dickson and Finlayson, 2009).

As a consequence of its strong anti-apoptotic effect, first and foremost, the peptide has been in the focus of neurotoxicity research and its neuroprotective potential has been extensively investigated in numerous neurodegenerative models (e.g. ischemia, ethanol toxicity, NO toxicity, oxidative stress) (Dejda et al., 2008). Apoptosis is a crucial mechanism by which multicellular organism control cell numbers and ensure the removal of damaged or potentially harmful cells (Wang, 2001; Peter and Krammer, 2003). The mechanism is governed by a family of cysteine proteases, the caspases, which have been divided into two functional sub-groups based upon their perceived roles in apoptosis. The initiator caspases (caspase 9, 12) are those that are responsible for initiating the caspase cascade by becoming aggregated upon receipt of a pro-apoptotic stimulus. The second group are the effector caspases (caspase 3, 6, 7), which in turn cleave intracellular substrates, resulting in the

dramatic morphological and biochemical changes of apoptosis (Boatright and Salvesen, 2003).

Although neuroprotective effect of PACAP1-38 has been demonstrated in monosodium glutamate (MSG) induced neurotoxicity in retina (Otori et al., 1998; Chen et al., 2001, Tamás et al., 2004), the underlying transduction pathways induced by PACAP1-38 in the *in vivo* MSG model remain unclear. Furthermore, the early expression of PACAP and PAC1-R pointed to the possibility of their involvement in development of a wide range of living organisms. The literature reporting the actions of PACAP and presence of PAC-1 receptor splicing variants in central nervous system is vast (Lu et al., 1998; Njaine et al., 2010), however, the paucity of detailed information on the PAC1-R expressing elements and function of exogenous PACAP in retinal development is striking.

Therefore, in the present thesis, we aimed to (i) dissect the PACAP1-38-activated signaling pathways mediating its anti-apoptotic effect in MSG induced excitotoxicity in immature rat retina, (ii) describe the expression of PAC1-R isoforms on precise developmental time scale, (iii) identify genes that are governed by PACAP, at last but not at least, (iv) to find those retinal cell types that are affected by PACAP in the developing rat retina.

2. Materials and methods

Animals and treatments

For all experiments albino Wistar rats were used. To study neuroprotective effect of PACAP and the underlying signaling pathways, MSG was injected (2 mg/g body weight) s.c to induce neurodegeneration. Concurrently, the following drugs were applied intravitreal (i.v); PACAP1-38 (100 pmol/eye), 2', 5'-dideoxy-adenosine (adenylate-cyclase inhibitor, DDA) (20 or 100 nmol/eye), ET-18-OCH_3 (phosphatidylinositol-specific PLC inhibitor) (5 or 10 μmol/eye), D609CAS (phosphatidylcholine-specific PLC inhibitor) (5 or 10 μmol/eye) and forskolin (adenylate-cyclase activator) (100 pmol/eye) in different combinations. Control animals received the same volume of 0.9% saline both s.c and i.v. As a separate experimental group, pups were i.v injected with PACAP1-38 (100 pmol/eye) to investigate PACAP regulated gene expression. The retinas were treated and dissected at different timepoints after the treatments, processed for either protein or RNA extraction.

RNA extraction, reverse transcription and RT-PCR

From the retinas, total RNA was extracted and purified using Rneasy Plus Mini Kit according to the manufacturer instructions. The amount of total RNA was determined with

BioPhotometer plus spectrophotometer by measuring optical density at 260 nm. Two micrograms of total RNA were used for reverse transcription (RT).

In order to increase specificity and yield, we employed a modification of RT-PCR called touchdown PCR in which the annealing temperature was gradually lowered to a more permissive temperature during the course of cycling, favoring amplification of the desired product. Amplification was carried out in a 25 µl reaction mixture containing 4 µl cDNA, 50 nM forward primer, 50 nM reverse primer, and Maxima Hot Start PCR Master Mix.

Sybergreen based real-time PCR was performed in Step-One Plus PCR instrument. Each sample was analyzed in triplicate. The results were analyzed by the $2^{-\Delta\Delta Ct}$ method after normalization. To normalize fluorescence signals, RPL13a and LacD were utilized as endogenous controls.

Western Blot

The treated retinas were homogenized in hypotonic lysis buffer or radioimmunprecipitation assay (RIPA) buffer. Samples were loaded and run on 4-12% NuPAGE, or 10, 12% SDS-polyacrylamide gels then transferred onto PVDF membranes under semi-dry condition. After blocking non-specific binding sites, membranes were probed overnight at 4 ºC with primary antibodies. Anti-β-tubulin (1:10.000) and anti-GAPDH (1:10.000) antibodies were used as loading controls. The binding of the primary antibodies were quantitated by using anti-mouse or anti-rabbit horse-radish peroxidase-conjugated secondary antibodies at a dilution of 1:10.000. The blotted proteins were developed using Western Lightning Chemiluminescence reagent Plus detection system.

Morphology

PACAP treatments were performed at P1 and P3 prior to dissection at P5. After eye removal, eyecups were fixed in buffered 4 % paraformaldehyde + 1% glutaraldehyde. After embedding procedure, eyecups were transferred into epoxy resin. Following polymerization, the semithin sections of 2-5 µm thickness were cut and stained with toluidine blue. Morphometric measurments were performed using Panoramic Viewer software.

For immunostaining, retinas were fixed in 4% PFA and proceed for cryostate sectioning or whole mount preparations. The 10-12 µm thick sections were incubated in mouse-anti-HPC-1, mouse-anti-Calbindin and sheep-anti-Chx-10 antibodies overnight at room temperature. After washing, sections were incubated in secondary antibodies (anti-mouse-IgG-Alexa 448; anti-sheep-IgG-Alexa 568), then were mounted in DAPI dissolved in Prolong. Sections were examined using an Olympus FV-1000 laser scanning confocal fluorescence microscope.

3. Results and discussion

Neuroprotective effect of PACAP and underlying signaling pathways

In our previous experiments, MSG-induced activation of caspase 9 and 3 were found to peak at 6 hours and terminate by 24 hours post-injection (Denes et al. 2011). Therefore, we investigated the anti-apoptotic effect of PACAP1-38 at 6 hour after MSG-injection. As a first step to indentify PACAP induced signaling pathways, we applied DDA, a cAMP analog AC inhibitor. However the effect of MSG was blocked by PACAP1-38; i.v administration of PACAP1-38 inhibited the activation of caspase 3 after concurrent injection of 5 µg (20 nmol) or 25 µg (100 nmol) DDA. Likewise, the level of cleaved caspase 9 was elevated following MSG injection and inhibited by PACAP1-38. Simultaneous administration of DDA and PACAP1-38 also resulted in a decreased level of the active enzyme regardless of DDA doses.

It is important to point out that metabotropic glutamate receptors (i.e. $mGlu_2$, $mGlu_3$, $mGlu_4$, $mGlu_6$, $mGlu_7$, $mGlu_8$) couple to Gi/Go-proteins inhibiting AC activity (Thoreson and Witkowsky, 1999). We hypothesized that DDA might be ineffective in this particular model because MSG itself could act as an AC inhibitor. To elucidate it further and to examine the effect of PACAP1-38 and MSG separately on the AC pathway. We performed quantitative phospho-protein kinase A (PKA) studies in a more precise temporary resolution. Interestingly, no significant changes were detected in phospho-PKA levels at 1, 3, 6 hours following PACAP1-38 injection. Moreover, no reduction in the active PKA level was observed in the samples that received MSG treatment alone. As a further test, the concentration of cAMP, the upstream activator of PKA was measured over a precise timescale using a competitive immunoassay following 100 pmol PACAP1-38 or 100 pmol forskolin i.v administration. Effects of the two substances were investigated with or without s.c MSG injection. The 100 pmol forskolin significantly increased the cAMP level 1 hour after i.v. injection regardless of the absence or presence of MSG. Surprisingly, PACAP1-38 did not evoke any increase in cAMP levels at any experimental time points.

Since our data indicated that PACAP did not induce the cAMP dependent signaling cascade thus we performed further experiments to identify the signaling pathway activated by PACAP. We used phosphatidylcholine-specific and phosphatidylinositol-specific PLC inhibitors, D609CAS and ET-18-OCH_3, respectively. D609CAS did not affect the level of caspase 3 alone. As expected, s.c. MSG injection increased whereas i.v. PACAP1-38 injection significantly decreased the activation of caspase 3. It was clearly shown that in the case of co-administration of PACAP1-38 and 10 µmol D609CAS, significant elevation of caspase 3 was detected. The other PLC inhibitor, ET-18-OCH_3 failed to prevent the effect of PACAP1-38

indicating that PACAP1-38 acts through coupling to a phosphatidylcholine-specific PLC only.

In summary, it seems that at picomolar concentrations applied in our study, PACAP1-38 has no ability to increase cAMP production and still exerts significant anti-apoptotic effect through PLC activation. We conclude that intracellular PACAP pathways might be determined by extracellular PACAP levels. Consequently, PACAP produce a variety of outcomes in a dose-dependent manner.

Identification and expression pattern of PAC1 receptor splice variants during postnatal retinal development.

Changes of transcript level during postnatal development were compared to the newborn (P0) retina as a reference. First, we used primers specific for all PAC1 isoforms. However, no remarkable overall changes could be detected in PAC1 receptor message level. The only statistically significant elevation was measured at P1. In later developmental stages, at P3, P5, P10, P15, and P20 the ratio of PAC1 receptor expression relative to that at P0 was constant.

Using splice variant specific primers, a sensitive touchdown-PCR was utilized to reveal the set of PAC1 isoforms expressed in rat retina. Four time points were chosen (i.e. P0, P5, P15, P20) in order to cover proportionally the postnatal development. However, no remarkable overall changes could be detected in PAC1 receptor message level. The only significant elevation was measured at P1. In later developmental stages the ratio of PAC1 receptor expression relative to that at P0 was constant.

Results obtained from the touchdown PCR indicated that expression of the individual PAC1 receptor isoforms might display unique profiles. Therefore, expression changes were mapped employing quantitative real-time PCR. As a first approach, retinas harvested at P0 were used as reference samples. Null isoform showed no impressive changes at P1, P3; but then manifested a decline from P5 to P10, Null message level fell at P15. The Hip isoform had a similar expression pattern, with a peak at P1, followed by a continuous downregulation by P20. The lowest expression level was detected at P15. Expression levels of Hop1 splice variant did not change at P1, P3 or P5, but thereafter, the Hop1 message level showed a significant increase at P10, P15 and P20. The expression analysis of Hiphop1 isoform showed one prominent but statistically not significant peak at P10.

Once the expression profile of each individual PAC1 isoform was determined, we investigated their expression in relation to the Null isoform at each time point to reveal the

ratios between the PAC1 isoforms. Since the previous experiment showed that none of the isoforms appeared to change between P3 and P5, we investigated only the following time points: P0, P1, P10, P15, P20. In early development, the Hip splice variant was expressed to the greatest extent at P0 and P1. Hop1 transcript became dominant at P10 and P15; and reached its highest level at P20. Although the Hiphop1 variant displayed considerable elevation at P10 compared to P0, among the PACAP variants it was present in the smallest amount in the rat retina. Looking at any time point from P0 to P20, Hiphop1 message level was lower than that of any other isoform.

To confirm the up or down regulation of mRNA, immunoblots with specific antibodies were performed. Unfortunately, there are no commercially available antibodies against PAC1 splice variants. However, due to the presence or absence of the cassettes inserted into the third intracellular loop, isoforms can be separated by polyacrylamide gel electrophoresis. The PAC1 receptor antibody used in our experiments recognizes the extracellular N-terminal sequence; consequently, it binds to all isoforms of the PAC1 receptor that share an identical N-terminal sequence. Since Hop1 and Hip variants have the same molecular weight (56 kDa), they could not be separated, and bands show an additive expression of Hip and Hop1 isoforms. An upregulation of the PAC1 receptor was clearly demonstrated at P15 and P20. Our antibody labeled a protein with the molecular mass of the Null isoform (53 kDa) as well, which disappeared by P15 and P20. Evidently, the band around 59 kDa corresponded with the largest PAC1 isoform, namely Hiphop1. The western blot result showed the presence of the Hiphop1 protein at all time points.

In sum, since each PAC1 receptor isoform displays a unique expression profile, we suggest that selective splicing and expression of PAC1 receptor may be a pivotal component of developmental processes.

Morphological consequences of PACAP injection in newborn rat retina

To detect morphological changes, one eye of P1 rats was injected i.v with PACAP1-38 whereas the other eye was treated with an equal volume of saline. Treatment was repeated at P3. Eyes were dissected at P5 and proceed for morphometric measurments or immunocytochemistry.

Our morphometric results showed a 2.65% increase in the thickness of the NBL compared to the control, whereas the thickness of the IPL appeared to decrease by 2.69%. Furthemore, in the GCL of control tissues, an average 15.02±1.51 cells/100μm were counted,

whereas in the treated samples 13.17±1.3/100μm cells were observed. These results show that PACAP administration caused a significant, 14.05% decrease in the ganglion cell number.

In order to identify the cell populations affected by PACAP, specific cell markers were employed. Development of the calbindin-positive horizontal cell population is affected by PACAP in terms of cell number and neurite outgrowth; cell number was upregulated accompanied by an underdeveloped arborization.

HPC-1 antibody can stain the amacrine cell bodies and the fibernetwork of IPL. The thickness of the seam of HPC-1 positive amacrine cells appeared to be increased compared to the control samples. Number of Chx10-immunreactive bipolar cells did not change upon PACAP injection. In contrast, more progenitor cells located in the ventricular zone of the developing retina were seen in the PACAP treated retina. However the number of migrating cells decreased as results of PACAP injection.

In sum, as a consequence of i.v PACAP1-38 injection, an increase was observed in the thickness of the neuroblast layer compared to that of the control tissue. In line of this observation, the number of the Chx-10 positive progenitor cells, horizontal and amacrin cells was also increased. In addition, neurite outgrowth of the horizontal cells was severely underdeveloped in PACAP treated retinas. In contrast, the cell number in the ganglion cell layer and the thickness of the inner plexiform layer appeared to decrease compared to the control retina. Consequently, the PACAP do has an ability to affect the morphogenesis of the retina, also, the number and differentiation of the retinal neurons.

Identification of PACAP regulated genes in the neonatal retina

In these experiments, P1 rats were treated i.v 100 pmol PACAP. The retinas were dissected 3, 6, 12 and 24 hours after the PACAP treatments. Our target genes were reported to play critical roles in neural and non-neural tissue development.

Symmetric/asymmetric cell division inducing genes (Dhh), as well as cell adhesion molecules (Acan, Cd44, L1CAM) displayed no changes were detected at any timepoints. Nevertheless, the exogenous PACAP could affect to the expression of Cul1 and Neurog1 message levels. Expression of these genes responsible for cell adhesion, cell communication and migration did not show significant changes in exogenous PACAP treated retinas.

The family of fibroblast growth factors (Fgf) consists of numerous members (Fgf1, 2, 4, 8, 9), which have been implicated in diverse cellular processes. Expression level of the first member, Fgf1 increased substantial by at 6 hours then this transcript level was further

elevated at 12 hours. The same changes were observed in the protein levels, Fgf1 showed a gradual elevation from 6 through 24 hours post-injection.

Among genes belonging to the family of growth differentiation factors, we investigated the expression levels of Bmp4, Bmp9 and Gdf3. Bmp4 message level was elevated at 6 hours following PACAP treatment. In 12 hours following PACAP injection, a more enhanced gene transcription was measured. Intrestingly, a remarkable increase was detected in protein level of Bmp4 only at 24 hours. Following a significant increase, Gdf3 transcription was hardly detectable after 6 hours. Then, it displayed a marked elevation at 12 hours after PACAP treatment. The changes in the transcript level was reflected in the protein level; after 12 hours a reduction, meanwhile after 24 hours a substantial elevation was observed.

Among genes involved in cell communication, Gjb1 transcript level showed a remarkable increase at all experimental time points. However, no changes was observed in Gjb1 protein level.

PACAP could remarkably affect the embrional cell line markers. PACAP1-38 injection resulted in upregulation of Nanog and T, at 3 and 6 hours, which are responsible for pluripotency and self-renewal. However following a significant elevation, T transcription was weakly detectable after 12 hours. The exogenous PACAP increased the expression of Msx1 at 6 hours, in the case of Oct4 and Pax6 we could not detect any changes.

Since the Wnt pathway plays an important role, in the ontogeny of many living organisms, we investigated the expression of Wnt1. PACAP1-38 caused a significant increase in expression of Wnt1 as early as 3 hours. At the protein level, effect of PACAP was observed at 12 hours after administration.

The retinal cell differentiation markers are important transcription factors playing an essential roles in the regulation of cell morphogenesis and differentiation processes. Following a slight increase, Mash1 transcription was decreased after 6 hours. Then, it displayed again an elevation at 12 hours after PACAP treatment. In the case of Math5, which regulates ganglion cell differentiation, the first significant change was measured at 6 hours, then the Math5 transcript level was further increased at 12 hours. At the protein levels, Math5 showed a remarkable elevation at 12 and 24 hours. Dlx2 was significantly increased at 3 and 12 hours compared to the control samples. Between the two time points a significant decrease was observed in the expression of Dlx2. At the protein level, no change was observed after 12 hours of PACAP administration, whereas a substantial upregulation was detected at 24 hours.

The transcription of Otx2 showed similar pattern to the Math5 at 6 and 12 hours. Other transcription factors (Dlx1, NeuroD1, Chx10) did not show any changes.

We could identify numerous genes and gene families, which are regulated by PACAP. The effects of exogenously applied PACAP might stimulate pluripotency due to upregulation of Wnt1, T and Gdf3. It could promote specific neuronal differentiation of ganglion cells and photoreceptors through upregulation of Math5, Dlx2 and Otx2, respectively. PACAP appears not regulate cell adhesion, cell migration, cell-cell communication (Gjb1) and axon outgrowth (Acan, CD44, L1CAM). In sum, PACAP affects retinogenesis directly as well as indirectly through regulation of secreted signaling proteins.

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I. Publications served as a basis of PhD thesis

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II. Posters and lectures served as a basis of PhD thesis

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III. Other scientific publications

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IV. Other conference participations

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Dénes V, Czotter N, **Lakk M**, Berta G, Gábriel R. PAC1 expressing structures of the neural retina alter their PAC1 isoform splicing during postnatal development. FENS Featured Regional Meeting, Prague, 2013. (poster)

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