THESIS OF THE DOCTORAL (PH.D.) DISSERTATION

The Role of PACAP in the Animal Model of Retinopathy of Prematurity

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I. Introduction

I/1. Retinopathy of Prematurity

Retinopathy of prematurity (ROP) is a vasoproliferative disorder of the immature retina in preterm infants and remains one of the leading causes of childhood visual impairment and preventable blindness worldwide. The condition was first recognized in the 1940s under the term retrolental fibroplasia, and it soon became apparent that its emergence was closely linked to oxygen therapy practices in neonatal care. This early observation fundamentally shaped subsequent investigations into the disease's underlying mechanisms (Terry, 1946; Bedrossian et al., 1954; Silverman, 1980; Robertson, 2003).

Advances in neonatal intensive care have markedly improved the survival of extremely premature and extremely low birth weight infants; however, these achievements have also been accompanied by an increasing burden of ROP. In high-income regions, structured screening programs, modern neonatal practices, and timely ophthalmic interventions have substantially reduced ROP-associated blindness. Conversely, in low- and middle-income countries, the disease continues to represent a significant public health challenge. Notably, its epidemiology correlates more strongly with healthcare quality and socioeconomic conditions than with geographical distribution, underscoring the essential link between ROP and the standard of perinatal care (Gilbert, 2008; Quinn et al., 2010; Kiranmayee et al., 2019).

ROP develops exclusively in preterm infants, with incidence inversely related to gestational age and birth weight. The most vulnerable population comprises extremely immature neonates, for whom ROP remains a major ophthalmic concern in contemporary perinatal medicine. Importantly, severe visual outcomes are largely preventable when the disease is identified promptly and managed according to established treatment guidelines (Good et al., 2005; Quinn et al., 2014; Quinn et al., 2016; Blencowe et al., 2013).

Understanding the normal development of the retinal vasculature is essential for interpreting ROP pathogenesis. Retinal vascularization during fetal life is a highly coordinated process involving sequential vasculogenesis and angiogenesis, driven primarily by the relative hypoxia of the developing retina. This hypoxic state promotes

the expression of angiogenic factors, particularly vascular endothelial growth factor (VEGF), and is mediated by glial elements and intracellular signaling pathways (Chang-Ling et al., 1995; Bai et al., 2009; Hartnett, 2015; Lutty & McLeod, 2018).

Because complete retinal vascularization occurs only near term, preterm infants are born with an incompletely vascularized retina. Exposure to the comparatively hyperoxic extrauterine environment disrupts physiological angiogenesis, leading to the initial vaso-obliterative phase of ROP. Subsequent retinal maturation increases metabolic demand; however, the insufficient vasculature leads to tissue hypoxia, triggering the second, vasoproliferative phase characterized by pathological neovascularization at the vascular–avascular junction. In its most severe form, this process culminates in extraretinal fibrovascular proliferation and tractional retinal detachment, ultimately causing blindness (Smith, 2003; Sapieha et al., 2010; Hellström et al., 2013; Sun & Smith, 2018; Graziosi et al., 2020).

ROP is inherently multifactorial. In addition to prematurity and oxygen exposure, oxidative stress, inflammation, dysregulated growth factor signaling, and metabolic disturbances all contribute to disease development. Hyperoxia promotes the formation of reactive oxygen and nitrogen species, causing endothelial injury and capillary loss, while preterm infants' limited antioxidant capacity increases vulnerability (Nielsen et al., 1988; Oguni et al., 1995; Gu et al., 2003; Beauchamp et al., 2004). Hypoxia-inducible factor-1α (HIF-1α) is a key regulator of hypoxic signaling and governs the expression of several molecules critical for retinal vascular development (Rey & Semenza, 2010; Hartnett & Penn, 2012).

VEGF plays a central pathogenic role by regulating endothelial proliferation, migration, and tubulogenesis. Erythropoietin (EPO), beyond its hematopoietic properties, also exerts angiogenic and cytoprotective effects. Moreover, the abrupt decline in insulin-like growth factor-1 (IGF-1) levels after preterm birth eliminates an important permissive factor for normal vascular maturation, strongly linking low IGF-1 levels to ROP susceptibility (Hellström et al., 2001; Hellström et al., 2003; Löfqvist et al., 2007).

In recent years, metabolic and inflammatory contributions - particularly neonatal hyperglycemia and conditions such as sepsis or necrotizing enterocolitis - have become increasingly recognized as potential aggravating factors for severe ROP (Garg et al.,

2003; Ertl et al., 2006; Tolsma et al., 2011; Lundgren et al., 2017; Turai et al., 2019; Lei et al., 2021).

Clinical management is guided by the International Classification of Retinopathy of Prematurity (ICROP), which standardizes the description of disease location, extent, and severity and forms the basis of treatment decision-making (Chiang et al., 2021; Nagy et al., 2023). Systematic ophthalmic screening remains essential, as timely detection of treatment-requiring ROP substantially improves visual prognosis.

Therapeutic strategies primarily target the vasoproliferative phase. Peripheral retinal laser photocoagulation remains an established standard of care, whereas anti-VEGF agents have broadened therapeutic possibilities, particularly for aggressive posterior disease. Nevertheless, concerns persist regarding their potential systemic effects in developing infants (Mintz-Hittner et al., 2011; Morin et al., 2016; Stahl et al., 2019). These limitations highlight the need for novel, targeted therapies that not only suppress pathological neovascularization but also confer neurovascular protection to the developing retina.

Animal models of oxygen-induced retinopathy (OIR) have proven indispensable to ROP research. Mouse and rat models enable detailed investigation of vascular and neuronal alterations and the preclinical evaluation of therapeutic approaches. At birth, rodents possess an immature visual system that is developmentally comparable to that of preterm infants born at around 25 weeks of gestation (Madan and Penn, 2003). While the mouse model is particularly suitable for molecular and genetic studies, the rat OIR model more closely mirrors the morphological and pathophysiological features of human ROP, making it especially valuable for translational research (Smith et al., 1994; Penn et al., 1994; Madan & Penn, 2003; Barnett et al., 2010).

I/2. PACAP

Pituitary adenylate cyclase-activating polypeptide (PACAP), identified in 1989, is a member of the secretin/VIP peptide family and is widely recognized for its neuromodulatory, trophic, and cytoprotective properties (Miyata et al., 1989; Vaudry et al., 2009). Its biological effects are mediated predominantly through the PAC1 receptor, though VPAC1 and VPAC2 receptors also participate in PACAP-induced signaling. PACAP is broadly expressed across multiple organ systems, particularly within the

nervous system, where it supports cell survival, differentiation, and anti-apoptotic and anti-inflammatory pathways (Reglodi et al., 2011; Harmar et al., 2012; Toth et al., 2020).

In the visual system, PACAP and its receptors are present in several retinal layers, including ganglion cells, amacrine and horizontal cells, and Müller glia. Their expression during retinal development suggests a role not only in injury responses but also in physiological retinogenesis (Seki et al., 1997; Atlasz et al., 2010; Denes et al., 2014; Patko et al., 2022). Experimental studies have consistently demonstrated the retinoprotective potential of PACAP in models of excitotoxic, ischemic, oxidative, and diabetic injury. PACAP administration preserves retinal structural integrity, attenuates apoptotic signaling, and improves functional outcomes (Tamas et al., 2004; Atlasz et al., 2007; Szabadfi et al., 2012; Szabadfi et al., 2014; Li et al., 2025).

The role of endogenous PACAP has also attracted growing interest. PACAP-deficient animal models exhibit heightened vulnerability, impaired stress adaptation, and more severe structural damage across multiple organ systems. In the retina, the absence of endogenous PACAP leads to increased cell death and more pronounced degenerative alterations under various injurious conditions (Hashimoto et al., 2001; Endo et al., 2011; Reglődi et al., 2012; Szabadfi et al., 2012). These findings highlight PACAP not only as a promising therapeutic agent but also as an intrinsic regulator of retinal homeostasis and stress resilience.

II. Aims

In retinopathy of prematurity (ROP), vascular and neuronal injury evolve in parallel; therefore, it is of particular interest to investigate molecules capable of influencing both components simultaneously. Given the well-established neuroprotective, anti-apoptotic, anti-inflammatory, and partially vasculotropic properties of PACAP, we hypothesized that it may contribute to the attenuation of oxygen-induced retinal damage. Accordingly, the objective of our studies was to explore the role of exogenous PACAP and the endogenous PACAP system in experimental models of oxygen-induced retinopathy (OIR).

The specific aims of the present work were as follows:

1. To examine the potential protective effects of exogenous PACAP in a rat model of oxygen-induced retinopathy, with particular emphasis on retinal structural alterations and the associated immunohistochemical and biochemical changes.
2. To assess the impact of endogenous PACAP deficiency in a mouse model of oxygen-induced retinopathy, employing immunohistochemical, biochemical, and functional methodologies.

III. Investigation of The Protective Effects of Exogeous PACAP in a Rat Model of Retinopathy of Prematurity

III/1. Materials and methods

All experiments were conducted on newborn Sprague–Dawley rats. To model retinopathy of prematurity, we employed the 50/10 oxygen-induced retinopathy (OIR) paradigm, in which pups were exposed to alternating hyperoxic and hypoxic environments every 24 hours during the first 14 postnatal days, followed by normoxic rearing until postnatal day (P) 19. Control animals were maintained in room air throughout the entire experimental period.

PACAP1-38 treatment was initially administered either intraperitoneally (1 mg/kg on postnatal days 1–7) or intravitreally (100 pmol/3 µl on postnatal days 11, 14, and 17). Overall, the study comprised the following groups:

- Control, n=18
- Control + intraperitoneal PACAP, n=6
- Control + intravitreal PACAP, n=25
- Control + intravitreal saline, n=25
- OIR, n=35
- OIR + intraperitoneal PACAP, n=26
- OIR + intravitreal PACAP, n=41
- OIR + intravitral saline, n=41

As systemic administration did not result in appreciable morphological improvement, subsequent experiments focused exclusively on the intravitreal treatment regimen.

To evaluate retinal vascular development, retinas isolated at P19 were stained with isolectin. Quantitative analyses included assessment of the peripheral avascular area, counting of vascular branch points, and measurement of neovascular tuft formation. Changes in inflammatory and angiogenic mediators were examined using cytokine array profiling. Retinal function was evaluated by dark-adapted electroretinography (ERG).

Statistical analyses were performed after testing for normality and homogeneity of variance. Depending on data distribution, parametric or non-parametric tests were applied. A significance threshold of $p < 0.05$ was used throughout.

III/2 Results

In the OIR model, characteristic retinopathic alterations developed, most notably the presence of a peripheral avascular retinal zone. In the control groups, complete retinal vascularization was observed, and PACAP treatment alone did not produce any detectable morphological changes.

Intravitreal PACAP administration significantly reduced the proportion of the peripheral avascular area compared with both untreated and saline-treated OIR groups, whereas intraperitoneal treatment exerted no meaningful effect. These findings indicate that local PACAP delivery favorably influences retinal vascularization.

In OIR animals, the number of vascular branch points increased relative to controls; however, this parameter was not modified by PACAP treatment. Similarly, neovascular tufts emerged in OIR retinas, yet their extent was not significantly altered following intravitreal PACAP administration.

Cytokine array analyses demonstrated upregulation of several inflammatory, adhesion-related, and angiogenic mediators in OIR, including CNTF, LIX, L-selectin, MIP-3α, TIMP-1, and VEGF. Intravitreal PACAP treatment attenuated the expression of most of these factors, whereas TIMP-1 levels increased further, suggesting a partial modulation of the inflammatory and angiogenic response.

Electroretinographic evaluations revealed reduced a- and b-wave amplitudes in OIR animals, indicating substantial functional impairment of the retina. Intravitreal PACAP treatment did not ameliorate this functional decline, nor were significant changes observed in implicit times.

III/3. Discussion

The aim of our study was to investigate the role of PACAP, a molecule with well-established neuro- and retinoprotective properties, in animal models of retinopathy of prematurity. Despite the availability of effective therapeutic strategies - including laser photocoagulation and anti-VEGF agents - ROP remains one of the leading causes of preventable childhood visual impairment. Given the limitations and potential adverse effects associated with current therapies, the exploration of novel, safer treatment approaches remain a pressing need. PACAP is a promising candidate in this regard.

In the rat OIR model, we demonstrated that intravitreal PACAP administration improved retinal vascularization by significantly reducing the extent of the peripheral avascular area. Concurrently, cytokine expression analysis revealed that PACAP attenuated the elevated levels of inflammatory and angiogenic mediators characteristic of OIR. These findings are consistent with previous observations showing the retinoprotective effects of PACAP across a variety of retinal injury models.

The biphasic pathomechanism of OIR - initial vaso-obliteration followed by pathological neovascularization - allowed for the temporally targeted administration of PACAP. Based on our results, PACAP appears to support reparative revascularization while simultaneously dampening the inflammatory responses associated with aberrant angiogenesis.

Neuronal involvement is also a key feature of ROP pathogenesis. The OIR model is known to exhibit inner retinal damage and Müller cell gliotic activation, leading to the release of inflammatory mediators and VEGF. Our present findings, together with earlier studies, suggest that PACAP can modulate these processes, as reflected by the reduced expression of L-selectin, LIX, CNTF, MIP-3α, and VEGF. The observed increase in TIMP-1, however, raises the possibility that PACAP may also influence tissue remodeling and extracellular matrix regulation.

Functional assessments revealed marked ERG deficits in OIR animals compared with controls, indicating substantial retinal dysfunction. Notably, PACAP treatment did not yield measurable functional improvement. This discrepancy suggests that structural regeneration and functional recovery may occur on different timescales, and that distinct retinal layers may exhibit differential sensitivity to treatment.

From a methodological perspective, systemic PACAP administration did not affect retinal vascular development, likely due to limitations in dosage, bioavailability, or route of administration. In contrast, local delivery was found to be more effective. Additionally, interpretation of the cytokine array data was influenced by inter-assay variability, necessitating the use of relative expression changes for reliable comparison.

Taken together, our findings indicate that locally administered PACAP mitigates vascular pathology in oxygen-induced retinopathy and reduces the expression of key inflammatory mediators. These results highlight the peptide’s potential therapeutic relevance in the context of ROP.

IV. Investigation of The Protective Role of Endogenous PACAP in a Mouse Model of Retinopathy of Prematurity

IV/1. Materials and Methods

For these experiments, we used CD-1 wild-type (Wt) and homozygous PACAP-deficient (KO) mice, housed under standard laboratory conditions in full compliance with institutional and national ethical guidelines. Retinopathy was induced using the classical Smith oxygen-induced retinopathy protocol. Pups were exposed to 75% oxygen from postnatal day (P) 7 to P12, followed by normoxic rearing until P17 (OIR-Wt $n = 15$; OIR-KO $n = 14$). Control groups were maintained in room air throughout the experiment (Control-Wt $n = 15$; Control-KO $n = 15$).

Morphological assessment of the retinal vasculature was performed at P17 following isolectin staining. Quantitative analysis included measurement of the avascular retinal area, the degree of overall vascularization, and the number of neovascular tufts, using dedicated image-processing software. To investigate intracellular signaling events, we assessed Akt phosphorylation by Western blot analysis. Expression of angiogenic factors was evaluated using a semiquantitative angiogenesis array. Retinal function was assessed by electroretinography (ERG), analyzing a- and b-wave amplitudes as well as implicit times. Statistical analyses were performed using parametric or non-parametric tests, with a significance threshold of $p < 0.05$.

IV/2. Results

Under normoxic conditions, both wild-type and PACAP-deficient mice exhibited complete retinal vascularization without pathological abnormalities. In contrast, the oxygen-induced retinopathy model produced characteristic vascular alterations, and PACAP-deficient mice displayed a significantly larger central avascular area compared with wild-type animals. The number of neovascular tufts was likewise increased in the KO group, whereas overall capillarization did not differ between genotypes.

Analysis of intracellular signaling revealed no differences in Akt phosphorylation under normoxic conditions. However, upon OIR induction, Akt activation increased in

wild-type mice, while PACAP-deficient mice exhibited significantly reduced Akt phosphorylation.

Angiogenesis array profiling showed elevated expression of several angiogenic molecules in wild-type OIR retinas. In PACAP-deficient OIR animals, an even broader range of angiogenic and inflammatory mediators displayed increased expression, whereas a subset of factors showed reduced levels. Under normoxic conditions, only minimal genotype-dependent differences were detected.

Functional assessments indicated that genotype had a significant impact on retinal performance. PACAP-deficient mice exhibited reduced a- and b-wave amplitudes even at baseline. In the OIR condition, b-wave amplitudes were further decreased in KO mice compared with retinopathic wild-type mice, while implicit times did not differ significantly.

IV/3. Discussion

In the second part of our study, we explored the role of endogenous PACAP in retinopathy of prematurity by employing PACAP-deficient mice. Our findings demonstrate that the absence of endogenous PACAP exacerbates the vascular pathology associated with oxygen-induced retinopathy, as shown by the enlarged central avascular area and increased neovascularization in KO animals. In normoxic conditions, no vascular differences were observed between genotypes, supporting earlier observations that the effects of PACAP deficiency primarily manifest under stress or injury.

Previous research has consistently shown that PACAP deficiency increases tissue vulnerability across a range of neural and retinal injury models. Our results extend these findings to the context of experimental ROP, suggesting that endogenous PACAP acts as a key protective factor against neonatal retinal injury. The minimal differences observed in control animals, contrasted with the pronounced changes under OIR conditions, suggest that PACAP plays a particularly significant regulatory role in angiogenesis during pathological stress.

Angiogenesis array profiling revealed enhanced expression of multiple pro- and anti-angiogenic factors in the ROP groups. In wild-type OIR retinas, molecules such as DLL4, CYR61, ADAMTS-1, IP-10, PDGF-AA, and IGFBP-3 - known to regulate vascular development, extracellular matrix remodeling, and inflammatory pathways -

were upregulated. PACAP-deficient OIR retinas exhibited further increases in several angiogenic and inflammatory mediators, suggesting that the absence of endogenous PACAP disrupts angiogenic balance and contributes to more severe pathological outcomes.

One of the key mediators of PACAP's cytoprotective properties is the Akt-dependent anti-apoptotic signaling pathway. Our Western blot results revealed enhanced Akt phosphorylation in wild-type OIR retinas but significantly reduced levels in PACAP-deficient mice. This is consistent with prior findings showing that PACAP promotes cell survival in various retinal injury models via Akt pathway activation. Our data therefore suggest that impaired Akt-mediated survival signaling may contribute to the more pronounced morphological abnormalities observed in PACAP-deficient animals.

Functional assessments further support this hypothesis. PACAP-deficient mice exhibited significantly reduced b-wave amplitudes in the OIR condition, indicating the increased dysfunction of the inner retina. Remarkably, even normoxic PACAP KO mice displayed lower baseline a- and b-wave amplitudes, suggesting that PACAP may play a role not only under pathological conditions but also during normal retinal functional development. The presence of PACAP receptors in the developing retina and their involvement in regulating retinal progenitor cell proliferation give further support to this possibility, although additional studies are needed to clarify this role.

Interpretation of functional results must consider the smaller sample size in ERG recordings, partly attributable to the increased fragility of PACAP-deficient animals. Another limitation is that while the OIR model reproduces numerous key features of human ROP, it does not fully capture the condition's clinical complexity or developmental dynamics.

Overall, our findings indicate that endogenous PACAP plays a crucial protective role in mitigating both vascular and functional damage in a mouse model of retinopathy of prematurity. PACAP deficiency leads to more severe pathological changes, potentially driven by disruption of angiogenic balance and diminished Akt-mediated cytoprotective signaling.

V. Novel scientific findings

Findings related to the effects of exogenous PACAP in the rat OIR model:

- We demonstrated that three intravitreal administrations of PACAP significantly reduced the size of the peripheral avascular area in the rat model of oxygen-induced retinopathy.
- We showed that intravitreal PACAP treatment attenuated the elevated expression of inflammatory and angiogenesis-related cytokines characteristic of the OIR model, while further increasing TIMP-1 levels.
- Using electroretinography, we confirmed that visual function was impaired in retinopathic animals, although PACAP treatment did not improve ERG wave morphology.

Findings related to the role of endogenous PACAP in the mouse OIR model

- We demonstrated that oxygen-induced retinopathy develops in a more severe form in PACAP-deficient mice, characterized by a larger avascular retinal area and increased neovascularization compared with wild-type animals.
- We found that PACAP deficiency resulted in more pronounced alterations in the expression of both pro- and anti-angiogenic factors in the OIR model.
- Western blot analysis revealed that the absence of endogenous PACAP is associated with reduced anti-apoptotic Akt phosphorylation under retinopathic conditions.
- Electroretinographic assessment showed that PACAP KO mice exhibit decreased b-wave amplitudes, indicating greater functional impairment of the inner retinal layers.

List of Publications

Publications on which the thesis is based:

Kvarik T, Mammel B, Reglodi D, Kovacs K, Werling D, Bede B, Vaczy A, Fabian E, Toth G, Kiss P, Tamas A, Ertl T, Gyarmati J, Atlasz T. (2016) PACAP Is Protective in a Rat Model of Retinopathy of Prematurity. J Mol Neurosci. 60(2):179-85. **IF: 2,229**

Kvarik T, Reglodi D, Werling D, Vaczy A, Kovari P, Szabo E, Kovacs K, Hashimoto H, Ertl T, Gyarmati J, Atlasz T. (2021) The Protective Effects of Endogenous PACAP in Oxygen-Induced Retinopathy. J Mol Neurosci. 71(12):2546-2557. **IF: 2,866**

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Kvarik T, Mammel B, Reglodi D, Antonelli MC, Farkas J, Tamas A, Ertl T, Atlasz T, Bodzai G., Kiss P, Gyarmati J. (2016) Effects of maternal stress during different periods of pregnancy on the early neurobehavioral response of rats. J Neurology and Neuroscience. 7(2):80. **IF: -**

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